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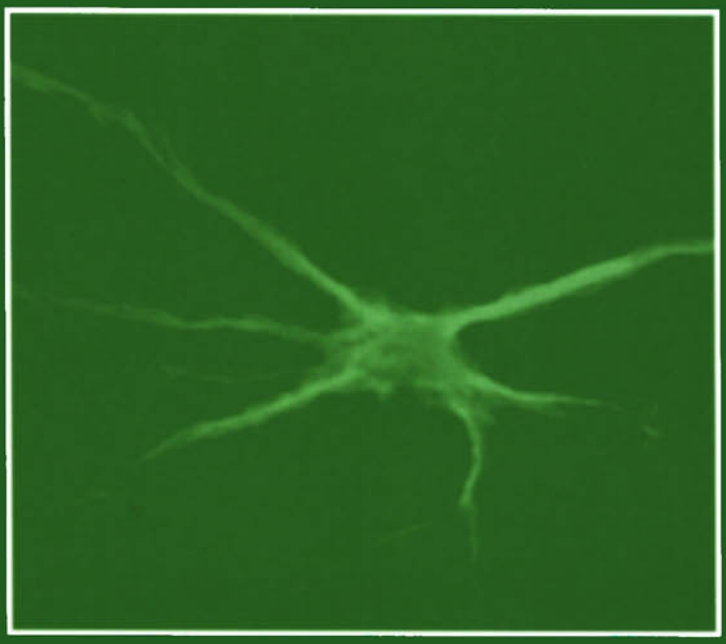
A SURVEY OF CELL BIOLOGY

Edited by
Kwang W. Jeon



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Front cover photograph: Merged confocal images of ISO-treated AS583-8 cells which demonstrate the difference in tyr-α–tubulin and acet-α–tubulin. (For more details see Chapter 5, Figure 9.)

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CONTENTS

Phosphoinositide Kinases and the Synthesis of Polyphosphoinositides in Higher Plant Cells

Bjørn K. Drøbak, Ralph E. Dewey, and Wendy F. Boss

Chlamydomonas Cell Cycle Mutants

John D. I. Harper

Cellular Aspects of Trophic Actions in the Nervous System

Daniela M. Vogt Weisenhorn, John Roback, Andrew N. Young, and Bruce H. Wainer

Conformational Changes of Contractile Proteins and Their Role in Muscle Contraction

Yurii S. Borovikov

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Differentiation-Related Changes in the Cell Cycle Traverse

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This review examines recent developments relating to the interface between cell proliferation and differentiation. It is suggested that the mechanism responsible for this transition is more akin to a "dimmer" than to a "switch," that it is more useful to refer to early and late stages of differentiation rather than to "terminal" differentiation, and examples of the reversibility of differentiation are provided. An outline of the established paradigm of cell cycle regulation is followed by summaries of recent studies that suggest that this paradigm is overly simplified and should be interpreted in the context of different cell types. The role of inhibitors of cyclin-dependent kinases in differentiation is discussed, but the data are still inconclusive. An increasing interest in the changes in G_2/M transition during differentiation is illustrated by examples of polyploidization during differentiation, such as megakaryocyte maturation. Although the retinoblastoma protein is currently maintaining its prominent role in control of proliferation and differentiation, it is anticipated that equally important regulators will be discovered and provide an explanation at the molecular level for the gradual transition from proliferation to differentiation.

KEY WORDS: Cell differentiation, Cell cycle, Cyclin-dependent kinases, p21/Cip1, p27/Kip1, pRb, E2F, polyploidization.

I. Introduction

The relationship between cell proliferation and differentiation has an intriguing complexity. In general, there is a reciprocity between these processes; cells which rapidly proliferate do not differentiate, and terminally differentiated cells cannot proliferate. However, between these two extremes there are gray areas. Although sometimes forgotten, cells can and do proliferate

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while differentiating, as in hematopoietic lineage selection followed by amplification, and differentiation is not always terminal but, in some forms of differentiation, is reversible. If there is a "switch" between proliferation and differentiation, is its mechanism a rheostat and should it be called a "dimmer" rather than a switch?

Another point that must be emphasized is that even though cell cycle machinery and many of its controls are remarkably conserved from yeast to human cells, there are nonetheless clear differences between species and cell types in cell cycle regulation. Thus, although paradigms for the cell cycle appear to be firmly established, it should be remembered that these paradigms need not apply to all cell types but depend on the cell's internal environment. An experimental demonstration of this phenomenon was provided by studies in which ectopic expression of wild-type p53 resulted in a reduction of growth in 32D murine myeloid leukemia cells transformed by the v-*abl* oncogene but induced monocytic differentiation in cells transformed by v-*src* (Soddu *et al.,* 1996). Differentiation and cell cycle are likely to be interrelated in a variety of different ways, depending on the cellular context of the preexisting regulatory molecules.

In this article, we discuss these relationships in the light of recent literature on *in vitro* differentiation systems, reported up to April 1998. While we have drawn on many publications, actual citations are of necessity more limited and perhaps rather arbitrarily selected since this review was deliberately prepared from our personal point of view. Our bias is based on investigations in our own laboratories on leukemia and colon differentiation cell systems and is reflected in the more detailed descriptions of these studies. We hope, however, that this perspective will provide a background of knowledge, as well as some generalizations and some cautions, which will permit the reader to assess critically current reports on *in vitro* differentiation of mammalian cells and perhaps suggest topics for further investigation.

II. Basic Definitions

A. Differentiation

In the normal development of an organism, cells acquire differing repertoires of gene expression which are reflected in diverse phenotypic characteristics. Developmentally regulated differentiation involves formation of a reservoir of stem cells, lineage selection, amplification transit stages of immature cells, and maturation to functioning, fully differentiated cells

(Fig. 1). When fully differentiated, cells generally no longer traverse the cell cycle and do not give rise to cells with new phenotypes; therefore, for an ill-defined combination of these reasons, these cells are usually referred to as terminally differentiated.

In model systems, this complexity is often simplified. It is unusual to find *in vitro* systems in which cells proliferate while they undergo intermediate phases of differentiation; instead, proliferation is generally immediately arrested, reinforcing the belief that growth cessation necessarily precedes differentiation. The maturation process is then followed by the development of one or several phenotypic features characteristic of the mature cell. Yet, despite these rather simplistic assumptions, *in vitro* model systems have provided a remarkable wealth of new information on various facets of mammalian cell differentiation.

B. Quiescence and Senescence

Terminal differentiation is related in rather subtle ways to replicative quiescence and to cellular senescence. In many situations the terms are overlapping because a terminally differentiated neuron is also quiescent and be-

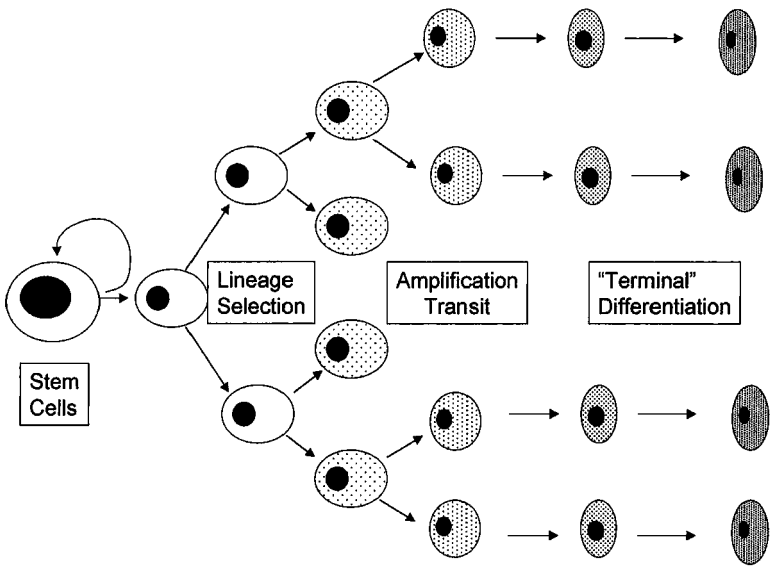

FIG. 1 Developmentally regulated differentiation. After the formation of stem cells, the process of differentiation begins with lineage selection, leading to amplification of the numbers of immature cells to functioning, fully differentiated cells.

lieved to be senescent. The distinctions between these three terms are both conceptual and operational. In theory, differentiation is characterized by a demonstrable function of a cell and a phenotype different from the phenotype of its precursor, quiescence by the absence of DNA replication and components of the replicative machinery, recognized by markers such as the Ki-67 antigen (Duchrow *et al.,* 1995), and senescence by an irreversible growth arrest in the absence of conventional markers of differentiation but in the presence of β-galactosidase (Dimri *et al.,* 1995; Vogt *et al.,* 1998). In practice, senescence is generally used to describe mesenchymal cells, usually fibroblasts in culture, which have reached the limit of their replicative life span and are unresponsive to treatment with mitogens (Hayflick and Moorhead, 1961). Among the characteristics that have been reported to distinguish senescent from cycling or quiescent cells are elevated levels of cyclin-dependent kinase inhibitors p21/Cip1, also known as senescent cell-derived inhibitor-1 (Sdi-1), and p16(INK4A) and increased expression of plasminogen activator inhibitor (McConnell *et al.,* 1998). At least four growth regulatory modulators of transcription have been reported to be repressed in senescent cells: c-fos, Id-1 and -2, and E2F-1 (Dimri *et al.,* 1996). Also, in contrast to immortal human fibroblasts, their senescent counterparts have a low content of 5-methylcytosine in the genome, lipofuscin granules, and flat morphology, as well as the previously mentioned β-galactosidase activity at pH 6 (Vogt *et al.,* 1998). The fact that human cells have a limited replicative life span and enter a state of irreversibly arrested growth after a finite number of divisions represents a significant barrier to the development of cell clones that develop into neoplastic growths.

In its current usage, there is no clear definition for the term replicative quiescence. Frequently taken to be synonymous with the G_0 phase of the cell cycle, its principal distinction from senescence and terminal differentiation is its reversibility, easily accomplished by the addition of factors required for growth (e.g., serum) if these are missing or the removal of growth restraining agents [e.g., transforming growth factor-β, TGF β]. However, since some cells can arrest in segments of the cell cycle other than G_0 [e.g., G_2 (Godyn *et al.,* 1994; Novitch *et al.,* 1996)], replicative quiescence need not imply residence in G_0. Important for this discussion, cell differentiation implies actual or eventual replicative quiescence.

C. Apoptosis

Truly terminal differentiation is followed by cell death. This is particularly obvious in epithelial surfaces from which the dead cells drop off, and the observation of this phenomenon suggested the term apoptosis (dropping off) for this or other physiologically programmed cell death (Kerr *et al.,*

1972). Enucleation of erythrocytes (Morioka *et al.,* 1998) and degradation of nuclei in lens fiber cells (Fromm and Overbeck, 1996; Bassnett and Mataic, 1997) also take place by processes that resemble, but are not identical to, apoptosis. Interestingly, cell differentiation alters the susceptibility of the differentiating cells to the induction of cell death by exogenous agents (Wang and Studzinski, 1997), suggesting that signaling pathways for differentiation and apoptosis interact.

III. Models of Cell Differentiation

A. Cell Types

A large variety of cell types have been used to study induced differentiation of mammalian cells and a partial summary and references are provided in Table I. There has been a tendency to select human cells in these studies because this is one of the avenues which may help to elucidate the mechanisms that block normal differentiation in neoplastic cells that comprise human cancers. This is illustrated by studies of the induction of differentiation of human hematopoietic cells or colon carcinoma cells. For some purposes, cells derived from animals have been used more extensively, for example, in numerous studies by Gary Stein's group on osteoblastic differentiation (Smith *et al.,* 1997) or the rat pheochromocytoma cell line PC12, which is an excellent model for neuronal differentiation induced by the nerve growth factor (Camps *et al.,* 1998; Sano and Katajima, 1998). Proliferating murine myoblasts can undergo differentiation into myotubules, and this system has been utilized in numerous studies of terminal differentiation (Franklin and Xiong, 1996; Zabludoff *et al.,* 1998; Datta *et al.,* 1998). A very interesting system is also provided by the ocular lens since the cells become enucleated as they differentiate but retain some of the components of cell cycle regulatory machinery (Fromm and Overbeck, 1996).

In vivo models are also used. The crypt–villus unit of the mouse small intestine is a specially organized continuum of proliferation, differentiation, and apoptosis which allows examination of the interrelationships between these processes in the natural setting (el-Deiry *et al.,* 1995), as is illustrated in Fig. 2.

B. Inducers of Differentiation

Induction of differentiation can be accomplished in some cases by removal of mitogens or by the incubation of susceptible cells with a wide array of

TABLE I

Examples of Cellular Models of Differentiation

Differentiation marker	Known function	Comments	Reference
	Hematopoietic (HL60, U937, K562, MEL)		
CD14	LPS cell surface binding protein	Early monocytic differentiation	Studzinski *et al.* (1985, 1996)
CD11b	Cell surface protein integrin mac-1	Late monocytic differentiation	Griffin *et al.* (1981)
NSE[a]	Cytoplasmic hydrolytic enzyme	Late monocytic differentiation	Yam *et al.* (1971)
NBT[b]	Component of oxidative burst	Phagocytic cells	Blair *et al.* (1985)
VDR[c]	Ligand-activated transcription factor	Steroid/retinoid receptor	Pan *et al.* (1991)
Hemoglobin	Functional protein	Erythroid differentiation	Fibach *et al.* (1983)
Vimentin	Filament protein	Early marker of differentiation	Ruis *et al.* (1990)
	Colon (HT29, Caco-2, WiDr, LS174T)		
Alkaline phosphatase	Brush border enzyme	Intestinal and placental isozymes	Gum *et al.* (1987)
Sucrase-isomaltase	Brush border enzyme	Increased with differentiation	Djelloul *et al.* (1997)
CEA[d]	Adhesion molecule	Early developmental protein	Saini *et al.* (1990)
Villin	Actin-binding protein of brush border	Expression increased along crypt–villus axis	Hodin *et al.* (1996)
Calretinin	Calcium-binding protein	Expression cell cycle dependent	Cargnello *et al.* (1996)
VDR	Ligand-activated transcription factor		Shabahang *et al.* (1993)
	Bone (MG-63, ROS 17/2.8, SaOS-2)		
Osteocalcin	Osteoblast-specific noncollagenous protein		van den Bemd *et al.* (1995), Franceschi *et al.* (1985)
Fibronectin	Extracellular adhesion molecule		

Alkaline phosphatase	Bone mineralization		
		Muscle (C2C12)	
MyoD	HLH transcription factor	Expressed in myoblasts	Yun and Wold (1996), Piette (1997), Coolican *et al.* (1997)
Myf5	HLH transcription factor	Expressed in myoblasts	
Myogenin	HLH transcription factor	Early marker	
Mrf4	HLH transcription factor		
Myosin heavy chain	Functional protein	Late marker	
MEF2	Transcription factor	Marker of late differentiation	
Creatine kinase	Functional protein		
Contractile myotubules			
		Nerve (PC-12, NIE-115)	
Neuronal B II tubulin	Functional protein	Early marker	Piette (1997)
Trk-A and B-receptors	Neurotropin receptor		
$p75^{LNGFR}$ receptor	Neurotropin receptor		
		Breast (MCF-7)	
Intracellular lipid	Storage/precursor material		Elstner *et al.* (1995)
		Fibroblast (3T3T)	
Adipocyte morphology			
P2P gene	Encodes hnRNP protein	Marker of terminal differentiation	Minoo *et al.* (1989)

[a] Nonspecific esterase.
[b] Nitroblue tetrazolium.
[c] Vitamin D receptor.
[d] Carcinoembryonic antigen.

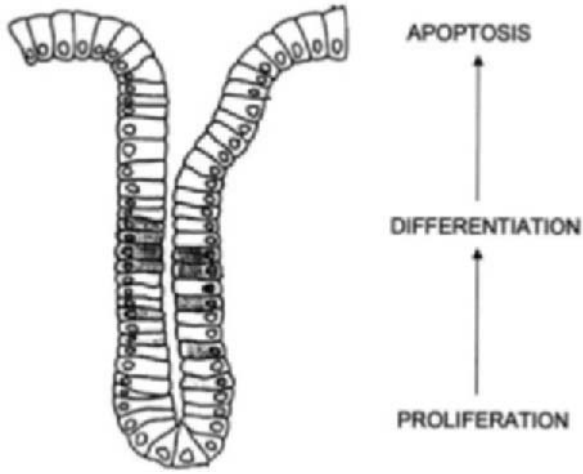

FIG. 2 The crypt–villus unit as a model of differentiation. *Ex vivo* analysis of intact enteric mucosa allows simultaneous evaluation of the entire crypt–villus axis and correlation of cell location with the degree of proliferation of differentiation.

compounds, including toxic chemicals such as the polar–planar compound dimethylsulfoxide and phorbol esters, hormones, morphogens, and cytokines such as 1,25-dihydroxyvitamin D_3 (1,25-D_3), retinoids, and interferon γ (IFN-γ). There is an expectation that forcible induction of differentiation by at least some of these agents will lead to improvements in treatment of human cancers, and this has already been achieved in the case of the morphogen retinoic acid (Thomas *et al.,* 1997). This compound and other inducers of hematopoietic differentiation are described in detail.

1. Inducers of Hematopoietic Lineage Differentiation

a. Retinoic Acid Retinoic acid (RA) is a physiologically active metabolite of vitamin A and has been shown to play a significant role in modulating cell growth and differentiation in the developing embryo. RA and its derivatives, collectively called retinoids, have a significant effect on the differentiation and growth of leukemia and a variety of other transformed cell types, including breast, melanoma, squamous cell cancers of the head and neck, and neuroblastomas (Beere and Hickman, 1993).

The biological activity of RA is believed to be mediated through the activation of a set of nuclear RA receptors (RARs and RXRs). The RARs and RXRs act as ligand-inducible, transcription enhancer factors and belong to the nuclear receptor superfamily which also includes steroid and thyroid hormone receptors (Hashimoto and Shudo, 1991). To date, three distinct

RARs (RAR-α, RAR-β, and RAR-γ) and three RXRs (RXR-α, RXR-β, and RXR-γ) have been identified. The distribution of these receptors is tissue specific, with RAR-α found mainly in hematopoietic cells, RAR-β predominating in the brain, and RAR-γ confined almost exclusively to the skin (Gallagher *et al.,* 1989; Zelent *et al.,* 1989). Similarly, cell type also determines RXR isoform distribution. For example, while RXR-α, -β, and -γ are detected in normal skin (Reichrath *et al.,* 1997) and kidney (Sugawara *et al.,* 1997), B lymphocytes express only RXR-α (Lomo *et al.,* 1998), and only RXR-α and -β are identified in uterine smooth muscle (Boettger-Tong *et al.,* 1997). In addition, cytoplasmic RA-binding proteins may also play a role in modulating the activity of RA-induced differentiation. The induction of cellular differentiation by RA is associated with a number of molecular events, including alteration in the production of growth factors IGF-1 (Lowe *et al.,* 1992), TGF-α (Dmitrovski *et al.,* 1990), and TGF-β (Weima *et al.,* 1989), modulation of the protein kinase–phosphoinositol system (Niles and Loewy, 1989), expression of EGF receptors (Zheng *et al.,* 1992), and effects on the expression of immediate early response genes, including c-*myc* (Taylor *et al.,* 1992), c-*fos,* c-*myb* (Onishi *et al.,* 1990), and c-*jun* (Momoi *et al.,* 1992).

b. Vitamin D_3 and Analogs Like RA, the physiological form of vitamin D, 1,25-D_3, is a naturally occurring compound with hormonal properties. Although its classical actions on calcium homeostasis have received the most attention, 1,25-D_3 also functions as a regulator of cell growth, survival, and differentiation. In addition to leukemia cells, its targets include the brush border cells of intestinal villi (McCarthy *et al.,* 1984; Delvin *et al.,* 1996), the development and function of osteoblasts (Farach-Carson and Ridall, 1998), keratinocytes (Jensen *et al.,* 1998), and multipotential cells of the hematopoietic system (Nakamura *et al.,* 1996; Lee *et al.,* 1996).

Although there is evidence that in intestinal cells actions of 1,25-D_3 can be mediated by a membrane response element, in hematopoietic cells the principal effector of 1,25-D_3 actions is an intracellular protein, VDR, which acts in the nucleus as a transcription factor in concert with one of the members of RXR family of steroid receptors. These bind to VDR elements (VDREs) in the promoter regions of target genes and modulate the transcription rates of these genes. This results in the upregulation of transcription in most target genes, such as osteocalcin, osteopontin, calbindin-28K, 24-hydroxylase, and β_3 integrin. Conversely, a VDRE in the 5′ flanking region of the parathyroid hormone gene mediates transcriptional repression by VDR (Demay *et al.,* 1992; Liu *et al.,* 1996c; Hawa *et al.,* 1996). Recent reviews on the diverse aspects of 1,25-D_3 signaling and references are available in two compendia on vitamin D (Feldman *et al.,* 1997; Norman *et al.,* 1997). For example, Hewison and O'Riordan (1997) succinctly sum-

marize the cell differentiation and immunomodulatory effects of 1,25-D_3 in one of these volumes. A recent report implicates the *HoxA10* gene in the mediation of the growth-suppressive effects of 1,25-D_3 on U937 and MCF-7 cells (Rots *et al.*, 1998).

Differentiating effects of 1,25-D_3 are accompanied by major changes in calcium homeostasis. To overcome this problem for potential therapeutic applications, a variety of chemical modifications have been introduced in the molecule. It appears that an unsaturated bond in position 16, hexafluorination of the methyl groups in the side chain, and epi configuration in the 20 position all greatly increase the differentiation potency without a corresponding increase in hypercalcemia induction (Elstner *et al.*, 1995; Uskokovic *et al.*, 1997; Binderup *et al.*, 1997). Several such compounds are in early stages of clinical trials for treatment of several types of human cancers, but the results remain to be evaluated.

c. Polar–Planar Compounds The polar–planar compounds represent a large, functionally heterogeneous group, including dimethylsulfoxide (DMSO), *N*-methylformamide, and hexamethylene bisacetamide (HMBA). All are inducers of differentiation, to a greater or lesser extent, in a variety of *in vitro* cell culture systems. HMBA (and various analogs) has been shown to induce terminal differentiation in malignant cell lines of hematopoietic and epithelial origin. U937 cells are induced to monocytic differentiation (Bernstein *et al.*, 1991), HL60 cells express markers consistent with granulocyte differentiation (Breitman and He, 1990), and MEL cells express an erythroid-like phenotype after exposure to HMBA (Marks *et al.*, 1987). HMBA has also been shown to induce differentiation in colon, breast, and kidney malignancies as well as neuroblastoma cells. The mechanism of action of HMBA is unclear. There is accumulating evidence that protein kinase C (PKC) may play a role in HMBA-induced differentiation (Melloni *et al.*, 1987). HMBA is also associated with changes in gene expression of a number of protooncogenes. After exposure to HMBA, c-*fos* RNA levels are increased, whereas expression of *p53*, c-*myb*, and c-*myc* is dramatically reduced (Richon *et al.*, 1989; Danish *et al.*, 1992).

d. Phorbol Esters Phorbol esters comprise a family of compounds with well-known tumor-promoting effects. The most commonly used phorbol ester is tetradecanolyl phorbol acetate (TPA), also known as phorbol 12-myristate 13-acetate. Its mechanism of action is believed to include the activation of PKC by acting as an analog to diacylglycerol, the natural activator of PKC (Ebeling *et al.*, 1985). Phorbol esters also have differentiating effects. TPA has been reported to induce differentiation of leukemia cells to monocytic phenotype (Zhang and Chellappan, 1996) and melanoma and breast cancer cells to more mature forms (Bertolotto *et al.*, 1998;

Shanmugam *et al.,* 1998), possibly through similiar mechanisms of PKC activation. While quite active *in vitro,* this compound may be difficult to exploit clinically due to its tumor-promoting effects.

2. Inducers of Colon Cell Differentiation

a. Butyrate Recently, the salutary effects of dietary fiber on colon cancer have been attributed to the production of short-chain fatty acids (SCFAs). SCFAs are produced during anaerobic fermentation of dietary fiber by endogenous intestinal bacteria that are natural constituents of the colonic lumen (Boffa *et al.,* 1992). Butyrate has been identified as one such SCFA that possesses both antiproliferative and differentiating properties (Barnard and Warwick, 1994; Heerdt *et al.,* 1994). Butyrate treatment of cultured cells is associated with changes in chromatin structure and cytoskeletal assembly, alterations in DNA synthesis, and various enzymatic activities (Darzynkiewicz *et al.,* 1981; Kruh, 1982). In addition, *in vitro* exposure of colon cancer cell lines to sodium butyrate results in reversible growth arrest and cellular differentiation as evidenced by morphologic changes and increased cellular expression of alkaline phosphatase (Gum *et al.,* 1987), CEA (Saini *et al.,* 1990), and villin (Hodin *et al.,* 1996). While the exact mechanisms of butyrate's effects are unknown, Sealy and Chalkley (1979) proposed that these effects are mediated through histone–DNA interactions. They suggested that hyperacetylation of histones associated with butyrate exposure was due to the inhibition of histone deacetylase. Other reports have indicated that butyrate can modulate gene expression through specific promoter regions. In murine erythroleukemia cells, a 156-bp promoter fragment was found to be necessary for butyrate-dependent activation of the ectopic chicken embryonic globin gene (Glauber *et al.,* 1991).

Butyrate's differentiation effects are noted only at concentrations of 0.3 m*M* or higher. Therefore, a short plasma half-life and large salt load limit butyrate's clinical applications. Derivatives and analogs, such as phenylbutyrate (Gore *et al.,* 1997; Samid *et al.,* 1992) and pivalyloxymethyl butyrate (Rephaeli *et al.,* 1991), have been developed which overcome some of these issues and are currently undergoing clinical trials.

b. Vitamin D_3 Analogs As discussed previously, 1,25-D_3 has been shown to have antiproliferative and differentiating effects on a variety of hematopoietic cells. In addition, 1,25-D_3 also has similar activity on solid tumors, including colon cancer. However, the therapeutic use of 1,25-D_3 is limited by its profound effects on calcium metabolism. Nonhypercalcemic analogs of vitamin D have been synthesized and have equal or increased differentiating effects on colon cancer cell lines without the hypercalcemic side effects (Cross *et al.,* 1992; Hulla *et al.,* 1995). Shabahang and colleagues (1994)

compared a panel of four synthetic vitamin D analogs to 1,25-D_3 in the colon cancer line HT-29 and demonstrated an increase in antiproliferative effects compared to 1,25-D_3. Using an *ex vivo* model, Thomas *et al.* (1992) biopsied human rectal mucosa and performed short-term incubations in 1,25-D_3 and its analog, calcipotriol. This resulted in a decrease in proliferation measured by crypt cell production rate and by Ki-67 labeling index. In addition to having an effect on established colon cancer, some vitamin D analogs have been shown to have a protective effect against carcinogen-induced colon cancer in rodent models (Otoshi *et al.*, 1995; Wali *et al.*, 1995).

C. Markers of Differentiation

Differentiation is monitored by the appearance of phenotypic markers characteristic of the mature cells of a given cell lineage. For instance, protein receptors displayed on immature cell surfaces are different from those in mature cells, and these can therefore be easily distinguished by immunophenotyping (Griffin *et al.*, 1981). Enzymes that provide a function in the mature cell also serve as useful markers, e.g., nonspecific esterase in monocytic differentiation (Yam *et al.*, 1971), nitroblue tetrazolium reduction in all phagocytic cells (Blair *et al.*, 1985), alkaline phosphatase in differentiation of colonic epithelial cells (Gum *et al.*, 1987), and muscle-specific creatine kinase in differentiation of muscle cells (Coolican *et al.*, 1997). Functioning proteins also serve as markers of differentiation; examples are myosin heavy chain in skeletal muscle differentiation (Bennet and Tonks, 1997), hemoglobin in erythroid differentiation of K562 cells (Fibach *et al.*, 1983), and crystallins in lens fiber cell differentiation (Ogino and Yasuda, 1998).

In some cases molecular markers of differentiation are not available, but cellular properties are altered in a characteristic way. This is illustrated by phorbol ester-induced macrophage differentiation of myeloid cells, which become adherent to surfaces (a feature of macrophages) and by fusion of myoblasts into contractile myotubules (Coolican *et al.*, 1997). Although these are more difficult to quantitate, such properties have been useful in many studies of differentiation.

It is also useful to distinguish between early and late markers of differentiation. For instance, in skeletal muscle differentiation myogenin appears early in the differentiation program, whereas the presence of myosin heavy chain indicates late differentiation (Novitch *et al.*, 1996). In the myeloid leukemia HL60 cell line the surface receptor CD14 appears very early in monocytic differentiation, it is followed by upregulation of the expression of CD11b and NSE markers, and an increase in adherence is noted even later (Studzinski *et al.*, 1985, 1996, unpublished observations).

IV. Overview of the Regulation of the Cell Cycle Traverse

A. Basic Mechanisms

1. Cell Cycle Compartments

Proliferation of eukaryotic cells results from the consecutive progression though four distinct phases of the cell cycle called G_1, S, G_2, and M. DNA replication occurs during S phase, chromosome separation (karyokinesis) takes place during the M phase and is followed by cell division (cytokinesis), and G_1 and G_2 are gap or growth phases. The G_1 phase has been further subdivided into early G_1 or postmitotic, mid-G_1, in which principal cell growth takes place, and late G_1, in which final preparations for DNA replication occur. The G_2 phase is thought to be necessary for monitoring of chromosome replication and preparations for mitotic spindle assembly (Nurse, 1990; Pardee, 1989; Sherr, 1994). As indicated previously, cells that are not actively dividing may either be permanently removed from these cycling phases by terminal differentiation, senescence, or apoptosis or be temporarily arrested in a noncycling quiescent state known as G_0 if the cells have the G_1 DNA content, though quiescence can also occasionally take place in the G_2 phase. While specific nuances have been described, the remarkable feature of the cell cycle is the conservation of the basic regulatory mechanisms and components from yeast to mammalian cells.

2. Checkpoints

a. Definition Traverse of the various compartments of the cell cycle is controlled by a series of regulatory steps referred to as checkpoints. A simple definition of a checkpoint is that it is a mechanism that prevents progression to the next part of the cell cycle unless and until the preceding part has been satisfactorily completed. Such checkpoints operate in each phase of the cell cycle, and although the precise mechanisms in most cases are unclear, several regulators, such as p53, have been shown to have great importance in checkpoint control. Interestingly, these regulators of cell cycle progression also influence cellular decisions to differentiate (Elledge, 1996).

b. The G_1 and S Phase Checkpoints Probably the first checkpoint to be clearly described is the restriction (R) point in mid- to late G_1 phase. Based on his own work and results in Baserga's and other laboratories, Pardee defined a transition in G_1 phase which commits a cell to initiate DNA replication (Pardee, 1989). Subsequent work indicated that phosphorylation

of the retinoblastoma susceptibility protein (pRb) may provide the principal mechanism for the transition through the R point (Dou *et al.,* 1993). Subsequent passage through the S phase is also controlled by the S phase checkpoints (O'Connor and Fan, 1996; Shao *et al.,* 1997), which are currently more nebulous in mammalian cells.

c. The G_2 and M Phase Checkpoints The final result of the cell cycle traverse is a faithful replication and accurate partitioning of genetic information. The fidelity of this partitioning is maintained by the G_2 and the M phase checkpoints. The G_2 phase checkpoints monitor the accuracy of DNA replication, the M phase checkpoints ensure correct chromosomal segregation and alignment, and each of these arrest cell cycle progress to allow editing and repair of genetic information. Overall, they ensure that each daughter cell receives a full complement of genetic information identical to the parental cell.

3. Mechanisms That Drive Cell Cycle Progression

a. Components Passage through the restriction points is believed to be propelled by the activity of a group of enzymes known as cyclin-dependent kinases (Cdks). Cdks, eight of which have been identified in mammals, are usually present throughout the cell cycle and work in concert with cyclins, which are nuclear proteins whose levels oscillate in a cell cycle-dependent manner (Hunter and Pines, 1994; Morgan, 1995).

b. Regulation In general, there are at least seven levels at which the activity of Cdks can be controlled (Fig. 3). The primary regulator of Cdks activity is cyclin binding because Cdks and cyclins need to form a complex prior to activation. Since the protein levels of the cyclins change during the cell cycle, the binding of Cdks and cyclins is related to cyclin availability. The abundance of cyclins, like all proteins, is dependent on the balance between their synthesis and their degradation, the latter occurring by ubiquitin-dependent proteolysis (Hoyt, 1997).

The activity of the cyclin–Cdk complexes also depends on both activating and inhibitory phosphorylations. A known kinase which can phosphorylate Cdks is cyclin-activating kinase (CAK). CAK is a multisubunit enzyme composed of cyclin H (regulatory subunit) and Cdk7 (catalytic subunit). This complex phosphorylates the threonines 161 and 160 on Cdc2 and Cdk2, respectively (Bartkova *et al.,* 1996). Phosphorylation of this site is necessary for the cyclin–Cdk complex to be activated. Cdk inhibitory phosphorylation sites include threonine 14 and tyrosine 15. Regulation of these sites is achieved by a group of proteins collectively called kinase/phosphatase cell cycle regulatory proteins. These include Cdc25A, -B, and

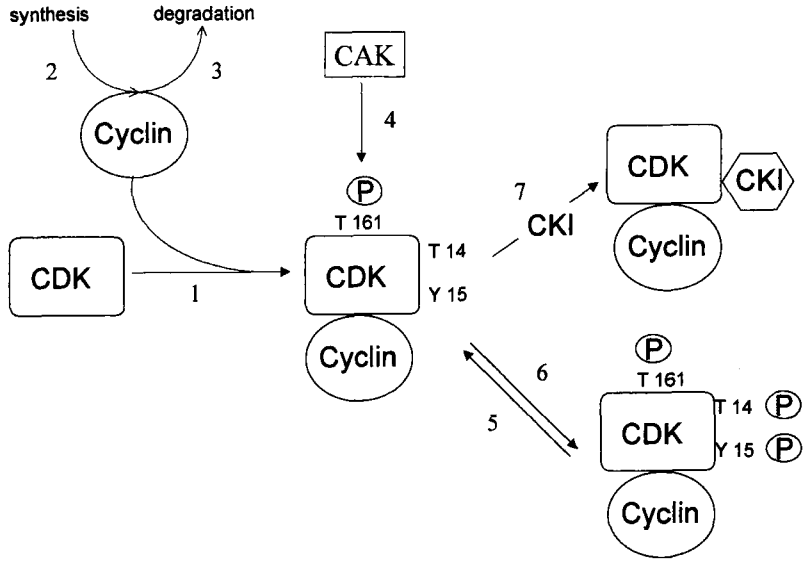

FIG. 3 Regulation of cyclin-dependent kinases. There are at least seven levels at which the activity of Cdks can be controlled: (1) complex formation with the appropriate cyclin, (2) synthesis and (3) degradation of the cyclin, (4) activation of the cyclin–Cdk complex by the cyclin-activating kinase (CAK), (5) activating dephosphorylation and (6) inhibitory phosphorylation of the Cdk, and (7) inhibitory binding of specific cyclin-dependent kinase inhibitors (CKIs).

-C (phosphatases), wee-1, and mik-1 (kinases). The phosphatases activate the cell cycle by cleaving phosphate groups on threonine 14 and tyrosine 15 residues of the Cdk, whereas the kinases are inhibitory by phosphorylating the same sites. Cdc25A and -B are involved in the G_1/S checkpoint (Jinno *et al.*, 1994), whereas Cdc25C and wee-1 regulate the traverse through the G_2/M phases (Gautier *et al.*, 1991; Heald *et al.*, 1993). Another component of Cdk regulation includes the Cdk inhibitory proteins (CDKIs), which prevent Cdk activation generally by binding to the kinases and thus preventing their activation by cyclins, as discussed in detail later.

4. Phase Transitions

While there are subtle variations on the general schema, the previously discussed sites of regulation are generally applicable to most models. Cyclins and Cdks are conventionally divided into two functional classes: those that primarily act in G_1 and at the G_1/S boundary (cyclins C, D, and E and kinases Cdk2, -4, and -6) and those specific to the G_2 and M phases (cyclin

B and kinase Cdc2). Cyclin A appears to be rather unique in that it has been reported to be required for G_1/S, S phase, and G_2/M transitions in a number of cell systems (Sherr, 1993).

a. The G_1 and S Phase Transitions Events during the G_1 phase allow an increase in cell mass and prepare the cell for DNA synthesis. For nontransformed cells to traverse the R point, specific growth factors are required. Once the R point is passed, the cell enters late G_1 and is committed to progress into the S phase. The cell cycle traverse is now mitogen independent. Thus, the restriction point can divide the G_1 phase into two stages: a growth factor-dependent stage (prerestriction point) and a growth factor-independent stage (postrestriction point) (Hartwell and Weinert, 1989).

The central event for the G_1/S transition appears to be the phosphorylation of the tumor suppressor, pRb, as shown in Fig. 4A (Bartek *et al.,* 1997; Chen *et al.,* 1989). pRb and other pRb-like "pocket" proteins (p130/Rb2 and p107) are believed to control the entry into the S phase by interacting with a member of the transcription factor E2F family. This family is composed of at least five proteins (E2F1–5) which are active when they form heterodimeric complexes with one of the E2F-related transcription factors—DP-1, -2, or -3. In its simplest form, the current hypothesis is that hypophosphorylated pRb binds to E2F, preventing cell entry into the S phase. Upon an increased level of phosphorylation, pRb frees E2F, which in its heterodimeric complex with DP is capable of activating genes necessary for S phase initiation. However, the actual situation appears to be more complicated (Fig. 4B). Recent reports (Brehm *et al.,* 1998; Magnaghi-Jaulin *et al.,* 1998) suggest that gene repression by pRb also involves modulation of the architecture of chromatin. The proposed mechanism rests on the finding that histone deacetylase HDAC1 physically interacts with pRb through the pocket domain and recruits HDAC1 to E2F. The complex of these three proteins binds to E2F target promoters. HDAC1 may then facilitate the removal of highly charged acetyl groups from core histones, leading to a tight association between the nucleosomes (Serrano *et al.,* 1993), which prevents the access of transcription factors to their cognate elements in the gene promoters.

Mitogenic signals that stimulate cell progress through G_1 coincide with increased expression of cyclins D. This extracellular regulation of cyclin D isoforms is unique and is not observed with other cyclin proteins. The current classical scenario is that cyclin D then forms a complex with Cdk 4 and/or Cdk 6, and its activity is regulated by the mechanisms described previously. The activated cyclin D–Cdk4/6 complexes then phosphorylate Rb and release E2F or the chromatin configuration constraints described above, leading to G_1 progression. The cyclin D complex alone does not dictate control of progression through G_1. Cyclin E is another G_1 cyclin

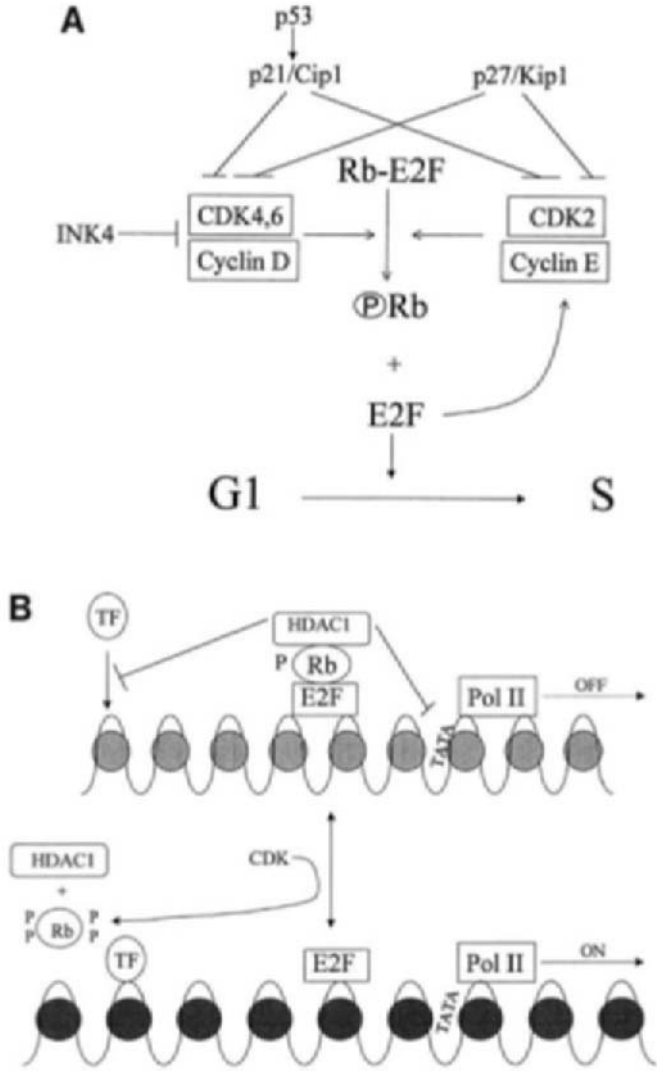

FIG. 4 (A) Regulation of the G_1/S traverse. The ability of Rb to control the G_1/S transition is mediated largely through its interaction with E2F. This interaction is controlled by the phosphorylation status of Rb, which in turn is affected by cyclin D–Cdk4,6 as well as cyclin E–Cdk2. p21/Cip1, p27/Kip1, and INK4 proteins act as inhibitors of these cyclin–Cdk complexes. (B) The role of Rb and histone deacetylation in cell cycle control. Histone deacetylase HDAC1 complexes with Rb and E2F and this complex binds to E2F target promoters, preventing gene expression. HDAC1 also may facilitate the removal of highly charged acetyl groups from core histones (darkly shaded, acetylated histones), preventing transcription factors (TF) from gaining access to the DNA. Activated Cdks phosphorylate pRb, thus releasing HDAC1 (reprinted by permission from *Nature.* DePinho, R. A. (1998). The cancer–chromatin connection. *Nature* **391,** 533–536, copyright 1998 Macmillan Magazines Ltd).

which is synthesized later in the cell cycle than cyclin D, peaking at the late G_1/S phase boundary. The expression of cyclin E is mitogen independent, and cyclin E forms an active complex with Cdk2. One level of regulation for the cyclin E–Cdk2 complex is through protein phosphatases Cdc25A and -B, which cleave the phosphate groups on the threonine 14 and tyrosine 15 residues of Cdk2 and activate the Cdk2–cyclin E complex (Hoffmann *et al.,* 1994). Cdc25A and -B activity is in turn regulated by phosphorylation. Levels of c-myc, raf, p21 ras, and cyclins have been shown to regulate the phosphorylation and therefore the activity of Cdc25A and -B (Fig. 5) (Hoffmann et al., 1993; Galaktionov et al., 1995). Cyclin D–Cdk4 and cyclin D–Cdk6 complexes are believed to trigger Rb phosphorylation, but the cyclin E–Cdk2 complex can also contribute to the phosphorylation of Rb in late G_1, leading to cell entry into the S phase.

Although the cyclin D–Cdk4/6 and cyclin E–Cdk2 complexes control entry into the S phase, these complexes are in turn controlled by families of G_1/S regulatory polypeptides, the CDKIs (Hunter and Pines, 1994). The cyclin D-, E-, and A-dependent kinases are negatively regulated by a family of CDKIs that consists of p21/Cip1, p27/Kip1, and p57/Kip2. Although all three of these inhibitors block progression through the G_1 phase, each is activated by different stimuli. The expression of p21/Cip1 is under the transcriptional control of *p53* tumor suppressor gene, activated by DNA damage (el-Deiry *et al.,* 1993). The increase in p21/Cip1 leads to the inhibition of cyclin E–Cdk2 activity, which contributes to the G_1 arrest. A second mechanism of p21/Cip1 may be related to its ability to bind to proliferating cell nuclear antigen (PCNA), a molecule involved in DNA replication and repair (Waga *et al.,* 1994). Like p21/Cip1, p27/Kip1 inhibits the activity of

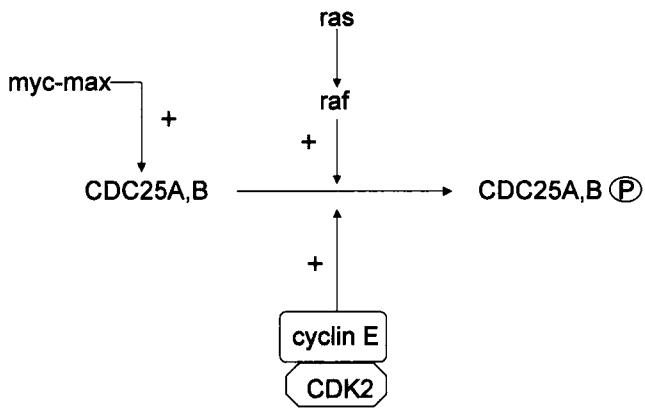

FIG. 5 Regulation of Cdc25A and -B. C-myc, ras/raf, and cyclin E regulate the phosphorylation status and the activity of Cdc25A and -B.

the G_1/S cyclin–Cdk complexes. For instance, p27/Kip1 participates in G_1 arrest produced by the exposure of fibroblasts derived from mink lung to the TGF-β and by cell–cell contact (Polyak *et al.*, 1994). In actively dividing cells, p27 is phosphorylated and its abundance is regulated by ubiquitin-mediated degradation. The inducers of p57/Kip2 are unknown.

Another family of regulatory peptides are the INK4 proteins, which include p16(INK4A), p15(INK4B), p18(INK4C), and p19(INK4D). These proteins specifically block cyclin D–Cdk4/6 activity leading to a G_1 phase arrest (Serrano *et al.*, 1993). The INK4 proteins inhibit Cdk4 and -6 by preventing the binding of cyclin D, but they also inhibit the activation of the formed Cdk4/6–cyclin D complexes. p15(INK4B) has been shown to be regulated by TGF-β and the treatment of human keratinocytes with TGF-β results in an increase in p15(INK4B) expression and its association with Cdk4 and -6 (Hannon and Beach, 1994).

b. The G_2 and M Phase Transitions Once the cell has faithfully replicated its genome, the next cellular function is to segregate this DNA into equivalent, or nearly equivalent, daughter cells. The central regulation for the transition from G_2 to mitosis is by the cyclin B–Cdc2 complex initially called maturation (mitosis) promoting factor (MPF) (Masui and Markert, 1971). In general, the activity of this complex is governed by similar factors responsible for the G_1/S transition, including CDK–cyclin association and activating phosphorylation by CAK. Until recently, the CDKIs were not known to play a major role in the control of G_2/M traverse, but studies in S. Reed's laboratory indicate that the situation is more complex than previously believed (Dulic *et al.*, 1998; Niculescu *et al.*, 1998).

Regulation of the Cdc2–cyclin B complex includes the G_2/M-specific phosphatase/kinase cell cycle regulatory proteins Cdc25C and wee-1. Cdc25C is a protein phosphatase which cleaves the inhibitory phosphate group at both tyrosine 15 and threonine 14 on Cdc2 (Sadhu *et al.*, 1990; Strausfeld *et al.*, 1994). Cdc25C requires phosphorylation to be activated and recent data support the notion that Cdc25C is phosphorylated and activated by the cyclin B–Cdc2 complex, thus forming a positive feedback loop (Hoffmann *et al.*, 1993) (Fig. 6). On the other hand, wee-1 is a protein kinase that phosphorylates these same sites and thus acts as an inhibitor of progression into mitosis (Parker *et al.*, 1995).

The Src-like protein kinase $p53/56^{lyn}$ may also be an important regulator of the G_2/M traverse. Originally described as a membrane-associated protein, recent data demonstrate $p53/56^{lyn}$ in the cell nucleus (Kharbanda *et al.*, 1996). Importantly, $p53/56^{lyn}$ physically associates with p34/Cdc2, acting as an inhibitory protein tyrosine kinase, phosphorylating tyrosine 15 much like the wee-1 kinase. Evidence comes from experiments linking a G_2/M block (induced by either ionizing radiation or chemotherapeutic agents)

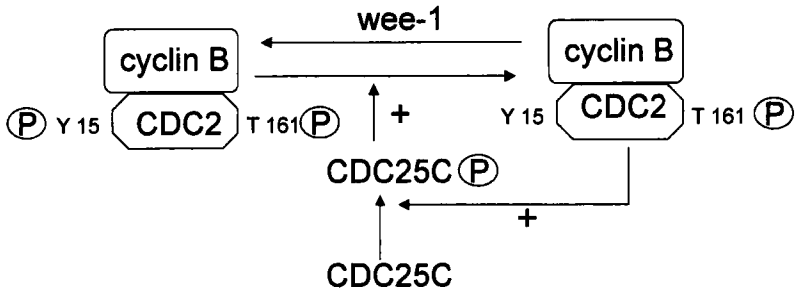

FIG. 6 Regulation of Cdc25C through activating phosphorylation. Cdc25C is a protein phosphatase which cleaves the inhibitory phosphate groups at both tyrosine 15 and threonine 14 on Cdc2. Cdc25C requires phosphorylation to be activated and recent data indicate that Cdc25C is phosphorylated and activated by the cyclin B–Cdc2 complex, thus forming a positive feedback loop. On the other hand, wee-1 is a protein kinase that phosphorylates these same sites on Cdc2 and thus acts as an inhibitor of G_2/M traverse.

with increased protein levels of $p53/56^{lyn}$, increased activation of $p53/56^{lyn}$, and decreased kinase activity of p34/Cdc2 (Kharbanda *et al.*, 1994a,b, 1996).

As with the G_1/S Cdks and cyclins, the level of Cdc2 is constant throughout the cell cycle, but levels of cyclin B are periodic, with the maximum level occurring just before the onset of mitosis. The ubiquitination pathway may play a pivotal role in cell cycle progression by degrading cyclin B at the beginning of anaphase, thus signaling the start of cytokinesis (Fig. 7).

The ubiquitination which activates the cyclin destruction machinery is controlled by the anaphase-promoting complex (APC) and is subject to a cell division checkpoint that is currently being elucidated in yeast and mammalian cells (King *et al.*, 1996; Elledge, 1998). It appears that the kinetochore binds a complex of proteins that includes members of the BUB ("budding uninhibited by benzimidazole") and MAD ("mitosis arrest deficient") families. When the chromosome is not properly aligned on the mitotic spindle, this complex of proteins inhibits the activity of an activator of APC, the Cdc20 protein in yeast, to initiate the destruction of cyclins. Defective functioning of this checkpoint can therefore lead to an unequal distribution of the chromosome complement at mitosis, resulting in aneuploidy.

B. Variations in the Mechanisms of Cell Cycle Control

The basic mechanisms regulating cell cycle traverse described above represent a reductionist consensus of a large number of studies that offers a useful initial approach to investigations of the cell cycle. Nonetheless, it

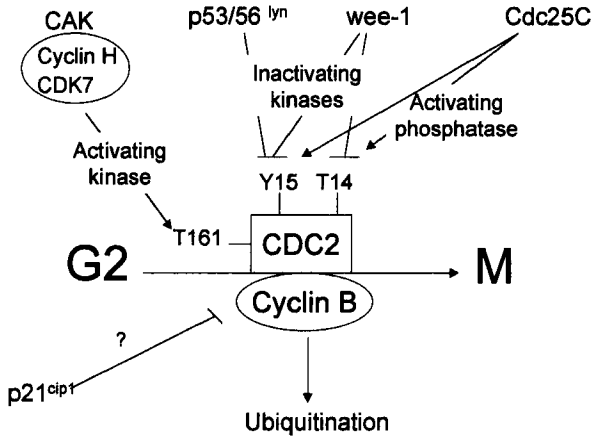

FIG. 7 Regulation of the G_2/M traverse. The central regulation of the transition from G_2 to mitosis is by the cyclin B–Cdc2 complex. In general, the activity of this complex is governed by factors similar to those responsible for the G_1/S transition, including Cdk–cyclin association and activating phosphorylation by CAK or a functionally equivalent kinase. Regulation of the Cdc2–cyclin B complex includes the G_2/M-specific kinase/phosphatase cell cycle regulatory proteins Cdc25C (phosphatase), wee-1, and $p53/56^{lyn}$ (kinases). It has been suggested that p21/Cip1 also plays a role in the G_2/M transition (Niculescu *et al.*, 1998).

is becoming increasingly clear that there are a number of variables that the simplified picture does not take into account. For instance, cell type-specific variations are frequently noted, and important cell cycle regulators appear to have functions not clearly related to the cell cycle progression. Several such examples are discussed here. Interestingly, these deviations from the established paradigm are often found in differentiating cells.

1. Cell Cycle-Independent Expression of Cdks and Cyclins

It has recently been reported that certain members of the Cdk family of kinases have functions unrelated to cell cycle regulation. Suggested alternative activities include roles in metabolic regulation (Kaffman *et al.*, 1994), apoptosis (Lahti *et al.*, 1995; Shi *et al.*, 1994; Zhang *et al.*, 1997), and basal transcription (Roy *et al.*, 1994; Rickert *et al.*, 1996). Active Cdk5 in particular has been found in several different types of terminally differentiated cells, including the central nervous system neurons (Nikolic *et al.*, 1996; Guidato *et al.*, 1998); rat oligodendrocytes (Tang *et al.*, 1998); the differentiating fiber cells of the embryonic lens (Gao *et al.*, 1995; He *et al.*, 1998), in which active Cdc2 has also been found (Gao *et al.*, 1997); human myeloblastic leukemia ML-1 cells (Li *et al.*, 1997b); and adult mouse muscle

(Lazaro *et al.,* 1997). Thus, it seems reasonable that Cdk5 plays a role in cell differentiation, particularly in neurons and in myoblasts, even though it interacts with at least two members of the cyclin D family (cyclin D1 and cyclin D2) and may be activated by these interactions (Li *et al.,* 1997b; Guidato *et al.,* 1998), just as Cdk4 and Cdk6 are activated by cyclins D but have been linked to cell cycle progression in numerous studies (Sherr, 1996).

Significant activity of two other Cdks has been reported in circumstances that appear unrelated to the cell cycle progression. Dobashi *et al.* (1996) described persistent activation of Cdk4 during neuronal differentiation of rat pheochromocytoma PC12 cells, and Cdk4 kinase activity was found to be increased during 1,25-D_3-induced monocytic differentiation of human leukemia cells HL60 (Q. Wang *et al.,* 1997). Cdk4 is also expressed in postmitotic lens fiber cells (Fromm and Overbeck, 1996) and in differentiating enterocytes in intestinal villi (Chandrasekaran *et al.,* 1996). Another cyclin-dependent kinase, Cdk1 (i.e., Cdc2), complexed with cyclin B1, is aberrantly expressed in degenerating neurons of Alzheimer's disease brains (Vincent *et al.,* 1997) and peak Cdc2–cyclin B activity correlates with changes in chromatin structure and nuclear envelope breakdown in lens fiber cells undergoing denucleation (He *et al.,* 1998).

Elevated levels of expression of cyclins D can also be found in nonproliferating and differentiating cells. In the examples discussed previously, increased Cdk4 levels and activity were usually accompanied by increases in D-type cyclins. In PC12 cells induced to differentiate by the addition of the nerve growth factor, cyclin D1 expression was increased (Yan and Zif, 1997; Van Grunsven *et al.,* 1996). Increased expression of cyclins D1 and D3 was also found in HL60 cells and in ML-1 human myeloblastic leukemia cells undergoing differentiation (Q. Wang *et al.,* 1997; Li *et al.,* 1997b), and increased expression of cyclin D3 was found during differentiation of myoblasts into quiescent myotubes (Kiess *et al.,* 1995). In the lens, cyclins D1 and D2 were found to be expressed in some postmitotic cells (Fromm and Overbeck, 1996). Upregulation of D-type cyclins followed the withdrawal of the differentiation inhibitory activity from mouse embryonic stem cells, a maneuver which initiates differentiation and leads to proliferative quiescence (Savatier *et al.,* 1996). These unexpected increases in cyclin D–Cdk4 complexes in differentiating cells may be explained by their actions on substrates other than pRB. Indeed, it was suggested that endogenous overexpression of cyclin D1 in lymphoma cells does not lead to pRb hyperphosphorylation (Zukerberg *et al.,* 1996), and ectopic expression of cyclin D1 in a murine epithelial cell line induces p27/Kip1 and can inhibit muscle gene expression even in the presence of a nonhyperphosphorylatable form of pRb (Skapek *et al.,* 1996). The possible targets of cyclins D include direct binding to the Rb protein (Kato *et al.,* 1993), to the estrogen receptor in cells of the breast (Neuman *et al.,* 1997; Zwijsen *et al.,* 1997), and to the

v-myb protein (Ganter *et al.,* 1998). In the latter case, the effects of cyclins D1 and D2 are independent of complex formation with a Cdk partner, implying that the role of D-type cyclins is not limited to the activation of Cdks, and that they may function as adaptor proteins that target kinase complexes to specific cellular regulators. Thus, one result of overexpression of cyclin D1 is G_1 arrest (Yan and Ziff, 1995).

The increased expression of D-type cyclins in differentiating cells appears to be either unique or near unique. Although an upregulation of cyclin G1 was reported to coincide with a loss of proliferative activity in rat Leydig cells during pubertal development (Ge and Hardy, 1997), and cyclin E-associated kinase activity increases in spontaneously differentiating normal osteoblasts (Smith *et al.,* 1997), roles unrelated to proliferation or involvement in differentiation per se have not been suggested for these cyclins. During differentiation cyclin E also becomes expressed as several proteins of different molecular weights (Q. Wang *et al.,* 1997). The different molecular weight cyclin E proteins noted in differentiating cells may be explained by alternative splicing of cyclin E mRNA (Mumberg *et al.,* 1997). Interestingly, the catalytic partner of cyclin E, Cdk2, can also be alternatively spliced, and the products have different catalytic activities at the G_1 transition into early S phase (Kwon *et al.,* 1998). Although the mechanisms responsible for the alternative splicing of cyclin E mRNA appear to be regulated by the cell cycle and/or differentiation, their nature and the significance of these phenomena are subjects for future investigations.

2. Additional Roles for Classical Effectors of the G_1 to S Phase Transition: The pRb and E2F Families of Proteins

The retinoblastoma susceptibility protein has been proposed to function as a molecular brake which restrains the cells' progress through the latter parts of the G_1 phase until all preparations for commitment to DNA replication, and subsequent cell division, are completed (Hatakeyama and Weinberg, 1995). This is accomplished by the variable level of phosphorylation of the pRb protein. Recently, however, the related "pocket proteins" p107 and p130 have been suggested to share in this responsibility, with a certain amount of specialization; p107 may control cell proliferation more directly, whereas p130 may regulate cell cycle exit and entry and pRb retains its central role in differentiation. It has been suggested that hypophosphorylated pRB functions to sustain, not to drive, the differentiated state (Yen and Varvayanis, 1994). Although the Rb protein is generally found in both growing and quiescent cells (Smith *et al.,* 1998), mitogen depletion can result in elevated levels of pRb (Tedesco *et al.,* 1995), and Rb mRNA increases during terminal differentiation of C2 myoblasts (Okuyama *et al.,* 1996). Paradoxically, variants of HL60 cells resistant to 1,25-D_3-induced

differentiation, which proliferate more rapidly than the parental cells, exhibit markedly increased levels (approximately 10-fold) of pRb (Wang *et al.,* 1998). Although the pRb is in the predominantly hyperphosphorylated state, this raises the question why these cells overproduce a negative cell cycle regulatory protein that then has to be inactivated by hyperphosphorylation. A possible answer is that pRb has functions which have a positive effect on cell growth when pRb is hyperphosphorylated. Association of hyperphosphorylated pRb with β-tubulin of mitotic spindles of human keratinocytes (Thomas *et al.,* 1996) is one example of novel activities of this protein. Furthermore, a cell cycle-regulated kinase capable of phosphorylating histone H1 associates with and phosphorylates in G_2/M phase the amino terminus of pRb, but not p107, and only minimally p130 (Sterner *et al.,* 1995, 1996). The importance of pRb for the regulation of G_2/M transition is also indicated by the finding that overproduction of pRb in cells that passed the G_1/S boundary results in a G_2 arrest (Karantza *et al.,* 1993). A model suggesting that pRb negatively regulates the promoter of the G_2 traverse-driving kinase, Cdc2, can provide a mechanism for this effect (Dalton, 1992). A role for pRb in suppressing apoptosis has also been suggested (Haas-Kogan *et al.,* 1995; J. Wang *et al.,* 1997) and may have direct involvement in differentiation. For instance, a number of laboratories (Gu *et al.,* 1993; Novitch *et al.,* 1996; Zacksenhaus *et al.,* 1996; Kobayashi *et al.,* 1998) have shown that pRb is required for differentiation of myoblasts, whereas Chen *et al.* (1996) reported that pRb positively regulates adipocyte differentiation by interacting directly with CCAAT/enhancer binding proteins.

Binding of pRb to E2F family members causes inhibition of the transcription of genes regulated by E2F transcription factors (Fattaey *et al.,* 1993). These genes are generally involved in DNA replication (e.g., the recently reported mammalian Cdc6 protein; Yan *et al.,* 1998). Some members of the E2F family also interact with p107 and p130 proteins (Beijersbergen *et al.,* 1994), and during differentiation the composition of these complexes can change (e.g., during the terminal differentiation of cardiac myocytes which switch their pocket protein partners; Flink *et al.,* 1998). The importance of E2F transcription factors in the control of differentiation is also illustrated by the observation that overexpression of E2F-1 in megakaryocytes blocked their maturation (Guy *et al.,* 1996). Two recent reports suggest that an autoregulatory loop in E2F function as a transcription factor may exist. Li *et al.* (1997a) noted that overexpression of p21/Cip 1 leads to inhibition of phosphorylation of E2F-1, and consequently its increased DNA binding, whereas Hiyama *et al.* (1998) reported transactivation of the *p21/Cip1* gene by E2F, though not by E2F4. The data of the latter group suggested that p21/Cip1 may function in cell cycle progression in addition to its well-known action in blocking the G_1/S phase transition.

3. Novel Functions of Cdk Inhibitors in the Cell Cycle Traverse

In addition to the previously mentioned study suggesting a positive participation of p21/Cip1 in the traverse of the cell cycle (Hiyama *et al.,* 1998), there are other indications that functions of proteins in the Cip/Kip family are not limited to the induction of G_1 arrest (Table II). Dulic *et al.* (1998) demonstrated an accumulation of p21/Cip1 protein in nuclei of fibroblasts entering mitosis and suggested that p21/Cip1 induces a transient pause in the late G_2 phase which may serve as a G_2 checkpoint control. In human breast carcinoma cells treated with genistein, Shao *et al.* (1998) reported induction of p21/Cip1 associated with G_2/M arrest, and Barboule *et al.* (1997) found an increase in p21/Cip1 following treatment with paclitaxel which was subsequent to mitotic arrest but was associated with the exit from abnormal mitosis, leading to formation of micronuclei. A recombinant adenovirus expressing p27/Kip1 also decreased cyclin B1–Cdc2 activity in human breast cancer cells (Craig *et al.,* 1997), and a functional interaction of p27/Kip1 with cyclin B–Cdc2 was reported during G_2/M progression in *Xenopus* oocytes (de Mora *et al.,* 1997). This finding was further explored, and recombinant p27/Kip1 in an *in vitro* system inhibited Cdc2 kinase activity, whereas a variant form of p27/Kip1, consisting of the carboxyl terminal of the molecule, enhanced Cdc2 kinase activity (Uren *et al.,* 1997). Conversely, the N-terminal form of p21/Cip1 was correlated with G_2/M arrest in TPA-treated U937 leukemia cells (Tchou *et al.,* 1996).

Although the original actions of Cdk inhibitors were described as an inhibition of Cdk enzymes, and in the case of p21/Cip1 as an interference of PCNA function, recent studies indicate additional mechanisms by which p21/Cip1 and p27/Kip can contribute to the proliferation arrest. For instance, p21/Cip1 can directly interact with E2F complexes and negatively influence the transcriptional activity of E2F (Afshari *et al.,* 1996). Similarly, p27/Kip1 appears to reduce the expression of the E2F-regulated genes by generating repressor complexes of E2F with p130/pRb2 (Shiyanov *et al.,* 1997). It is also worth noting that the functions of Cdk inhibitors are regulated by phosphorylations, which remain to be more fully explored.

V. Cell Cycle Control and Differentiation

A. The G_1/S Block

1. Involvement of the Cip/Kip Family of Cdk Inhibitors

Various exceptions notwithstanding, the principal block in the cell cycle of differentiating cells is evident in the G_1 phase. However, despite the numer-

TABLE II

Reported Effects of CDK Inhibitors on the Cell Cycle Traverse and Differentiation

Differentiation	Cell cycle	Comments	Reference
		Hematopoietic	
p21/CIP1			
U937/1,25-D_3 or retinoic acid	Transcriptional activation of p21	Increased markers of differentiation	Liu *et al.*, (1996a,b)
HL60/1,25-D_3	G_1 arrest and transient upregulation of p21	Increased markers of differentiation	Wang *et al.*, (1996)
Antisense to p21 in HL60 cells		Attenuated TPA-induced differentiation	Freemerman *et al.*, (1997)
Overexpression of p21		Increased cell surface markers of differentiation	Liu *et al.*, (1996c)
B lymphocytes to plasma cells	Upregulation of p21	Terminal differentiation	Morse *et al.*, (1997)
TPO-induced differentiation and ectopic expression of p21	Upregulation of p21	Megakaryocyte differentiation	Matsumura *et al.*, (1997)
U937/TPA	Upregulation of p21 and G_2/M arrest		Tchou *et al.*, (1996)
p27/KIP1			
HL60/1,25-D_3	G_1 arrest and upregulation of p27	Increased markers of differentiation	Q. Wang *et al.*, (1996, 1997)
U937/TPA	Increased p27/Kip1 levels	Increased markers of differentiation	Asiedu *et al.*, (1997)
Antisense to p27 in HL60 cells	Reduced G_1 block after 1,25-D_3	No effect on markers of differentiation	Wang *et al.*, (1998)
p16/INK4A			
Acute lymphoblastic leukemia	Increased expression of p16	Promotes differentiation	Urashima *et al.*, (1997)
p15/INK4B			
HL60 cells/TPA	Increased expression of p15	Monocyte differentiation	Schwaller *et al.*, (1997)
p18/INK4C			
Differentiation of B lymphocytes to plasma cells	Upregulation of p18	Terminal differentiation	Morse *et al.*, (1997)
p19/INK4D			
Murine myeloid cells	Induction of p19	Macrophage differentiation	Adachi *et al.*, (1997)

Keratinocytes			
p21/CIP1			
Primary keratinocytes derived from p21 knockout mice	Increased proliferation	Decreased modulation of differentiation markers	Missero *et al.*, (1996)
Keratinizing form of keratinocytes	Increased p21 transcription	Increased differentiation	Arany *et al.*, (1996)
Overexpression of p21	Growth arrest correlated with increased p21	No induction of markers of differentiation	Harvat *et al.*, (1998)
p27/KIP1			
Antisense for p27	Did not prevent growth arrest	Inhibited differentiation	Hauser *et al.*, (1997)
Intestine			
p21/CIP1			
$CaCo_2$ at confluence	p21 levels increased	Increased differentiation	Gartel *et al.*, (1996)
Nerve			
p21/CIP1			
Inducible p21 in PC12 cells	Permanent growth arrest	No induction of differentiation	Erhardt and Pittman (1998)
Glioma cells	G_1 block	Increased differentiation	Kokunai *et al.*, (1998)
p27/KIP1			
Glial cells	Growth arrest	No change in differentiation	Tikoo *et al.*, (1997), Bonnefile *et al.*, (1997), Durand *et al.*, (1997)
Muscle			
p21/CIP1			
Ectopic expression in rhabdomyosarcoma	Growth arrest	Markers of differentiation not detected	Knudsen *et al.*, (1998)
C2C12/ectopic MyoD expression	Upregulation of p21 and growth arrest	Increased differentiation	Puri *et al.*, (1997a,b)
p27/KIP1			
Ectopic expression in rhabdomyosarcoma	Growth arrest	Markers of differentiation not detected	Knudsen *et al.*, (1998)
p16/INK4A			
Ectopic expression in rhabdomyosarcoma	Growth arrest	Markers of differentiation not detected	Knudsen *et al.*, (1998)
p18/INK4AC			
C2C12 myoblast cells	50-fold increase in p18 protein levels	Increased differentiation	Franklin and Xiong (1996)

ous systems studied, there are few, if any, instances in which the mechanisms for the G_1 arrest are fully understood. Considerable excitement was generated when a downstream target of p53 tumor suppressor protein, the Cdk inhibitor p21/Cip1, was found to be upregulated in a number of differentiation systems (Steinman *et al.,* 1994; Jiang *et al.,* 1994) as well as in DNA damage-induced growth arrest and in senescence (Ponten *et al.,* 1995; McConnell *et al.,* 1998). It was suggested that p21/Cip1 and/or p27/Kip1 not only promote the G_1 arrest but also contribute to differentiation (Parker *et al.,* 1995). It appears, however, that these Cdk inhibitors are not solely responsible for the G_1 block, and data regarding their role in differentiation are conflicting. For instance, mice lacking p21/Cip1 undergo normal development (Deng *et al.,* 1995), though $p21^{-/-}$ embryonic fibroblasts show impaired arrest in G_1 in response to DNA damage. An imbalance between growth and differentiation can be demonstrated in these cells and in other *in vitro* cell differentiation systems with p21/Cip1 knockouts. Keratinocytes which are $p21/Cip1^{-/-}$, and to a lesser extent those with p27/Kip1 knockouts, have increased proliferative potential (Missero *et al.,* 1996). Late stages of differentiation are also affected in p21/Cip1-deficient but not in p27/Kip1-deficient mouse keratinocytes. The involvement of p21/Cip1 in what was suggested to be the terminal phase of differentiation was indicated by a report that induction of differentiation in keratinizing, but not in nonkeratinizing keratinocytes, increases p21/Cip1 transcription (Arany *et al.,* 1996). Kallassy *et al.* (1998) found that retrovirus-mediated expression of p21/Cip1 inhibited the growth of immortalized human normal keratinocytes and of ras-transformed keratinocytes, and Harvat *et al.* (1998) reported that irreversible growth arrest of normal human epidermal keratinocytes correlates with increased levels of p21/Cip1, p27/Kip1, and p16(INK 4A). However, they also showed that growth arrest resulting from overexpression of these Cdk inhibitors, using a recombinant adenovirus, is not sufficient to induce the expression of markers of differentiation, whereas Di Cunto *et al.* (1998) inhibited the expression of markers of teminal differentiation by forced expression of p21/Cip1 in mouse primary keratinocytes. On the other hand, Hauser *et al.* (1997) reported that in primary mouse keratinocytes induced to differentiate by placing them into suspension culture, upregulation of p27/Kip1 is linked to the differentiation program rather than to growth arrest. More surprisingly in the context of previous findings, terminal differentiation is induced by stable transfection with RAR-β of squamous cell carcinoma cell lines which have no detectable expression of Cdk inhibitors (Crowe, 1998).

In other cell systems there is an established role for the Cip/Kip family of inhibitors in the G_1 block, but participation in the induction of differentiation is more uncertain. In differentiation of cells of intestinal origin, p21/Cip1 was detected in cells that ceased to proliferate and began to differenti-

ate (Gartel *et al.,* 1996). With regard to nervous tissue cells, Poluha *et al.* (1997) suggested that p21/Cip1 is required for neuronal differentiation of PC12 cells in a novel pathway that involves nitric oxide, whereas Erhardt and Pittman (1998) transfected an inducible p21/Cip1 vector into PC12 cells and found permanent growth arrest but no direct induction of the differentiated phenotype. However, the differentiation response to nerve growth factor was greatly accelerated, and Kokunai *et al.* (1998) reported that overexpression of p21/Cip1 in human glioma cells resulted in G_1 block and changes suggesting differentiation. Similarly, p27/Kip1 was found to be more strictly linked to growth arrest than to differentiation in glial cells and oligodendrocyte precursors (Tikoo *et al.,* 1997; Casaccia-Bonnefil *et al.,* 1997; Durand *et al.,* 1997). Using another system, Onishi and Hruska (1997) reached a similar conclusion following induction of differentiation with parathyroid hormone in osteoblast-like cells.

Both p21/Cip1 and p27/Kip1 are involved in myogenesis. During skeletal muscle differentiation, MyoD, one of the master regulatory factors, induces expression of p21/Cip1 (Halevy *et al.,* 1995), which leads to permanent cell cycle arrest (Puri *et al.,* 1997a,b,c). p27/Kip1 was found to be expressed in a transient wave in developing mouse embryo myotomes, followed by the expression of p18(INKC), p21/Cip1, and p57/Kip2 in the postmitotic state (Zabludoff *et al.,* 1998). However, ectopic expression of p21/Cip1, p27/Kip1, or p16(INK4A) in rhabdomyosarcoma cells, a tumor of skeletal muscle origin, resulted in growth arrest of these cells though expression of differentiation markers was not detected (Knudsen *et al.,* 1998). Differentiation of leukemic cells is also known to be accompanied by increased levels of Cdk inhibitors. Early reports indicated that an exposure of HL60 human leukemia cells to TPA or to other inducers of differentiation upregulates the expression of p21/Cip1 by a p53-independent pathway and takes place as an "immediate early" response that can be uncoupled from the G_1 arrest (Jiang *et al.,* 1994; Steinman *et al.,* 1994). On the other hand, Mercer's group found that there is an inducer-specific response in human leukemia cells. Some inducers, such as TPA, okadaic acid, or IFN-γ, do induce expression of p21/Cip1, which correlates with both growth arrest and monocytic differentiation, whereas other inducers, e.g., RA, 1,25-D_3, or DMSO, do not (Zhang *et al.,* 1995).

There also appears to be cell specificity among leukemic cell lines with regard to the Cdk inhibitor, which is upregulated by a given inducer. Freedman's laboratory associated monocytic differentiation in U937 cells induced by 1,25-D_3 or by RA with transcriptional activation of p21/Cip1 (Liu *et al.,* 1996a,b), but upregulation of p21/Cip1 in HL60 cells in response to 1,25-D_3 appeared to be transient and not temporally related to a G_1 arrest (Wang *et al.,* 1996). Instead, an increase in the protein levels of p27/Kip1 was found in HL60 cells (Wang *et al.,* 1996; 1997), and it did not appear

to be transcriptional (Q. Wang and G. Studzinski, unpublished data). p27/Kip1 was also found to be increased in TPA-treated U937 cells (Asiedu *et al.,* 1997). In murine myeloid cells 32Dc13, induction of p19(INK4D) is associated with macrophage differentiation (Adachi *et al.,* 1997).

The role of Cip/Kip proteins in leukemia cell differentiation is still ill defined. Antisense expression to p21/Cip1 attenuated TPA-induced monocytic differentiation in HL60 cells (Freemerman *et al.,* 1997), whereas transient overexpression of p21/Cip1 in U937 cells resulted in cell surface expression of some monocytic markers (Liu *et al.,* 1996a). However, there are no data showing unequivocally that any one of the previously discussed Cdk inhibitors directly controls a differentiation-related gene, and increased levels of p27/Kip1 have been demonstrated in 1,25-D_3-resistant sublines which do not differentiate (Wang *et al.,* 1998). Furthermore, transfection of HL60 cells with p27/Kip1 antisense oligonucleotides or an expression vector with p27/Kip1 in an antisense orientation reduced the G_1 block induced by 1,25-D_3 but had no effect on the expression of differentiation markers (Wang *et al.,* 1998). Thus, the role of Cip/Kip proteins in leukemia cell differentiation, and perhaps in keratinocyte differentiation (Di Cunto *et al.,* 1998), may simply be to slow down the G_1 to S phase transition and thus facilitate mechanisms (unknown) which upregulate the expression of the differentiation programs.

The third member of the Cip/Kip family, p57/Kip2, has been implicated as a participant in differentiation systems much less frequently. Delayed cell cycle exit during chondrocyte development has been reported in $p57^{-/-}$ mice, along with complex developmental defects (Y. Yan *et al.,* 1997).

2. The INK4 Family

The Cdk inhibitors with specificity for G_1-associated cyclin–Cdk complexes, in particular Cdk4 and Cdk6, are also upregulated in some differentiation systems. These smaller proteins, p15(INK4B), p16(INK4A), p18(INKC), and p19(INKD), are characterized structurally by ankyrin repeat motifs and, along with the differentially spliced variant of the *p16* gene, *p19ARF,* are candidate suppressor genes. Several members of this group are suspected to have a major role in human cancer (Quelle *et al.,* 1995; Serrano *et al.,* 1996). This growth-suppressive effect appears to be transduced principally by the pRb pathway. p19ARF binds to MDM2 protein and accelerates its degradation (Zhang *et al.,* 1998; Pomerantz *et al.,* 1998), thus promoting stabilization and accumulation of p53, which can induce a G_1 arrest (Levine, 1997).

It was previously noted that overexpression of p16(INK4A) or of p21/Cip1 and p27/Kip1 is insufficient to induce squamous or myogenic differentiation (Harvat *et al.,* 1998; Knudsen *et al.,* 1998). However, p16(INK4A)

does appear to promote differentiation in some cell types (e.g., in cells of human acute lymphoblastic leukemia; Urashima *et al.,* 1997) and has been linked to replicative quiescence during differentiation of the embryonic teratocarcinoma cell line NT2 into postmitotic neurons (Lois *et al.,* 1995). Lois *et al.,* also noted that the expression of p15(INK4B) was downregulated during lymphocyte activation, whereas Schwaller *et al.* (1997) observed induction of p15(INK4B) in HL60 cells induced to monocytic differentiation by TPA. Surprisingly, the TPA-induced upregulation of p15(INK4B) was accompanied by a downregulation of p18(INK4C) and p19(INK4D), whereas granulocytic differentiation by DMSO was accompanied by increased expression of p18(INK4C) and p19(INK4D). This disparate regulation of the INK genes occurred at the posttranscriptional level. On the other hand, in mouse myeloid cells 32Dc13-induced expression of p19(INK4D) facilitated the appearance of the macrophage phenotype (Adachi *et al.,* 1997), and in terminal differentiation of B lymphocytes to plasma cells p18(INK4C), as well as p21/Cip1, were upregulated (Morse *et al.,* 1997).

A special role for p18(INK4) in myogenic differentiation has been suggested by studies of Xiong and colleagues. During induced myogenesis of C2C12 myoblast cells several Cdk inhibitors increase transiently or gradually, but p18(INK4C) protein levels increase most dramatically, showing a 50-fold increase. Interestingly, there was sequential formation of complexes of p18(INK4C) with Cdks—first with Cdk6 and later with Cdk4 (Franklin and Xiong, 1996). More detailed analysis of this system showed that the *p18(INK4C)* gene expresses two mRNA transcripts. As the myoblasts differentiate into myotubules the abundance of the larger (2.4 kb) transcript decreases, and the levels of the smaller (1.2 kb) transcript, expressed from a downstream promoter, increase and this transcript becomes the predominant form in the myotubes. The 1.2-kb mRNA is more efficiently translated, possibly because of the absence of the long 5′ UTR in exon 1, which attenuates translation of the larger transcript (Phelps *et al.,* 1998). This provides an interesting glimpse into the mechanisms through which a differentiation program alters promoter switching and the translational control of the abundance of a cell cycle regulatory protein.

3. Additional Controls of the G_1 Phase Traverse

The overwhelming attention accorded to the Cdk inhibitors as factors that force the differentiating cell to interrupt its cell cycle traverse should not lead to the conclusion that these are the only regulators of G_1 transition. The obvious factors are the G_1 cyclins and the Cdks, but there are no clear indications that these, or other factors, have important roles in differentiation-related G_1 arrest. Decreases in the levels of Cdks have been reported; for instance, in murine erythroleukemia cells treated with hexamethylene

bisacetamide, a polar–planar compound, erythroid differentiation was accompanied by modulation of a number of cell cycle components including a downregulation of Cdk4 (Rifkind *et al.,* 1996), and in human promyelocytic leukemia HL60 cells monocytic differentiation was associated with reduced protein levels of both Cdk4 and Cdk6 (Wang *et al.,* 1998). It seems likely that Cdk inhibitors act in concert with other factors whose presence or activity is altered during differentiation, and the identification of these factors may provide additional clues to the molecular basis of differentiation.

B. The S Phase

The effects of differentiation-associated processes on DNA replication are generally thought to be indirect, resulting from the inability of the differentiating cell to exit the G_1 or, in some cases, the G_2 phase. Experimental manipulations which abrogate the natural barriers in the late G_1 to a cell's progression into S phase result in cell cycle abnormalities, such as a prolongation of the S phase. For instance, induced expression of p27/Kip1 antisense in HL60 cells induced to differentiate with 1,25-D_3 results in a reduced G_1 block but causes increased duration of the S phase (Wang *et al.,* 1998). Similarly, skeletal muscle cells which lack pRb accumulate in S and G_2 phases and when induced to enter mitosis by administration of caffeine undergo "mitotic catastrophe" (Novitch *et al.,* 1996). It seems likely that mechanisms which prevent entry of a cell into the S phase include deficiencies in, but not the total absence of, DNA replication machinery and associated chromatin components, including histones. The obvious participants in these events, complexes of Cdk2 with cyclin A, have been studied in some differentiation systems. For instance, during HMBA-induced differentiation of human embryonal carcinoma cell line NEC14 the levels of cyclin A transcripts decrease sharply, and the ATF/CRE and GC box sites in its promoter appear to be responsible (Nakamura *et al.,* 1995). In HMBA-induced murine erythroleukemia cell differentiation, persistent suppression of cyclin A expression and reduced histone H1 kinase activity have also been reported (Kiyokawa *et al.,* 1992). Cyclin A levels are also reduced in U937 and HL60 cells induced to macrophage differentiation by TPA but, interestingly, not in granulocytic differentiation of HL60 cells induced by DMSO (Horiguchi-Yamada *et al.,* 1994; Burger *et al.,* 1994; Asiedu *et al.,* 1997). In differentiating C2C12 myocytes cyclin A, as well as cyclins B and D1, is downregulated to undetectable levels (Jahn *et al.,* 1994; Wang and Nadal-Ginard, 1995). Similarly, during terminal differentiation of embryonic chicken lens cyclin A mRNA and protein are detected in the proliferating but not the differentiating cells (He *et al.,* 1998). Conversely, in the

developing eye of *Drosophila melanogaster,* forced expression of cyclin A protein drives cells into the S phase (Dong *et al.,* 1997). The potential targets of cyclin A–Cdk2 complex include the 34-kDa subunit of replication protein A (Cardoso *et al.,* 1993) and perhaps histones.

In proliferating cells transcription of most of the histone genes (the "main type histones") is upregulated at the onset of S phase, whereas the "replacement histones" can be synthesized in the absence of DNA replication. Such variant forms include the histone *H3.3* genes, which encode histone proteins during male germ cell differentiation (Bramlage *et al.,* 1997). It was recently shown that the *H3.3 B* gene promoter binds Oct-1, CREB/ATF, and AP-1 transcription factors (Witt *et al.,* 1997), thus showing one of the links between the differentiation and the general transcription control machineries. Control of the expression of the replication-linked histones H4, H3, H2A, H2B, and H1, which is activated at the G_1/S transition, can be exerted by a specific histone gene transcription factor complex *HiNF-D* (van Wijnen *et al.,* 1996) as well as by general transcription factors such as E2F, which binds specifically to a recognition motif in the H2A-1 promoter region (Oswald *et al.,* 1996). These and numerous similar studies have led to the generalization that transcriptional control of cell cycle progression and histone gene expression provide an important paradigm for proliferation–differentiation transitions (Stein *et al.,* 1996).

A further link to differentiation-related transcription factors and replication is provided by the finding that ectopic expression of the erythroid-specific factor GATA-1 in fibroblasts results in the prolongation of their S phase (Dubart *et al.,* 1996). Thus, the onset of differentiation sets mechanisms in motion that not only produce a G_1/S block but also slow down progression through the S phase of cells not subjected to the G_1 block.

C. The G_2/M Block

The major kinase activity that promotes the onset of mitosis is MPF, or the Cdc2(Cdk1)–cyclin B complex. There are only a few reports that clearly relate regulation of MPF to differentiation; this is not surprising given that all protein kinases and phosphatases, which operate upstream, downstream, or in parallel with Cdc2, have not been identified in mammalian cells. It was noted by Novitch *et al.* (1996) that differentiated myocytes lacking pRb accumulate in S and G_2 phases and express high levels of the G_2 cyclins, cyclins B and A, as well as Cdc2 kinase but fail to proceed into mitosis. Removal of the inactivating phosphorylations on the Cdc2–cyclin B complexes by administration of caffeine induces a form of mitotic catastrophe in these myocytes, suggesting that differentiation may impose a G_2 block at the level of MPF. Along the same lines, MPF activity was noted by He

et al. (1998) in terminally differentiating primary lens fiber cells in the eye of newborn rats, which correlated with changes in chromatin structure and nuclear envelope breakdown that leads to enucleation. Furthermore Godyn *et al.* (1994) observed that in 1,25-D_3-induced monocytic differentiation of HL60 cells, a dual cell cycle block takes place; the well-known G_1 block was accompanied by a G_2 arrest (microscopic examination excluded a mitotic arrest) and the G_2 arrest was associated with the appearance of cells with multiple nuclei and higher ploidy levels (Studzinski *et al.*, 1996). Thus, differentiation imposes a G_2 block, probably by inactivating the MPF activity, which may be reversible, but forced exit from G_2 may lead to destruction of the nucleus or polyploidization.

D. Polyploidization

Several forms of developmental differentiation are accompanied by multinucleation and/or acquisition of a higher ploidy level. These include osteoclast differentiation, which is believed to be under the control of 1,25-D_3 (Baron *et al.*, 1993; Roodman, 1996), though this control may be indirect (David *et al.*, 1997). Generation of polypoid cells is also a normal developmental program in megakaryopoiesis, and cytokines such as thrombopoietin (TPO) have been reported to contribute to these processes (McDonald and Jackson, 1994).

Polyploidization during megakaryocytic differentiation has been studied in model systems using leukemia cell lines such as HEL, K562, and CHRF (Hudson *et al.*, 1996). The inducers of ploidy increase include TPA (Murate *et al.*, 1993), cytotoxic necrotizing factor which has a dominant action over hemin-induced erythroid differentiation of K562 cells (Denko *et al.*, 1997), and nocodazole (Kikuchi *et al.*, 1997). In HL60 cells, an increase in ploidy is induced by a long-term exposure to 1,25-D_3 (Studzinski *et al.*, 1996). In this system only one round of endomitosis takes place, which can be explained by an increased stringency of the G_1 block in the tetraploid cells (Zhang *et al.*, 1996).

The mechanism of polyploidization is believed to be endoreduplication, also known as endomitosis, whereby two or more rounds of DNA replication take place without an intervening mitosis, thus doubling, quadrupling, etc. the chromosome complement. There appear to be two components that are required for endoreduplication to occur; the activity of MPF must be inhibited, and the restraints on DNA rereplication need to be removed. Inhibition of MPF may be due to several causes; one is a marked reduction in the Cdc2 protein level, which occurs during TPA-induced megakaryocytic differentiation of HEL cells resulting in low Cdc2 kinase activity. Surprisingly, these cells have an elevated expression of cyclin B1, which translocates

to the nucleus (Datta *et al.*, 1996). Somewhat similar results were obtained when protein kinase inhibitor K252a was used in place of TPA in this system (Yokoe, 1997). Another factor important for endoreduplication which can inhibit MPF activity and has recently gained prominence in the literature (discussed previously) is the Cdk inhibitor p21/Cip1. It was noted by Waldman *et al.* (1996) that human cells with damaged DNA and no p21/Cip1 arrest in G_2 but then undergo endoreduplication of DNA, producing abnormal polyploid nuclei. This was followed by the observation that TPO-induced megakaryocytic differentiation of human leukemia CMK cells is accompanied by an immediate early upregulation of p21/Cip1, but not of p27/Kip1 or of the INK4 Cdk inhibitors. Ectopic expression of p21/Cip1 or p27/Kip1, but not of p16(INK4A), led to megakaryocytic differentiation of these cells. It was suggested that TPO induced activating tyrosine phosphorylation of the transcription factor STAT 5, which binds to the binding sites of the p21/Cip1 promoter (the p21-SIE sites), thereby transactivating the *p21* gene (Matsumura *et al.*, 1997). Similar results were reported by Kikuchi *et al.* (1997) using another human megakaryocytic leukemia cell line (UT-7). The latter group also suggested that the second condition necessary for DNA replication without an intervening mitosis, the lifting of the unknown restraint on DNA reduplication, is provided by a hypothetical megakaryocyte-specific licensing factor. Steven Reed's group suggested that pRb is a critical determinant for blocking DNA endoreduplication (Niculescu *et al.*, 1998). This suggestion was based on their experiments in which p21/Cip1 was ectopically expressed in a panel of cell lines, resulting in a general G_1 and G_2 arrest in cells with either intact or defective pRb, but in pRb-negative cell lines DNA endoreduplication also took place. While it is unlikely that current knowledge is sufficient to form a coherent picture of the mechanisms that lead to polyploidy, there is no doubt that this is an area of research that should lead to a better understanding of the aberrant differentiation that characterizes the aggressive, aneuploid/polyploid forms of cancer.

VI. Transcription Factors and Differentiation

A. General Transcription Factors

Transcription factors act as the nuclear effectors of developmental and environmental cues that alter gene readout and are therefore important components of the differentiation machinery. Directly or indirectly, transcription factors interact with the cell cycle control pathways, and reference has previously been made to the general, ubiquitous transcription factors

as they are known to impinge on the expression of genes involved in cell cycle progression, particularly the E2F family. The *jun/fos* families, which encode the components of the AP-1 transcription factor, and the Sp-1 factor have been shown to be involved in monocytic differentiation (Kolla and Studzinski, 1994; Rao *et al.,* 1998). Several other transcription factors have also been closely linked to the growth–differentiation interface, but the specifics of exactly how this transition is accomplished are still elusive.

B. The myc/max/mad Superfamily

One of the earliest candidates for the molecular switch (or rheostat) between proliferation and differentiation was the c-*myc* gene. The impetus for this was provided in part by the discovery that c-*myc* is rapidly downregulated by 1,25-D_3 induction of monocytic differentiation of HL60 cells (Reitsma *et al.,* 1983), which was elaborated upon in a number of other laboratories (Brelvi and Studzinski, 1986; Simpson *et al.,* 1987). The differentiation-related downregulation of c-*myc* can take place posttranscriptionally, as in RA-induced differentiation of F9 cells (Dean *et al.,* 1986), or during myogenesis (Wisdom and Lee, 1990).

The regulation and known actions of the c-*myc* transcription factor provide a clear rationale for its proliferation-promoting effects. For instance, in mouse 3T3 cells with ectopic expression of CSF-1 receptor, the expression of the c-*myc* gene is under the dual control of the growth/survival factors–regulated ets transcription factor as well as E2F-1 (Roussel *et al.,* 1994), which fits with the Cdk/Rb/E2F pathway, whereas in a panel of cancer cells exogenous expression of p21/Cip1 downregulated the c-myc protein (Blagosklonny *et al.,* 1997). The targets of c-*myc* include ornithine decarboxylase (ODC), a key enzyme in polyamine synthesis required for DNA replication (Nishiguchi *et al.,* 1993; Takeda *et al.,* 1991), and Cdc25A, a protein phosphatase essential for the G_1 to S phase transition (Jinno *et al.,* 1994; Galaktionov *et al.,* 1996). Cdc25A promotes this transition by dephosphorylation of the Cdk2–cyclin E complex (Hoffmann *et al.,* 1994) and by inhibition of the Cdk2–cyclin E complex by its association with p21/Cip1 (Saha *et al.,* 1997). C-myc has also been reported to activate cyclin E gene transcription and to inhibit p27/Kip1 association with Cdk2–cyclin E complexes (Perez-Roger *et al.,* 1997).

Recent reports generally confirm that inactivation of c-*myc* promotes differentiation, though it is not entirely clear if this is independent of its antiproliferative effect. On the one hand, constitutive expression of a member of the *myc* family, N-*myc,* blocked neuronal differentiation, and this was attributed to the inhibition of the exit from the cell cycle (Bogenmann *et al.,* 1995); on the other hand, both Selvakumaran *et al.* (1996) and Ryan

and Birnie (1997a) concluded that c-*myc* can block differentiation by a mechanism different than that used to promote the cell cycle progression or the expression of ODC. Furthermore, Gandarillas and Watt (1997) reported that c-*myc* indirectly promotes keratinocyte differentiation by driving keratinocyte stem cells into the transit amplifying compartment (Fig. 1).

The evidence regarding the role of another member of this family, *max*, indicates that it is the partner of both *myc* and *mad-1*, forming hetorodimers with c-*myc* that activate transcription, whereas mad-1 heterodimers with max repress transcription from the same promoters and produce a G_1 block (Roussel *et al.*, 1996). Mad-1 is a basic helix–loop–helix zipper protein which is induced in a number of differentiating cell types, including HL60 leukemia cells (X. Wang *et al.*, 1997), differentiating suprabasal skin keratinocytes (Lymboussaki *et al.*, 1996), and in the developing mouse in the postmitotic differentiated cells in the apices of colonic crypts (Chin *et al.*, 1995). Mad-1 is thought to function by antagonizing the proliferation-promoting actions of c-*myc* because exogneously expressed c-*myc* blocks inducer-mediated differentiation of MEL cells without interfering with expression of endogenous *mad-1* (Cultraro *et al.*, 1997). Although Ryan and Birnie (1997b) showed that HL60 cells could be induced to express some markers of differentiation without concomitant upregulation of *mad-1* mRNA, *mad-1*-containing complexes were detected in the later stages of differentiation. While this suggested that *mad-1* is important for the maintenance of the differentiated state and not its induction, recent work in Eisenman's laboratory has shown that different members of the MAD protein family are expressed during distinct steps of differentiation. Mad-3 transcripts and proteins are present in proliferating P19 neuronal cells and Maxi-1 and Mad-4 in cells in the middifferentiation pathway, whereas Mad-1 is primarily expressed in late differentiation (Queva *et al.*, 1998). It was also shown that Mad-1 regulates cell cycle withdrawal during granulocytic differentiation (Foley *et al.*, 1998).

C. MyoD

In differentiating myocytes withdrawal from the cell cycle and muscle-specific gene transcription are regulated by the basic helix–loop–helix protein MyoD (Yun and Wold, 1996) as well as by the pocket proteins. A downstream effector of MyoD is p21/Cip1, which upon activation of MyoD in C2C12 cells prevents the reassociation of the complex of Cdk2–cyclin A with E2F4, which normally follows serum stimulation (Puri *et al.*, 1997c, 1998). This may be responsible for the permanent cell cycle arrest in differentiating myocytes.

The Id (inhibitor of DNA binding and differentiation) proteins have the helix–loop–helix structure and regulate transcriptional activities of basic HLH transcription factors in a dominantly negative fashion. The Id proteins oppose the action of MyoD in myogenesis (Langlands *et al.,* 1997) but are also expressed in other cell types including leukemia (Ishiguro *et al.,* 1995, 1996).

D. p53

The tumor suppressor protein p53 serves multiple functions, including a role in cell differentiation. An interesting example is the induction of monocytic differentiation by exogenous expression of wild-type p53 in 32D leukemia cells transformed with either v-*src* or c-*jun* (Martinelli *et al.,* 1997). Although p53 is an upstream regulator of p21/Cip1, it is not certain if it has additional, directly differentiating effects.

VII. Proliferation–Differentiation Interfaces

A. Reversibility of Differentiation

The term "terminal differentiation" has often been used inappropriately. It is obvious that when the nucleus is degraded and/or extruded from the cell, as in erythroid (Morioka *et al.,* 1998) or lens fiber cell differentiation (He *et al.,* 1998), there is no possibility of reentry into the cell cycle. In other differentiation systems, cells with intact nuclei that are quiescent, but not senescent, can be shown to be capable of at least partial entry into the cell cycle. For instance, although forced expression of cell cycle regulatory genes, such as *E2F-1,* v-*myc* or *Id-1,* in terminally differentiated skeletal muscle myotubules does not result in DNA synthesis, serum stimulation of the myotubules produces a typical immediate early response, including the upregulation of c-*fos,* c-*jun,* c-*myc,* and *Id-1* genes. This is followed by an elevated expression of cyclin D1 after 4 h of serum treatment (Tiainen *et al.,* 1996a), consistent with a transition from G_0 to mid-G_1 phase of these cells. An interesting challenge will be to determine if manipulations other than the introduction of viral proteins such as the large T antingen (Ohkubo *et al.,* 1994) can force such cells further into the cell cycle.

Complete reversal of quiescence of fully mature, differentiated cells is not uncommon. Physiological maturation of macrophages does not involve truly terminal changes since these cells, like lymphocytes, are capable of reentry into the cell cycle after prolonged periods of quiescence (Hume

and Gordon, 1982; Stewart, 1984; Van Hal *et al.,* 1995). *In vitro,* 1,25-D_3-induced monocytic/macrophage differentiation of HL60 cells has also been shown to be reversible (Bar-Shavit *et al.,* 1986; Studzinski and Brelvi, 1987), as has proliferation of monocyte-derived macrophage cocultured with aortic endothelial cells (Antonov *et al.,* 1997), human hepatoma cells (Glaise *et al.,* 1998), and human melanoma cells (Jiang *et al.,* 1995).

Several mechanisms have been proposed for cell cycle reentry of differentiated cells. The absence of high levels of p21/Cip1 seems to be a permissive condition since in the examples discussed previously p21/Cip1 protein is not detected in 1,25-D_3-differentiated HL60 cells and actually disappeared during hepatoma cell differentiation, whereas the failure to irreversibly arrest the cell cycle of doxorubicin-treated myocytes was also attributed to lack of p21/Cip1 (Puri *et al.,* 1997c). Persistence of the expression of D cyclins is another possible factor allowing reversal of 1,25-D_3-HL60 cell differentiation (Q. Wang *et al.,* 1997), and cyclin E was found to be readily upregulated upon contact of aortic macrophages with the endothelial cells (Antonov *et al.,* 1997). Jiang *et al.* (1995) found a difference in the p130/pRb2/E2F complexes in reversibly compared to irreversibly differentiated melanoma cells, though the nature of this difference was not specified.

Viral proteins can also reactivate cell cycle progression. For instance, adenoviral protein E1A expressed in terminally differentiated muscle cells activates the expression of late G_1 genes, including cyclin E and Cdk2 (Tiainen *et al.,* 1996b). Also, the human papillomavirus E7 oncoprotein uncouples cellular differentiation from proliferation by allowing cellular DNA synthesis in differentiated keratinocytes (Jones *et al.,* 1997). Clearly, a precise definition of what constitutes terminal differentiation would benefit studies of differentiation.

B. Reciprocity of Proliferation and Differentiation

It has been frequently stated that cells need to withdraw from the cell cycle when they begin to differentiate (Zavitz and Zipursky, 1997), and it is sometimes suggested that proliferation arrest precedes differentiation and perhaps that it is needed to initiate differentiation (Tanaka *et al.,* 1997).

As pointed out earlier in this article, this is a popular misconception. For instance, hematopoietic cell differentiation in its early stages actually requires cell proliferation, as in the activation of the stem cells followed by passage through the amplification stage of differentiation (Fig. 1). *In vitro,* the many examples of concurrent differentiation and proliferation include 1,25-D_3-induced monocytic differentiation of HL60 cells (Zhang *et al.,* 1994) and simultaneous signaling of proliferation and differentiation in skeletal muscle cells by the insulin-like growth factor (Coolican *et al.,* 1997).

It would probably be more useful for reporting of experimental results if the phrase "terminal differentiation" was replaced by "late-stage differentiation" and distinguished from "early stages of differentiation." Some authors already make this distinction: Kaliman *et al.* (1996) reported that phosphotidylinositol 3-kinase is dispensable for myoblast proliferation or the initial events of differentiation but is essential for terminal differentiation. Also, Puri *et al.* (1997b) found that the formation of E2F4–p130/pRb2-containing complexes is an early event in myogenic differentiation, which does not interfere with DNA replication.

As differentiation procedes to its late stages, DNA replication becomes incompatible with differentiation, as recently discussed (Piette, 1997). The Steins' group has studied in detail the proliferation–differentiation transition using both normal diploid osteoblasts and osteosarcoma cells (Stein *et al.*, 1990; McCabe *et al.*, 1994). One of their seminal observations was that transcription factor occupancy of the AP-1 site contiguous with the vitamin D response element site in the promoter of the osteocalcin gene could determine if proliferation or differentiation takes place (Owen *et al.*, 1990; Bortell *et al.*, 1992). Other examples of DNA binding site competition, at contiguous or overlapping sites, were recently reported (Huang *et al.*, 1997; Hirano *et al.*, 1998; Lim *et al.*, 1998). This is an attractive concept that helps to explain the incompatibility of rapid proliferation with late-stage differentiation.

VIII. Conclusions

Rapid cell proliferation is not compatible with cell differentiation probably because of competition for scarce cellular resources, yet during the initial stages of cell and tissue specialization proliferation is necessary. Thus, the relationships between these processes are complex and not completely understood. The complexity is increased by the recent realization that growth signaling pathways, such as the MAP kinase pathway (e.g., Marcinkowska *et al.*, 1997; Camps *et al.*, 1998) and the survival regulating pathways (Wang and Studzinski, 1997), also interact with signals for differentiation.

The major control over differentiation decisions versus proliferation is generally believed to be exerted by the retinoblastoma susceptibility protein, which appears to integrate signals transmitted predominantly by the activated cyclin-dependent kinases (Bartek *et al.*, 1997). However, this paradigm merits critical scrutiny and may be strictly applicable to only some situations, with alternative controls governing proliferation–differentiation transitions in different cell types.

Thus, despite a plethora of data, the molecular basis for the differentiation-related changes in cell cycle traverse is incompletely understood. However, there are strong indications that this will not be the case for very long.

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References

Adachi, M., Roussel, M. F., Havenith, K., and Sherr, C. J. (1997). Features of macrophage differentiation induced by p19INK4d, a specific inhibitor of cyclin D-dependent kinases. *Blood* **90,** 126–137.

Afshari, C. A., Nichols, M. A., Xiong, Y., and Mudryj, M. (1996). A role for a p21–E2F interaction during senescence arrest of normal human fibroblasts. *Cell Growth Differ.* **7,** 979–988.

Antonov, A. S., Munn, D. H., Kolodgie, F. D., Virmani, R., and Gerrity, R. G. (1997). Aortic endothelial cells regulate proliferation of human monocytes in vitro via a mechanism synergistric with macrophage colony-stimulating factor. Convergence at the cyclin E/p27(Kip1) regulatory checkpoint. *J. Clin. Invest.* **99,** 2867–2876.

Arany, I., Brysk, M. M., Calhoun, K. H., and Tyring, S. K. (1996). Differentiation and IFN gamma regulate WAF1/CIP1 transcription in p53-independent and p53-dependent pathways in epithelial cells. *In Vivo* **10,** 119–123.

Asiedu, C., Biggs, J., Briggs, J., and Kraft, A. S. (1997). Complex regulation of CDK2 during phorbol ester-induced hematopoietic differentiation. *Blood* **90,** 3430–3437.

Barboule, N., Chadebech, P., Baldin, V., Vidal, S., and Valette, A. (1997). Involvement of p21 in mitotic exit after paclitaxel treatment in MCF-7 breast adenocarcinoma cell line. *Oncogene* **15,** 2867–2875.

Barnard, J., and Warwick, G. (1994). Butyrate rapidly induces growth inhibition and differentiation in HT-29 cells. *Cell Growth Differ.* **4,** 495–501.

Baron, R., Racesloot, J. H., Neff, L., Chakraborty, M., Chatterjee, D., Lomri, A., and Horne, W. C. (1993). *In* "Cellular & Molecular Biology of Bone." Academic Press, San Diego.

Bar-Shavit, Z., Kahn, A. J., Stone, K. R., Trial, J., Hilliard, T., Reitsma, P. H., and Teitelbaum, S. L. (1986). Reversibility of vitamin D-induced human leukemia cell-line maturation. *Endocrinology* **118,** 679–686.

Bartek, J., Bartkova, J., and Lukas, J. (1997). The retinoblastoma protein pathway in cell cycle control and cancer. *Exp. Cell Res.* **237,** 1–6.

Bartkova, J., Zemanova, M., and Bartek, J. (1996). Expression of CDK7/CAK in normal and tumor cells of diverse histogenesis, cell-cycle position and differentiation. *Int. J. Cancer* **66,** 732–737.

Bassnett, S., and Mataic, D. (1997). Chromatin degradation in differentiating fiber cells of the eye lens. *J. Cell Biol.* **137,** 37–49.

Beere, H., and Hickman, J. (1993). Differentiation: A suitable strategy for cancer chemotherapy? *Anti-Cancer Drug Design* **8,** 299–322.

Beijersbergen, R. L., Kerkhoven, R. M., Zhu, L., Carlee, L., Voorhoeve, P. M., and Bernards, R. (1994). E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. *Genes Dev.* **8,** 2680–2690.

Bennett, A. M., and Tonks, N. K. (1997). Regulation of distinct stages of skeletal muscle differentiation by mitogen-activated protein kinases. *Science* **278,** 1288–1291.

Bernstein, S., Sherman, M., Hass, R., Nakamura, T., Kharbanda, S., and Kufe, D. (1991). Effects of hexamethylene biscetamide on induction of monocytic differentiation of human U-937 myeloid leukemia cells. *Biochem. Pharmacol.* **42,** 403–407.

Bertolotto, C., Bille, K., Ortonne, J., and Ballotti, R. (1998). In B16 melanoma cells, the inhibition of melanogenesis by TPA results from PKC activation and diminution of microphthalmia binding to the M-box of the tyrosinase promoter. *Oncogene* **16,** 1665–1670.

Binderup, L., Binderup, E., and Godtredsen, W. O. (1997). Development of new vitamin D analogs. *In* "Vitamin D" (D. Feldman, Glorieux F. H., and Pike J. W., eds.), pp. 1027–1044. Academic Press, San Diego.

Blagosklonny, M. V., Prabhu, N. S., and El-Deiry, W. S. (1997). Defects in p21WAF1/CIP1, Rb, and c-myc signaling in phorbol ester-resistant cancer cells. *Cancer Res.* **57,** 320–325.

Blair, O. C., Carbone, R., and Sartorelli, A. C. (1985). Differentiation of HL60 promyelocytic leukemia cells monitored by flow cytometric measurement of nitro blue tetrazolium (NBT) reduction. *Cytometry* **6,** 54–61.

Boettger-Tong, H., Shipley, G., Hsu, C., and Stancel, G. (1997). Cultured human uterine smooth muscle cells are retinoid responsive. *Proc. Soc. Exp. Biol. Med.* **215,** 59–65.

Boffa, L., Lupton, J., Mariani, M., Ceppi, M., Newmark, H., Scalmati, A., and Lipkin, M. (1992). Modulation of colonic epithelial cell proliferation, histone acetylation, and luminal short chain fatty acids by variation of dietary fiber (wheat bran) in rats. *Cancer Res.* **52,** 5906–5912.

Bogenmann, E., Torres, M., and Matsushima H. (1995). Constitutive N-myc gene expression inhibits trkA mediated neuronal differentiation. *Oncogene* **10,** 1915–1925.

Bortell, R., Owen, T. A., Bidwell, J. P., Gavazzo, P., Breen, E., van Wijnen, A. J., DeLuca, H. F., Stein, J. L., Lian, J. B., and Stein, G. S. (1992). Vitamin D-responsive protein–DNA interactions at multiple promoter regulatory elements that contribute to the level of rat osteocalcin gene expression. *Proc. Natl. Acad. Sci. USA* **89,** 6119–6123.

Bramlage, B., Kosciessa, U., and Doenecke, D. (1997). Differential expression of the murine histone genes H3.3A and H3.3B. *Differentiation* **62,** 13–20.

Brehm, A., Miska, E. A., McCance, D. J., Reid, J. L., Bannister, A. J., and Kouzarides, T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature* **391,** 597–600.

Breitman, T., and He, R. (1990). Combinations of retinoic acid with either sodium butyrate, dimethyl sulfoxide, or hexamethylene bisacetamide synergistically induce differentiation of the human myeloid leukemia cell line HL60. *Cancer Res.* **50,** 6268–6273.

Brelvi, Z. S., and Studzinski, G. P. (1986). Changes in the expression of oncogenes encoding nuclear phosphoproteins but not c-Ha-ras have a relationship to monocytic differentiation of HL60 cells. *J. Cell Biol.* **102,** 2234–2243.

Burger, C., Wick, M., and Muller, R. (1994). Lineage-specific regulation of cell cycle gene expression in differentiating myeloid cells. *J. Cell Sci.* **107,** 2047–2054.

Camps, M., Chabert, C., Muda, M., Boschert, U., Gillieron, C., and Arkinstall, S. (1998). Induction of the mitogen-activated protein kinase phosphatase MKP3 by nerve growth factor in differentiating PC12. *FEBS Lett.* **425,** 271–276.

Cardoso, M. C., Leonhardt, H., and Nadal-Ginard, B. (1993). Reversal of terminal differentiation and control of DNA replication: Cyclin A and Cdk2 specifically localize at subnuclear sites of DNA replication. *Cell* **74,** 979–992.

Cargnello, R., Celio, M., Schwaller, B., and Gotzos, V. (1996). Change of calretinin expression in the human colon adenocarcinoma cell line HT29 after differentiation. *Biochim. Biophys. Acta* **1313,** 201–208.

Casaccia-Bonnefil, P., Tikoo, R., Kiyokawa, H., Friedrich, V., Jr., Chao, M. V., and Koff, A. (1997). Oligodendrocyte precursor differentiation is perturbed in the absence of the cyclin-dependent kinase inhibitor p27/Kip1. *Genes Dev.* **11,** 2335–2346.

Chandrasekaran, C., Coopersmith, C. M., and Gordon, J. I. (1996). Use of normal and transgenic mice to examine the relationship between terminal differentiation of intestinal epithelial cells and accumulation of their cell cycle regulators. *J. Biol. Chem.* **271,** 28414–28421.

Chen, P., Scully, P., Shew, J., Wang, J., and Lee, W. (1989). Phosphorylation of the retinoblastoma gene product is modulated during cell cycle and cellular differentiation. *Cell* **58,** 1193–1198.

Chen, P. L., Riley, D. J., Chen, Y., and Lee, W. H. (1996). Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. *Genes Dev.* **10,** 2794–2804.

Chin, L., Schreiber-Agus, N., Pellicer, I., Chen, K., Lee, H. W., Dudast, M., Cordon-Cardo, C., and DePinho, R. A. (1995). Contrasting roles for myc and Mad proteins in cellular growth and differentiation. *Proc. Natl. Acad. Sci. USA* **92,** 8488–8492.

Coolican, S. A., Samuel, D. S., Ewton, D. Z., McWade, F. J., and Florini, J. R. (1997). The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. *J. Biol. Chem.* **272,** 6653–6662.

Craig, C., Wersto, R., Kim, M., Ohri, E., Li, Z., Katayose, D., Lee, S. J., Trepel, J., Cowan, K., and Seth, P. (1997). A recombinant adenovirus expressing p27Kip1 induces cell cycle arrest and loss of cyclin-Cdk activity in human breast cancer cells. *Oncogene* **14,** 2283–2289.

Cross, H., Pavleka, M., Slavik, J., and Peterlik, M. (1992). Growth control of human cancer cells by vitamin D and calcium in vitro. *J. Natl. Cancer Inst.* **84,** 1355–1357.

Crowe, D. L. (1998). Retinoic acid receptor beta induces terminal differentiation of squamous cell carcinoma lines in the absence of cyclin-dependent kinase inhibitor expression. *Cancer Res.* **58,** 142–148.

Cultraro, C. M., Bino, T., and Segal, S. (1997). Function of the c-myc antagonist Mad 1 during a molecular switch from proliferation to differentiation. *Mol. Cell Biol.* **17,** 2353–2359.

Dalton, S. (1992). Cell cycle regulation of the human cdc2 gene. *EMBO J.* **11,** 1797–1804.

Danish, R., El-awar, O., Weber, B., Langmore, J., Turka, L., Ryan, J., and Clarke, M. (1992). c myb effects on kinetic events during MEL cell differentiation. *Oncogene* **7,** 901–907.

Darzynkiewicz, Z., Traganos, F., Xue, S., and Melamed, M. (1981). Effect of n-butyrate on cell cycle progression and in situ chromatin structure of L1210 cells. *Exp. Cell Res.* **136,** 279–293.

Datta, B., Min, W., Burma, S., and Lengyel, P. (1998). Increase in p202 expression during skeletal muscle differentiation: Inhibition of MyoD protein expression and activity by p202. *Mol. Cell. Biol.* **18,** 1074–1083.

Datta, N. S., Williams, J., Caldwell, J., Curry, A. M., Ashcraft, E. K., and Long, M. W. (1996). Novel alterations in CDK1/cyclin B1 kinase complex formation occur during the acquisition of a polyploid DNA content. *Mol. Biol. Cell* **7,** 209–223.

David, J. P., Neff, L., Rincon, M., and Baron, R. (1997). Osteoclast differentiation: Evidence for an indirect mechanism of action of $1\alpha,25(OH)_2D_3$. *In* "Proceedings of the Tenth Workshop on Vitamin D—Strasburg, France" (A. W. Norman, R. Buillon, and M. Thomasset, eds.), pp. 673–674. Univ. of California Press, Riverside.

Dean, M., Levine, R. A., and Campisi, J. (1986). c-myc regulation during retinoic acid-induced differentiation of F9 cells is posttranscriptional and associated with growth arrest. *Mol. Cell. Biol.* **6,** 518–524.

Delvin, P. P., Lopez, V., Levy, E., and Menard, D. (1996). Calcitriol differentially modulates mRNA encoding calcitriol receptors and calcium-binding protein 9 kDa in human fetal jejunum. *Biochem. Biophys. Res. Commun.* **224,** 544–548.

Demay, M. B., Kiernam, M. S., DeLuca, H. F., and Kronenberg, H. M. (1992). Sequences in the human parathyroid hormone gene that bind the 1,25-dihydroxyvitamin D_3 receptor and mediate transcriptional repression in response to 1,25-dihydroxyvitamin D_3. *Proc. Natl. Acad. Sci. USA* **89,** 8097–8101.

de Mora, J. F., Uren, A., Heidaran, M., and Santos, E. (1997). Biological activity of p27kip1 and its amino- and carboxy-terminal domains in G2/M transition of Xenopus oocytes. *Oncogene* **15,** 2541–2551.

Deng, C., Zhang, P., Harper, J. W., Elledge, S. L., and Leder, P. (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82,** 675–684.

Denko, N., Langland, R., Barton, M., and Lieberman, M. A. (1997). Uncoupling of S-phase and mitosis by recombinant cytotoxic necrotizing factor 2 (CNF2). *Exp. Cell Res.* **234,** 132–138.

DePinho, R. A. (1998). The cancer–chromatin connection. *Nature* **391,** 533–536.

Di Cunto, F., Topley, G., Calautti, E., Hsiao, J., Ong, L., Seth, P. K., and Dotto, G. P. (1998). Inhibitory function of p21Cip1/Waf1 in differentiation of primary mouse keratinocytes independent of cell cycle control. *Science* **280,** 1069–1072.

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, *et al.* (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. USA* **20,** 9363–9367.

Dimri, G. P., Testori, A., Acosta, M., and Campisi, J. (1996). Replicative senescence, aging and growth-regulatory transcription factors. *Biol. Signals* **5,** 154–162.

Djelloul, S., Forgue-Lafitte, M., Hermelin, B., Mareel, M., Bruyneel, E., Baldi, A., Giordano, A., Chastre, E., and Gespach, C. (1997). Enterocyte differentiation is compatible with SV40 large T expression and loss of p53 function in human colonic Caco-2 cells. *FEBS Lett.* **406,** 234–242.

Dmitrovsky, E., Moy, D., Miller, W. H., Jr., Li, A., and Masui, H. (1990). Retinoic acid causes a decline in TGF-alpha expression, cloning efficiency, and tumorigenicity in a human embryonal cancer cell line. *Oncogene Res.* **5,** 233–239.

Dobashi, Y., Kudoh, T., Toyoshima, K., and Akiyama, T. (1996). Persistent activation of CDK4 during neuronal differentiation of rat phenochromocytoma PC12 cells. *Biochem. Biophys. Res. Commun.* **221,** 351–355.

Dong, X., Zavitz, K. H., Thomas, B. J., Lin, M., Campbell, S., and Zipursky, S. L. (1997). Control of G1 in the developing drosophila eye: RCA 1 regulates cyclin A. *Genes Dev.* **11,** 94–105.

Dou, Q. P., Levin, A. H., Zhao, S., and Pardee, A. B. (1993). Cyclin E and cyclin A as candidates for the restriction point protein. *Cancer Res.* **53,** 1493–1497.

Dubart, A., Romeo, P. H., Vainchenker, W., and Dumenil, D. (1996). Constitutive expression of GATA-2 interferes with the cell-cycle regulation. *Blood* **87,** 3711–3721.

Duchrow, M., Schluter, C., Key, G., Kubbutat, M. H., Wohlenberg, C., Flad, H. D., and Gerdes, J. (1995). Cell proliferation-associated nuclear antigen defined by antibody Ki-67: A new kind of cell cycle-maintaining proteins. *Arch. Immunol. Ther. Exp. (Warsz)* **43,** 117–121.

Dulic, V., Stein, G. H., Far, D. F., and Reed, S. I. (1998). Nuclear accumulation of p21Cip1 at the onset of mitosis: A role at the G2/M-phase transition. *Mol. Cell. Biol.* **18,** 546–557.

Durand, B., Gao, F. B., and Raff, M. (1997). Accumulation of the cyclin-dependent kinase inhibitor p27/Kip1 and the timing of oligodendrocyte differentiation. *EMBO J.* **16,** 306–317.

Ebeling, J., Vandenbark, G., Kuhn, L., Ganong, B., Bell, R., and Niedel, J. (1985). Diacylglycerols mimic phorbol diester induction of leukemic cell differentiation. *Proc. Natl. Acad. Sci. USA* **82,** 815–819.

el-Deiry, W., Tokino, T., Velculescu, V., Levy, D., Parsons, R., Trent, J., Lin, D., Mercer, W., Kinzler, K., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell* **75,** 817–825.

el-Deiry, W. S., Tokino, T., Waldman, T., Oliner, J. D., Velculescur, V. E., Burrell, M., Hill, D. E., Healy, E., Rees, J. L., Hamilton, S. R., *et al.* (1995). Topological control of p21WAF1/CIP1 expression in normal and neoplastic tissues. *Cancer Res.* **55,** 2910–2919.

Elledge, S. (1996). Cell cycle checkpoints: Preventing an identity crisis. *Science* **274,** 1664–1672.

Elledge, S. (1998). Mitotic arrest: Mad 2 prevents sleepy from waking up the APC. *Science* **279,** 999–1000.

Elstner, E., Linker-Israeli, M., Said, J., Umiel, T., de Vos, S., Shintaku, P., Heber, D., Binderup, L., Uskokovic, M., and Koeffler, H. (1995). 20-epi-vitamin D_3 analogues: A novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cells. *Cancer Res.* **55,** 2822–2830.

Erhardt, J. A., and Pittman, R. N. (1998). p21WAF1 induces permanent growth arrest and enhances differentiation, but does not alter apoptosis in PC12 cells. *Oncogene* **16,** 443–451.

Farach-Carson, M. C., and Ridall, A. L. (1998). Dual 1,25-dihydroxyvitamin D3 signal response pathways in osteoblasts: Cross talk between genomic and membrane-initiated pathways. *Am. J. Kidney Dis.* **31,** 729–742.

Fattaey, A., Helin, K., and Harlow, E. (1993). Transcriptional inhibition by the retinoblastoma protein. *Philos. Trans. R. Soc. London B Biol. Sci.* **340,** 333–336.

Feldman, D., Glorieux, F. H., and Pike, J. W. (1997). "Vitamin D." Academic Press, San Diego.

Fibach, E., Treves, A., and Rachmilewitz, E. A. (1983). Spontaneous commitment of murine erythroleukemic cells to terminal differentiation. *Cancer Res.* **43,** 4136–4141.

Flink, I. L., Oana, S., Maitra, N., Bahl, J. J., and Morkin, E. (1998). Changes in E2F complexes containing retinoblastoma protein family members and increased cyclin-dependent kinase inhibitor activities during terminal differentiation of cardiomyocytes. *J. Mol. Cell. Cardiol.* **30,** 563–578.

Foley, K. P., McArthur, G. A., Queva, C., Hurlin, P. J., Soriano, P., and Eisenman, R. N. (1998). Targeted disruption of the MYC antagonist MAD1 inhibits cell cycle exit during granulocyte differentiation. *EMBO J.* **17,** 777–785.

Franceschi, R., James, W., and Zerlauth, G. (1985). 1α,25 dihydroxyvitamin D_3 specific regulation of growth, morphology and fibronectin in a human osteosarcoma cell line. *J. Cell. Physiol.* **123,** 401–409.

Franklin, D. S., and Xiong, Y. (1996). Induction of p18INK4c and its predominant association with CDK4 and CDK6 during myogenic differentiation. *Mol. Biol. Cell* **7,** 1587–1599.

Freemerman, A. J., Vrana, J. A., Tombes, R. M., Jiang, H., Chellappan, S. P., Fisher, P. B., and Grant, S. (1997). Effect of antisense p21 (WAF1/CIP1/MDA6) expression on the induction of differentiation and drug-mediated apoptosis in human myeloid leukemia cells (HL60). *Leukemia* **11,** 504–513.

Fromm, L., and Overbeck, P. A. (1996). Regulation of cyclin and cyclin-dependent kinase gene expression during lens differentiation requires the retinoblastoma protein. *Oncogene* **12,** 69–75.

Galaktionov, K., Jessus, C., and Beach, D. (1995). Raf1 interaction with cdc25 phosphatase ties mitogenic signal transduction to cell cycle activation. *Genes Dev.* **9,** 1046–1058.

Galaktionov, K., Chen, X., and Beach, D. (1996). Cdc25 cell cycle phosphatase as a target of c-myc. *Nature* **382,** 511–517.

Gallagher, R., Said, F., Papenhausen, P., Paietta, E., and Wiernick, P. (1989). Expression of retinoic acid receptor alpha gene in acute promyelocytic leukemia. *Leukemia* **3,** 789–795.

Gandarillas, A., and Watt, F. M. (1997). C-myc promotes differentiation of human epidermal stem cells. *Genes Dev.* **11,** 2869–2882.

Ganter, B., Fu, S. I., and Lipsick, J. S. (1998). D-type cyclins repress transcriptional activation by the v-Myb but not the c-Myb DNA-binding domain. *EMBO J.* **17,** 255–268.

Gao, C. Y., Bassnett, S., and Zelenka, P. S. (1995). Cyclin B, p34cdc2, and H1-kinase activity in terminally differentiating lens fiber cells. *Dev. Biol.* **169,** 185–194.

Gao, C. Y., Zakeri, Z., Zhu, Y., He, H., and Zelenka, P. S. (1997). Expression of Cdk5, p35, and Cdk5-associated kinase activity in the developing rat lens. *Dev. Genet.* **20,** 267–275.

Gartel, A. L., Serfas, M. S., Gartel, M., Goufman, E., Wu, G. S., el-Deiry, W. S., and Tyner, A. L. (1996). p21 (WAF1/CIP1) expression is induced in newly nondividing cell in diverse epithelia and during differentiation of the $CaCo_2$ intestinal cell line. *Exp. Cell Res.* **227,** 171–181.

Gautier, J., Solomon, M., Booher, R., Bazan, F., and Kirschner, M. (1991). cdc25 is a specific tyrosine phosphatase that directly activates $p34^{cdc2}$. *Cell* **67,** 197–211.

Ge, R. S., and Hardy, M. P. (1997). Decreased cyclin A2 and increased cyclin G1 levels coincide with loss of proliferative capacity in rat Leydig cells during pubertal development. *Endocrinology* **138,** 3719–3726.

Glaise, D., Ilyin, G. P., Loyer, P., Cariou, S., Nilodeau, M., Lucas, J., Puisieux, A., Oxturk, M., and Guguen-Guillouzo, C. (1998). Cell cycle gene regulation in reversibly differentiated new human hepatoma cell lines. *Cell Growth Differ.* **9,** 165–176.

Glauber, J., Wandersee, N., Little, J., and Ginder, G. (1991). 5′ flanking sequences mediate butyrate stimulation of embryonic globin gene expression in adult erythroid cells. *Mol. Cell. Biol.* **11,** 4690–4697.

Godyn, J. J., Xu, H., Zhang, F., Kolla, S., and Studzinski, G. P. (1994). A dual block to cell cycle progression in HL60 cells exposed to analogues of vitamin D_3. *Cell Prolif.* **27,** 37–46.

Gore, S., Samid, D., and Weng, L. (1997). Impact of the putative differentiating agents sodium phenylbutyrate and sodium phenylacetate on proliferation, differentiation, and apoptosis of primary neoplastic myeloid cells. *Clin. Cancer Res.* **3,** 1755–1762.

Griffin, J. D., Ritz, J., Nadler, L. M., and Schlossman, S. F. (1981). Expression of myeloid differentiation antigens on normal and malignant myeloid cells. *J. Clin. Invest.* **68,** 932–941.

Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. (1993). Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell* **72,** 309–324.

Guidato, S., MsLoughlin, D. M., Grierson, A. J., and Miller, C. C. (1998). Cyclin D2 interacts with cdk-5 and modulates cellular cdk-5/p35 activity. *J. Neurochem.* **70,** 335–340.

Gum, J., Kam, W., Byrd, J., Hicks, J., Sleisenger, M., and Kim, Y. (1987). Effects of sodium butyrate on human colonic adenocarcinoma cells. *J. Biol. Chem.* **262,** 1092–1097.

Guy, C. T., Zhou, W., Kaufman, S., and Robinson, M. O. (1996). E2F-1 blocks terminal differentiation and causes proliferation in transgenic megakaryocytes. *Mol. Cell. Biol.* **16,** 685–693.

Haas-Kogan, D. A., Kogan, S. C., Levi, D., Dazin, P., T'Ang, A., Fung, Y. K., and Israel, M. A. (1995). Inhibition of apoptosis by the retinoblastoma gene product. *EMBO J.* **14,** 461–472.

Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D., and Lassar, A. B. (1995). Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267,** 1018–1021.

Hannon, G., and Beach, D. (1994). p15(INK4) is a potential effector of TGF-beta induced cell cycle arrest. *Nature* **371,** 257–261.

Hartwell, L., and Weinert, T. (1989). Checkpoints: Controls that ensure the order of cell cycle events. *Science* **246,** 629–634.

Harvat, B., Wang, A., Seth, P., and Jetten, A. (1998). Up-regulation of p27(Kip1), p21(WAF1/Cip1) and p16(Ink4a) is associated with, but not sufficient for, induction of squamous differentiation. *J. Cell Sci.* **111,** 1185–1196.

Hashimoto, Y., and Shudo, K. (1991). Retinoids and their nuclear receptors. *Cell Biol. Rev.* **25,** 209–230.

Hatakeyama, M., and Weinberg, R. A. (1995). The role of RB in cell cycle control. *Prog. Cell Cycle Res.* **1,** 9–19.

Hauser, P. J., Agrawal, D., Flanagan, M., and Pledger, W. J. (1997). The role of p27kip1 in the in vitro differentiation of murine keratinocytes. *Cell Growth Differ.* **8,** 203–211.

Hawa, N. S., O'Riordan, J. L., and Farrow, S. M. (1996). Functional analysis of vitamin D response elements in the parathyroid hormone gene and a comparison with the osteocalcin gene. *Biochem. Biophys. Res. Commun.* **228,** 352–357.

Hayflick, L., and Moorhead, P. S. (1961). The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25,** 585–621.

He, H. Y., Gao, C., Vrensen, G., and Zelenka, P. (1998). Transient activation of cyclin B/Cdc2 during terminal differentiation of lens fiber cells. *Dev. Dyn.* **211,** 26–34.

Heald, R., McLoughlin, M., and McKeon, F. (1993). Human wee-1 maintains mitotic timing by protecting the nucleus from cytoplasmically activated cdc2 kinase. *Cell* **74,** 463–474.

Heerdt, B., Houston, M., and Augenlicht, L. (1994). Potentiation by short-chain fatty acids on differentiation and apoptosis in human colonic carcinoma cell lines. *Cancer Res.* **54,** 3288–3294.

Hewison, M., and O'Riordan, J. L. H. (1997). Immunomodulatory and cell differentiation effects of vitamin D. *In* "Vitamin D" (D. Feldman, F. H. Glorieux, and J. W. Pike, eds.), pp. 447–462. Academic Press, San Diego.

Hirano, F., Tanaka, H., Hirano, Y., Hiramoto, M., Handa, H., Makino, I., and Scheidereit, C. (1998). Functional interference of Sp1 and NF kappaB through the same DNA binding site. *Mol. Cell. Biol.* **18,** 1266–1274.

Hiyama, H., Iavarone, A., and Reeves, S. A. (1998). Regulation of the cdk inhibitor p21 gene during cell cycle progression is under the control of the transcription factor E2F. *Oncogene* **16,** 1513–1523.

Hodin, R., Meng, S., Archer, S., and Tang, R. (1996). Cellular growth state differentially regulates enterocyte gene expression in butyrate-treated HT-29 cells. *Cell Growth Differ.* **7,** 647–653.

Hoffmann, I., Clarke, P., Marcote, M., Karsenti, E., and Draetta, G. (1993). Phosphorylation and activation of human cdc25c by cdc2–cyclin B and its involvement in the self-amplification of MPF at mitosis. *EMBO J.* **12,** 53–63.

Hoffmann, I., Draetta, G., and Karsenti, E. (1994). Activation of the phosphatase activity of human cdc25A by a cdk2–cyclin E dependent phosphorylation at the G_1/S transition. *EMBO J.* **13,** 4302–4310.

Horiguchi-Yamada, J., Yamada, H., Nakada, S., Ochi, K., and Nemoto, T. (1994). Changes of G1 cyclins, cdk2, and cyclin A during the differentiation of HL60 cells induced by TPA. *Mol. Cell. Biochem.* **132,** 31–37.

Hoyt, M. (1997). Eliminating all obstacles: Regulated proteolysis in the eukaryotic cell cycle. *Cell* **91,** 149–151.

Huang, R. P., Fan, Y., Ni, Z., Mercola, D., and Adamson, E. D. (1997). Reciprocal modulation between Sp1 and Egr-1. *Cell Biochem.* **66,** 489–499.

Hudson, K. M., Denko, N. C., Schwab, E., Oswald, E., Weiss, A., and Lieberman, M. A. (1996). Megakaryocytic cell line-specific hyperploidy by cytotoxic necrotizing factor bacterial toxins. *Blood* **88,** 3465–3473.

Hulla, W., Kallay, E., Krugluger, W., Peterlik, M., and Cross, H. (1995). Growth control of human colon-adenocarcinoma-derived Caco-2 cells by vitamin D compounds and extracellular calcium in vitro: Relation to c myc oncogene and vitamin D receptor expression. *Int. J. Cancer* **62,** 711–716.

Hume, D. A., and Gordon, S. (1982). Regulation of bone-marrow macrophage proliferation. *Adv. Exp. Med. Biol.* **155,** 261–266.

Hunter, T., and Pines, J. (1994). Cyclins and cancer II: Cyclin D and CDK inhibitors come of age. *Cell* **79,** 573–582.

Ishiguro, A., Spirin, K. S., Shiohara, M., Tobler, A., Norton, J. D., Rigolet, M., Shimbo, T., and Koeffler, H. P. (1995). Expression of Id2 and Id3 mRNA in human lymphocytes. *Leukemia Res.* **19,** 989–996.

Ishiguro, A., Spirin, K. S., Shiohara, M., Tobler, A., Gombart, A. F., Israel, M. A., Norton, J. D., and Koeffler, H. P. (1996). Id2 expression increases with differentiation of human myeloid cells. *Blood* **87,** 5225–5231.

Jahn, L., Sadoshima, J., and Izumo, S. (1994). Cyclins and cyclin-dependent kinases are differentially regulated during terminal differentiation of C2C12 muscle cells. *Exp. Cell Res.* **212,** 297–307.

Jensen, T. J., Henriksen, L. O., Solvsten, H., and Kragballe, K. (1998). Inhibition of the 1,25-dihydroxyvitamin D3-induced increase in vitamin D receptor (VDR) levels and binding of VDR-retinoid X receptor (RXR) to a direct repeat (DR)-3 type response element by an RXR-specific ligand in human keratinocyte cultures. *Biochem. Pharmacol.* **55,** 767–773.

Jiang, H., Lin, J., Su, Z. Z., Collart, F. R., Huberman, E., and Fisher, P. B. (1994). Induction of differentiation in human promyelocytic HL60 leukemia cells activates p21, WAF/CIP1, expression in the absence of p53. *Oncogene* **11,** 3389–3396.

Jiang, H., Lin, J., Young, S. M., Goldstein, N. I., Waxman, S., Davila. V., Chellapan, S. P., and Fisher, P. B. (1995). Cell cycle gene expression and E2F transcription factor complexes in human melanoma cells induced to terminally differentiate. *Oncogene* **11,** 1179–1189.

Jinno, S., Suto, K., Nagata, A., Igarashi, M., Kanaoka, Y., Nojima, H., and Okayama, H. (1994). Cdc25A is a novel phosphatase functioning early in the cell cycle. *EMBO J.* **13,** 1549–1556.

Jones, D. L., Alani, R. M., and Munger, K. (1997). The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. *Genes Dev.* **11,** 2101–2111.

Kaffman, A., Herskowitz, I., Tjian, R., and O'Shea, E. K. (1994). Phosphorylation of the transcription factor PHO4 by a cyclin–CDK complex, PHO80–PHO85. *Science* **263,** 1153–1156.

Kaliman, P., Vinals, F., Testar, X., Palacin, M., and Zorzano, A. (1996). Phosphatidylinositol 3-kinase inhibitors block differentiation of skeletal muscle cells. *J. Biol. Chem.* **271,** 19146–19151.

Kallassy, M., Martel, N., Damour, O., Yamasaki, H., and Nakazawa, H. (1998). Growth arrest of immortalized human keratinocytes and suppression of telomerase activity by p21WAF1 gene expression. *Mol. Carcinogen.* **21,** 26–36.

Karantza, V., Maroo, A., Fay, D., and Sedivy, J. M. (1993). Overproduction of Rb protein after the G1/S boundary causes G2 arrest. *Mol. Cell. Biol.* **13,** 6640–6652.

Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E., and Sherr, C. J. (1993). Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK-4. *Genes Dev.* **7,** 331–334.

Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26,** 239–257.

Kharbanda, S., Yuan, Z., Taneja, N., Weichselbaum, R., and Kufe, D. (1994a). p56/53^{lyn} tyrosine kinase activation in mammalian cells treated with mitomycin C. *Oncogene* **9,** 3005–3011.

Kharbanda, S., Yuan, Z., Rubin, E., Weichselbaum, R., and Kufe, D. (1994b). Activation of src-like p56/53^{lyn} tyrosine kinase by ionizing radiation. *J. Biol. Chem.* **269,** 20739–20743.

Kharbanda, S., Saleem, A., Yuan, Z., Kraeft, S., Weichselbaum, R., Chen, L., and Kufe, D. (1996). Nuclear signaling induced by ionizing radiation involves colocalization of the activated p56/53^{lyn} tyrosine kinase with p34^{cdc2}. *Cancer Res.* **56,** 3617–3621.

Kiess, M., Gill, R. M., and Hamel, P. A. (1995). Expression of the positive regulator of cell cycle progression, cyclin D3, is induced during differentiation of myoblasts into quiescent myotubes. *Oncogene* **10,** 159–166.

Kikuchi, J., Furukawa, Y., Iwase, S., Terui, Y., Nakamura, M., Kitagawa, S., Kitagawa, M., Komatsu, N., and Miura, Y. (1997). Polyploidization and functional maturation are two distinct processes during megakaryocytic differentiation: Involvement of cyclin-dependent kinase inhibitor p21 in polyloidization. *Blood* **89,** 3980–3990.

King, R. W., Deshaies, R., Peters, J., and Kirschner, M. (1996). How proteolysis drives the cell cycle. *Science* **274,** 1652–1659.

Kiyokawa, H., Ngo, L., Kurosaki, T., Rifkind, R. A., and Marks, P. A. (1992). Changes in p34cdc2 kinase activity and cyclin A during induced differentiation of murine erythroleukemia cells. *Cell Growth Differ.* **3,** 377–383.

Knudsen, E. S., Pazzagli, C., Born, T. L., Bertolaet, B. L., Knudsen, K. E., Arden, K. C., Henry, R. R., and Feramisco, J. R. (1998). Elevated cyclins and cyclin-dependent kinase activity in the rhabdomyosarcoma cell line RD. *Cancer Res.* **58,** 2042–2049.

Kobayashi, M., Yamauchi, Y., and Tanaka, A. (1998). Stable expression of antisense Rb-1 RNA inhibits terminal differentiation of mouse myoblast C2 cells. *Exp. Cell Res.* **239,** 40–49.

Kokunai, T., Izawa, I., and Tamaki, N. (1998). Overexpression of p21WAF1/CIP1 induces cell differentiation and growth inhibition in a human glioma cell line. *Int. J. Cancer* **75,** 643–648.

Kolla, S. S., and Studzinski, G. P. (1994). Constitutive DNA binding of the low mobility forms of the AP-1 and SP-1 transcription factors in HL60 cells resistant to 1-beta-D-arabinofuranosylcytosine. *Cancer Res.* **54,** 1418–1421.

Kruh, J. (1982). Effects of sodium butyrate, a new pharmacological agent, on cell culture. *Mol. Cell. Biochem.* **42,** 65–82.

Kwon, T. K., Buchholz, M. A., Jun, D. Y., Kim, Y. H., and Nordin, A. A. (1998). The differential catalytic activity of alternatively spliced cdk2α and cdk2β in the G1/S transition and early S phase. *Exp. Cell Res.* **238,** 128–135.

Lahti, J. M., Xiang, J., and Kidd, V. J. (1995). Cell cycle-related protein kinases and T cell death. *Adv. Exp. Med. Biol.* **376,** 247–258.

Langlands, K., Yin, X., Anand, G., and Prochownik, E. V. (1997). Differential interactions of Id proteins with basic-helix–loop–helix transcription factors. *J. Biol. Chem.* **272,** 19785–19793.

Lazaro, J. B., Kitzmann, M., Poul, M. A., Vandromme, M., Lamb, N. J., and Fernandez, A. (1997). Cyclin dependent kinase 5, cdk5, is a positive regulator of myogenesis in mouse C2 cells. *J. Cell Sci.* **110,** 1251–1260.

Lee, Y. Y., Kim, E. S., Seol, J. G., Kim, B. K., Binderup, L., Elstner, E., Park, D. J., and Koeffler, H. P. (1996). Effect of a vitamin D3 analog, EB1089, on hematopoietic stem cells from normal and myeloid leukemic blasts. *Leukemia* **10,** 1751–1757.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. *Cell* **88,** 323–331.

Li, W. W., Fan, J., Hochhauser, D., and Bertino, J. R. (1997a). Overexpression of p21waf1 leads to increased inhibition of E2F-1 phosphorylation and sensitivity to anticancer drugs in retinoblastoma-negative human sarcoma cells. *Cancer Res.* **57,** 2193–2199.

Li, Z. R., Hromchak, R., and Bloch, A. (1997b). Differential expression of proteins regulating cell cycle progression in growth vs. differentiation. *Biochim. Biophys. Acta* **1356,** 149–150.

Lim, D. A., Grossen, M., Lehman, C. W., and Botchan, M. R. (1998). Competition for DNA binding sites between the short and long forms of E2 dimers umderlies repression in bovine papillomavirus type 1 DNA replication control. *J. Virol.* **72,** 1931–1940.

Liu, M., Iavarone, A., and Freedman, L. P. (1996a). Transcriptional activation of the human p21(WAF1/CIP1) gene by retinoic acid receptor. Correlation with retinoid induction of U937 cell differentiation. *J. Biol. Chem.* **271,** 31723–31728.

Liu, M., Lee, M. H., Cohen, M., Bommakanti, M., and Freedman, L. P. (1996b). Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev.* **10,** 142–153.

Liu, S. M., Koszewski, N., Lupex, M., Malluche, H. H., Olivera, A., and Russel, J. (1996c). Characterization of a response element in the 5′ flanking region of the avian (chicken) PTH gene that mediates negative regulation of gene transcription by 1,25-dihydroxyvitamin D_3 and binds the vitamin D receptor. *Mol. Endocrinol.* **10,** 206–215.

Lois, A. F., Cooper, L. T., Geng, Y., Nobori, T., and Carson, D. (1995). Expression of the p16 and p15 cyclin-dependent kinase inhibitors in lymphocytic activation and neuronal differentiation. *Cancer Res.* **55,** 4010–4013.

Lomo, J., Smeland, E., Ulven, S., Natarajan, V., Blomhoff, R., Gandhi, U., Dawson, M., and Blomhoff, H. (1998). RAR, not RXR, ligands inhibit cell activation and prevent apoptosis in B-lymphocytes. *J. Cell. Physiol.* **175,** 68–77.

Lowe, W., Meyer, T., Karpen, C., and Lorentzen, L. (1992). Regulation of insulin-like growth factor I production in rat C6 glioma cells: Possible roles as an autocrine/paracrine growth factor. *Endocrinology* **130,** 2683–2691.

Lymboussaki, A., Kaipainen, A., Hatva, E., Vastrik, I., Jeskanen, M., Werner, S., Stenback, F., and Alitalo, R. (1996). Expression of Mad, an antagonist of Myc oncoprotein function, in differentiating keratinocytes during tumorigenesis of the skin. *Br. J. Cancer* **73,** 1347–1355.

Magnaghi-Jaulin, L., Groisman, R., Naguibneva, I., Robin, P., Loralin, S., LeVillain, J. P., Troalen, F., Trouche, D., and Harel-Bellan, A. (1998). Retinoblastoma protein represses transcription by recruiting a histone deacetylase. *Nature* **391,** 601–605.

Marcinkowska, E., Wiedlocha, A., and Radzikowski, C. (1997). 1,25-Dihydroxyvitamin D3 induced activation and subsequent nuclear translocation of MAPK is upstream regulated by PKC in HL60 cells. *Biochem. Biophys. Res. Commun.* **241,** 419–426.

Marks, P., Sheffery, M., and Rifkin, R. (1987). Induction of transformed cells to terminal differentiation and the modulation of gene expression. *Cancer Res.* **47,** 659–666.

Martinelli, R., Blandino, G., Scardigli, R., Crescenzi, M., Lombardi, D., Sacchi, A., and Soddu, S. (1997). Oncogenes belonging to the CSF-1 transduction pathway direct p53 tumor suppressor effects to monocytic differentiation in 32D cells. *Oncogene* **15,** 607–611.

Masui, Y., and Markert, C. L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J. Exp. Zool.* **177,** 129–146.

Matsumura, I., Ishikawa, J., Nakajima, K., Oritani, K., Tomiyama, Y., Miyagawa, J., Kato, T., Miyazaki, H., Matsuzawa, Y., and Kanakura, Y. (1997). Thrombopoietin-induced differentiation of a human megakaryoblastic leukemia cell line, CMK, involves transcriptional activation of p21(WAF1/Cip1) by STAT5. *Mol. Cell. Biol.* **17,** 2933–2943.

McCabe, L. B., Last, T. J., Lynch, M., Lian, J., Stein, J., and Stein, G. (1994). Expression of cell growth and bone phenotypic gene during the cell cycle of normal diploid osteoblasts and osteosarcoma cells. *J. Cell Biochem.* **56,** 274–282.

McCarthy, J. T., Barham, S. S., and Kumar, R. (1984). 1,25-Dihydroxyvitamin D_3 rapidly alters the morphology of the duodenal mucosa of rachitic chicks: Evidence for novel effects of 1,25-dihydroxyvitamin D_3. *J. Steroid Biochem.* **21,** 253–258.

McConnell, B. B., Starborg, M., Brookes, S., and Peters, G. (1998). Inhibitors of cyclin-dependent kinases induce features of replicative senescence in early passage human diploid fibroblasts. *Curr. Biol.* **8,** 351–354.

McDonald, T. P., and Jackson, C. W. (1994). Mode of inheritance of the higher degree of megakaryocyte polyploidization in C3H mice. I. Evidence for a role of genomic imprinting in megakaryocyte polyploidy determination. *Blood* **83,** 1493–1498.

Melloni, E., Pontremoli, S., Michetto, M., Sacco, O., Cakiroglu, A., Jackson, J., Rifkin, R., and Marks, P. (1987). Protein kinase C activity and hexamethylacetamide-induced erythroleukemia cell differentiation. *Proc. Natl. Acad. Sci. USA* **84,** 5282–5286.

Minoo, P., Sullivan, W., Solomon, L., Martin, T., Toft, D., and Scott, R. (1989). Loss of proliferative potential during terminal differentiation coincides with the decreased abundance of a subset of hnRNP proteins. *J. Cell Biol.* **109,** 1937–1946.

Missero, C., Di Cunto, F., Kiyokawa, H., Koff, A., and Dotto, G. P. (1996). The absence of p21Cip1/WAF1 alters keratinocyte growth and differentiation and promotes ras-tumor progression. *Genes Dev.* **10,** 3065–3075.

Momoi, T., Kawai, Y., Momoi, M., and Etoh, Y. (1992). Activin synergistically increased c jun mRNA in P19 embryonal carcinoma cells in the presence of retinoic acid. *Biochem. Biophys. Res. Commun.* **184,** 1350–1356.

Morgan, D. (1995). Principles of CDK regulation. *Nature* **374,** 131–134.

Morioka, K., Tone, S., Mukaida, M., and Takano-Ohmuro, H. (1998). The apoptotic and nonapoptotic nature of the terminal differentiation of erythroid cells. *Exp. Cell Res.* **240,** 206–217.

Morse, L., Chen, D., Franklin, D., Xiong, Y., and Chen-Kiang, S. (1997). Induction of cell cycle arrest and B cell terminal differentation by CDK inhibitor p18(INK4c) and IL-6. *Immunity* **6,** 47–56.

Mumberg, D., Wick, M., Burger, C., Haas, K., Funk, M., and Muller, R. (1997). Cyclin ET, a new splice variant of human cyclin E with a unique expression pattern during cell cycle progression and differentiation. *Nucleic Acids Res.* **25,** 2098–2105.

Murate, T., Saga, S., Hotta, T., Asano, H., Ito, T., Kato, K., Tsushita, K., Kinoshita, T., Ichikawa, A., and Yoshida, S. (1993). The close relationship between DNA replication and the selection of differentiation lineages of human erythroleukemia cell lines K562, HEL, and TF1 into either erythroid or megakaryocytic lineages. *Exp. Cell Res.* **208,** 35–43.

Nakamura, K., Takahashi, T., Sasaki, Y., Tsuyuoka, R., Okuno, Y., Kurino, M., Ohmoir, K., Iho, S., and Nakao, K. (1996). 1,25-Dihydroxyvitamin D3 differentiates normal neutrophilic promyeloctes to monocytes/macrophages in vitro. *Blood* **87,** 2693–2701.

Nakamura, T., Okuyama, S., Okamoto, S., Nakajima, T., Sekiya, S., and Oda, K. (1995). Down-regulation of the cyclin A promoter in differentiating human embryonal carcinoma cells is mediated by depletion of ATF-1 and ATF-2 in the complex at the ATF/CRE site. *Exp. Cell Res.* **216,** 422–430.

Neuman, E., Ladha, M. H., Lin, N., Upton, T. M., Miller, S. J., DiRenzo, J., Pestell, R. G., Hinds, P. W., Dowdy, S. F., Brown, M., and Ewen, M. E. (1997). Cyclin D1 stimulation of estrogen receptor transcriptional activity independent of cdk4. *Mol. Cell. Biol.* **17,** 5338–5347.

Niculescu, A. B., Chen, X., Smeets, M., Hengst, L., Prives, C., and Reed, S. I. (1998). Effects of p21 (Cip1/Waf1) at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. *Mol. Cell. Biol.* **18,** 629–643.

Nikolic, M., Dudek, H., Kwon, Y. T., Ramos, Y. F., and Tsai, L. H. (1996). The cdk5/p35 kinase is essential for neurite outgrowth during neuronal differentiation. *Gene Dev.* **10,** 816–825.

Niles, R. M., and Loewy, B. P. (1989). Induction of protein kinase C in mouse melanoma cells by retinoic acid. *Cancer Res.* **49,** 4483–4487.

Nishiguchi, Y., Hibasami, H., Komada, Y., Sakurai, M., and Nakashima, K. (1993). Human promyelocytic cell line HL60 has the specific binding sites for prolactin and its ornithine decarboxylase, DNA synthesis and cellular proliferation are induced by prolactin. *Leukemia Res.* **17,** 633–637.

Norman, A. W., Bouillon, R., and Thomasset, M. (1997). "Vitamin D. Chemistry, Biology and Clinical Application of the Steroid Hormone." Univ. of California Press, Riverside.

Novitch, B. G., Mulligan, G. J., Jacks, T., and Lassar, A. B. (1996). Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. *J. Cell. Biol.* **135,** 441–456.

Nurse, P. (1990). Universal control mechanism regulating the onset of M-phase. *Nature* **344,** 503–508.

O'Connor, P.M., and Fan, S. (1996). DNA damage checkpoints: Implications for cancer therapy. *Prog. Cell Cycle Res.* **2,** 165–173.

Ogino, H., and Yasuda, K. (1998). Induction of lens differentiation by activation of a bZIP transcription factor, L-Maf. *Science* **280,** 115–118.

Ohkubo, Y., Kishimoto, T., Nakata, T., Yasuda, H., and Endo, T. (1994). SV40 large T antigen reinduces the cell cycle in terminally differentiated myotubes through inducing Cdk2, Cdc2, and their partner cyclins. *Exp. Cell Res.* **214,** 270–278.

Okuyama, Y., Sowa, Y., Fujita, T., Mizuno, T., Nomura, H., Nikaido, T., Endo, T., and Sakai, T. (1996). ATF site of human RB gene promoter is a responsive element of myogenic differentiation. *FEBS Lett.* **397,** 219–224.

Onishi, H., Kamano, H., Tanaka, T., Okabe, A., and Irino, S. (1990). p75 c-myb expression in leukemia–lymphoma cells correlated with proliferation and differentiation. *Leukemia Res.* **14,** 423–432.

Onishi, T., and Hruska, K. (1997). Expression of p27Kip1 in osteoblast-like cells during differentiation with parathyroid hormone. *Endocrinology* **138,** 1995–2004.

Oswald, F., Dobner, T., and Lipp, M. (1996). The E2F transcription factor activates a replication-dependent human H2A gene in early S phase of the cell cycle. *Mol. Cell. Biol.* **16,** 1889–1895.

Otoshi, T., Iwata, H., Kitano, M., Nishizawa, Y., Morii, H., Yano, Y., Otani, S., and Fukushima, S. (1995). Inhibition of intestinal tumor development in rat multi-organ carcinogenesis by 22-oxa-calcitrol, a synthetic analogue of 1α,25 dihydroxyvitamin D_3. *Carcinogenesis* **16,** 2091–2097.

Owen, T. A., Aronow, M., Shalhoub, V., Barone, L. M., Wilming, L., Tassinari, M. S., Kennedy, M. B., Pockwinse, S., Lian, J. B., and Stein, G. S. (1990). Progressive development of the rat osteoblast phenotype in vitro: Reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J. Cell. Physiol.* **143,** 420–430.

Pan, P., Reddy, K., Lee, S., and Studzinski, G. (1991). Differentiation-related regulation of 1,25-dihydroxyvitamin D_3 receptor mRNA in human leukemia cells HL60. *Cell Prolif.* **24,** 159–170.

Pardee, A. (1989). G1 events and regulation of cell proliferation. *Science* **246,** 603–608.

Parker, L., Sylvestre, P., Byrnes, M., Liu, F., and Piwnica-Worms, H. (1995). Identification of a 95-kDa WEE-1-like tyrosine kinase in HeLa cells. *Proc. Natl. Acad. Sci. USA* **92,** 9638–9642.

Parker, S. B., Eichele, G., Zhang, P., Rawls, A., Sands, A. T., Bradley, A., Olson, E. N., Harper, J. W., and Elledge, S. J. (1995). p53-independent expression of p21 Cip1 in muscle and other terminally differentiating cells. *Science* **267,** 1024–1027.

Perez-Roger, I., Solomom, D. L., Sewing, A., and Land, H. (1997). Myc activation of cyclin E/Cdk2 kinase involves induction of cyclin E gene transcription and inhibition of p27 (Kip1) binding to newly formed complexes. *Oncogene* **14,** 2373–2381.

Phelps, D. E., Hsiao, K. M., Li, Y., Hu, N., Franklin, D. S., Westphal, E., Lee, E. Y., and Xiong, Y. (1998). Coupled transcriptional and translational control of cyclin-dependent kinase inhibitor p18INK4c expression during myogenesis. *Mol. Cell. Biol.* **18,** 2334–2343.

Piette, J. (1997). The transition from proliferation to differentiation in nerve cells: What can we learn from muscle? *Exp. Cell Res.* **234,** 193–204.

Poluha, W., Schonhoff, C. M., Harrington, K. S., Lachyankar, M. B., Crosbie, N. E., Bulseco, D. A., and Ross, A. H. (1997). A novel, nerve growth factor-activated pathway involving nitric oxide, p53, and p21WAF1 regulates neuronal differentiation of PC12 cells. *J. Biol. Chem.* **272,** 24002–24007.

Polyak, K., Kato, J., Solomon, M., Sherr, C., Massague, J., Roberts, J., and Koff, A. (1994). p27/Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes Dev.* **8,** 9–22.

Pomerantz, J., Schriber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H. W., Gordon-Cardo, C., and DePinho, R. A. (1998). The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* **92,** 713–723.

Ponten, F., Berne, B., Ren, Z. P., Nister, M., and Ponten, J. (1995). Ultraviolet light induces expression of p53 and p21 in human skin: Effect of sunscreen and constitutive p21 expression in skin appendages. *J. Invest. Dermatol.* **105,** 402–406.

Puri, P. L., Avantaggiati, M. L., Balsano, C., Sang, N., Graessmann, A., Giordano, A., and Levrero, M. (1997a). p300 is required for MyoD-dependent cell cycle arrest and muscle-specific gene transcription. *EMBO J.* **16,** 369–383.

Puri, P. L., Balsano, C., Burgio, V. L., Chirillo, P., Natoli, G., Ricci, L., Mattei, E., Graessmann, A., and Levrero, M. (1997b). MyoD prevents cyclinA/cdk2 containing E2F complexes formation in terminally differentiated myocytes. *Oncogene* **14,** 1171–1184.

Puri, P. L., Medaglia, S., Cimino, L., Maselli, C., Germani, A., DeMarzio, E., Levrero, M., and Balsano, C. (1997c). Uncoupling of p21 induction and MyoD activation results in the failure of irreversible cell cycle arrest in doxorubicin-treated myocytes. *J. Cell. Biochem.* **66,** 27–36.

Puri, P. L., Cimino, L., Fulco, M., Zimmerman, C., LaThangue, N. B., Giordano, A., Graessmann, A., and Levrero, M. (1998). Regulation of E2F4 mitogenic activity during terminal differentiation by its heterodimerization partners for nuclear translocation. *Cancer Res.* **58,** 1325–1331.

Quelle, D. E., Zindy, F., Ashmun, R. A., and Sherr, C. J. (1995). Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* **83,** 993–1000.

Queva, C., Hurlin, P. J., Foley, K. P., and Eisenman, R. N. (1998). Sequential expression of the MAD family of transcriptional repressors during differentiation and development. *Oncogene* **16,** 967–977.

Rao, J., Zhang, F., Donnelly, R. J., Spector, N. L., and Studzinski, G. P. (1998). Truncation of Sp1 transcription factor by myeloblastin in undifferentiated HL60 cells. *J. Cell. Physiol.* **175,** 121–128.

Reichrath, J., Mittman, M., Kamradt, J., and Muller, S. (1997). Expression of retinoid-X receptors (-alpha, -beta, -gamma) and retinoic acid receptors (-alpha, -beta, -gamma) in normal human skin: An immunohistochemical evaluation. *Histochem. J.* **29,** 127–133.

Reitsma, P. H., Rothberg, P. G., Astrin, S. M., Trial, J., Bar-Shavit, Z., Hall, A., Teitelbaum, S. L., and Kahn, A. J. (1983). Regulation of myc gene expression in HL-60 leukemia cells by a vitamin D metabolite. *Nature* **306,** 492–494.

Rephaeli, A., Rabizadeh, E., Aviram, A., Shaklai, M., Ruse, M., and Nudelman, A. (1991). Derivatives of butyric acid as potential antineoplastic agents. *Int. J. Cancer* **49,** 66–72.

Richon, V., Ramsay, R., Rifkin, R., and Marks, P. (1989). Modulation of the c myb, c myc and p53 mRNA and protein levels during induced murine leukemia cell differentiation. *Oncogene* **4,** 165–173.

Rickert, P., Seghezzi, W., Shanahan, F., Cho, H., and Lees, E. (1996). Cyclin C/CDK8 is a novel CTD kinase associated with RNA polymerase II. *Oncogene* **12,** 2631–2640.

Rifkind, R. A., Richon, V. M., and Marks, P. A. (1996). Induced differentiation, the cell cycle, and the treatment of cancer. *Pharmacol. Ther.* **2,** 97–102.

Roodman, G. D. (1996). Advances in bone biology: The osteoclast. *Endocr. Rev.* **17,** 308–332.

Rots, N. Y., Liu, M., Anderson, E. C., and Freedman, L. P. (1998). A differential screen for ligand-regulated genes: Identification of HoxA10 as a target of vitamin D3 induction in myeloid leukemic cells. *Mol. Cell. Biol.* **18,** 1911–1918.

Roussel, M. F., Davis, J. N., Cleveland, J. L., Ghysdael, J., and Hiebert, S. W. (1994). Dual control of myc expression through a single DNA binding site targeted by ets family proteins and E2F-1. *Oncogene* **9,** 405–415.

Roussel, M. F., Ashmun, R. A., Sherr, C. J., Eisenman, R. N., and Ayer, D. E. (1996). Inhibition of cell proliferation by the *Mad1* transcriptional repressor. *Mol. Cell. Biol.* **16,** 2796–2801.

Roy, R., Adamczewski, J. P., Seroz, T., Vermeulen, W., Tassan, J. P., Schaeffer, L., Nigg, E. A., Hoeijmaker, J. H., and Egly, J. M. (1994). The MO15 cell cycle kinase is associated with the TFIIH transcription–DNA repair factor. *Cell* **79,** 1093–1101.

Ruis, C., Cabanas, C., and Aller, P. (1990). The induction of vimentin gene expression by sodium butyrate in human promonocytic leukemia U937 cells. *Exp. Cell Res.* **188,** 129–134.

Ryan, K. M., and Birnie, G. D. (1997a). Cell-cycle progression is not essential for c-myc to block differentiation. *Oncogene* **14,** 2835–2843.

Ryan, K. M., and Birnie, G. D. (1997b). Analysis of E-box DNA binding during myeloid differentiation reveals complexes that contain Mad but not Max. *Biochem. J.* **325,** 79–85.

Sadhu, K., Reed, S., Richardson, H., and Russell, P. (1990). Human homolog of fission yeast cdc25 mitotic inducer is predominantly expressed in G_2. *Proc. Natl. Acad. Sci. USA* **87,** 5139–5143.

Saha, P., Eichbaum, Q., Silberman, E. D., Mayer, B. J., and Dutta, A. (1997). p21CIP1 and Cdc25A: Competition between an inhibitor and an activator of cyclin-dependent kinases. *Mol. Cell. Biol.* **17,** 4338–4345.

Saini, K., Steel, G., and Thomas, P. (1990). Induction of carcinoembryonic antigen gene expression in human colorectal carcinoma by sodium butyrate. *Biochem. J.* **272,** 541–544.

Samid, D., Shack, S., and Sherman, L. (1992). Phenylacetate: A novel nontoxic inducer of cell differentiation. *Cancer Res.* **52,** 1988–1992.

Sano, M., and Kitajima, S. (1998). Activation of mitogen-activated protein kinases is not required for the extension of neurites from PC12D cells triggered by nerve growth factor. *Brain Res.* **785,** 299–308.

Savatier, P., Lapillonne, H., vanGrunsven, L. A., Rudkin, B. B., and Samarut, J. (1996). Withdrawal of differentiation inhibitory activity/leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells. *Oncogene* **12,** 309–322.

Schwaller, J., Pabst, T., Koeffler, H. P., Niklaus, G., Loetscher, P., Fey, M. F., and Tobler, A. (1997). Expression and regulation of G1 cell-cycle inhibitors (p16INK4A, p15INK4B, p18INK4C, p19INK4D) in human acute myeloid leukemia and normal myeloid cells. *Leukemia* **11,** 54–63.

Sealy, L., and Chalkley, R. (1979). Modification of histones immediately following synthesis. *Arch. Biochem. Biophys.* **197,** 78–82.

Selvakumaran, M., Liebermann, D., and Hoffman, B. (1996). The proto-oncogene c-myc blocks myeloid differentiation independently of its target gene ornithine decarboxylase. *Blood* **88,** 1248–1255.

Serrano, M., Hannon, G., and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CKD4. *Nature* **366,** 704–707.

Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., and De Pinho, R. A. (1996). Role of the INK4a locus in tumor suppression and cell mortality. *Cell* **85,** 27–37.

Shabahang, M., Buras, R., Davoodi, F., Schumaker, L., Nauta, R., and Evans, S. (1993). 1,25 Dihydroxyvitamin D_3 receptor as a marker of human colon carcinoma cell line differentiation and growth inhibition. *Cancer Res.* **53,** 3712–3718.

Shabahang, M., Buras, R., Davoodi, F., Schumaker, L., Nauta, R., Uskokovic, M., Brenner, R., and Evans, S. (1994). Growth inhibition of HT-29 human colon cancer cells by analogues of 1,25 dihydroxyvitamin D_3. *Cancer Res.* **54,** 4057–4064.

Shanmugam, M., Krett, N., Peters, C., Maizels, E., Murad, F., Kawakatsu, H., Rosen, S., and Hunzicker-Dunn, M. (1998). Association of PKC delta and active Src in PMA-treated MCF-7 human breast cancer cells. *Oncogene* **16,** 1649–1654.

Shao, R. G., Cao, C. X., Shimizu, T., O'Connor, P. M., Kohn, K. W., and Pommier, Y. (1997). Abrogation of an S-phase checkpoint and potentiation of camptothecin cytotoxicity by 7-hydroxystaurosporine (UCN-01) in human cancer cells lines, possibly influenced by p53 function. *Cancer Res.* **57,** 4029–2035.

Shao, Z. M., Alpaugh, M. L., Fontana, J. A., and Barsky, S. H. (1998). Genistein inhibits proliferation similarly in estrogen receptor-positive and negative human breast carcinoma cell lines characterized by p21WAF1/CIP1 induction, G2/M arrest, and apoptosis. *J. Cell. Biochem.* **69,** 44–54.

Sherr, C. (1993). Mammalian G_1 cyclins. *Cell* **73,** 1059–1065.

Sherr, C. (1994). G1 phase progression: Cycling on cue. *Cell* **79,** 551–555.

Sherr, C. J. (1996). Cancer cells cycles. *Science* **274,** 1672–1677.

Shi, L., Nishioka, W. K., Th'ng, J., Bradbury, E. M., Litchfield, D. W., and Greenberg, A. H. (1994). Premature p34cdc2 activation required for apoptosis. *Science* **263,** 1143–1145.

Shiyanov, P., Hayes, S., Chen, N., Pestov, D. G., Lau, L. F., and Raychaudhuri, P. (1997). p27Kip1 induces an accumulation of the repressor complexes of E2F and inhibits expression of the E2F-regulated genes. *Mol. Biol. Cell* **8,** 1815–1827.

Simpson, R. U., Todd, H., Begley, D. A., Mitchell, B. S., and Alizadeh, B. N. (1987). Transcriptional regulation of the c-myc protooncogene by 1,25-dihydroxyvitamin D3 in HL60 promyelocytic leukemia cells. *J. Biol. Chem.* **262,** 4104–4108.

Skapek, S. X., Rhee, J., Kim, P. S., Novitch, B. G., and Lassar, A. B. (1996). Cyclin-mediated inhibition of muscle gene expression via a mechanism that is independent of pRB hyperphosphorylation. *Mol. Cell. Biol.* **16,** 7043–7053.

Smith, E., Frankel, B., MacLachlan, T. K., Giordano, A., Stein, J. L., Lian, J. B., and Stein, G. S. (1997). Post-proliferative cyclin E-associated kinase activity in differentiated osteoblasts: Inhibition by proliferating osteoblasts and osteosarcoma cells. *J. Cell. Biochem.* **66,** 141–152.

Smith, E. J., Leone, G., and Nevins, J. R. (1998). Distinct mechanisms control the accumulation of the Rb-related p107 and p130 proteins during cell growth. *Cell Growth Differ.* **9,** 297–303.

Soddu, S., Blandino, G., Scardigi, R., Martinelli, R., Rizzo, M. G., Crescenzi, M., and Sacchi, A. (1996). Wild type p53 induces diverse effects in 32D cells expressing different oncogenes. *Mol. Cell. Biol.* **16,** 487–495.

Stein, G. S., Lian, J. B., and Owen, T. A. (1990). Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. *FASEB J.* **4,** 3111–3123.

Stein, G. S., Stein, J. L., Van Wijnen, A. J., and Lian, J. B. (1996). Transcriptional control of cell cycle progression: The histone gene is a paradigm for the G1/S phase and proliferation/differentiation transitions. *Cell Biol. Int.* **20,** 41–49.

Steinman, R. A., Hoffman, B., Iro, A., Guillouf, C., Liebermann, D. A., and el-Houseini, M. E. (1994). Induction of p21 (WAF-1/CIP1) during differentiation. *Oncogene* **9,** 3389–3396.

Sterner, J. M., Murata, Y., Kim, H. G., Kennett, S. B., Templeton, D. J., and Horowitz, J. M. (1995). Detection of a novel cell cycle-regulated kinase activity that associates with the amino terminus of the retinoblastoma protein in G2/M phases. *J. Biol. Chem.* **270,** 9281–9288.

Sterner, J. M., Tao, Y., Kennett, S. B., Kim, H. G., and Horowitz, J. M. (1996). The amino terminus of the retinoblastoma (Rb) protein associates with a cyclin-dependent kinase-like kinase via Rb amino acids required for growth suppression. *Cell Growth Differ.* **7,** 53–64.

Stewart, C. C. (1984). Regulation of mononuclear phagocyte proliferation. *In* "The Reticuloendothelial System" (S. M. Reichard and J. P. Filkins, Eds.), Vol. 7A, pp. 37–56. Plenum, New York.

Strausfeld, U., Fernandez, A., Capony, J., Girad, F., Lautredou, N., Derancourt, J., Labbe, J., and Lamb, N. (1994). Activation of $p34^{cdc2}$ protein kinase by microinjection of human cdc25c into mammalian cells. *J. Biol. Chem.* **269,** 5989–6000.

Studzinski, G. P., and Brelvi, Z. S. (1987). Changes in proto-oncogene expression associated with reversal of macrophage-like differentiation of HL60 cells. *JNCI* **79,** 67–76.

Studzinski, G. P., Bhandal, A., and Brelvi, Z. (1985). A system for monocytic differentiation of leukemic cells HL60 by a short exposure to 1,25-dihydroxycholecalciferol. *Proc. Soc. Exp. Biol. Med.* **179,** 288–295.

Studzinski, G. P., Rathod, B., Rao, J., Kheir, A., Wajchman, H. J., Zhang, F., Finan, J. B., and Nowell, P. C. (1996). Transition to tetraploidy in 1,25-dihydroxyvitamin D_3-resistant HL60 cells is preceded by reduced growth factor dependence and constitutive up-regulation of Sp1 and AP-1 transcription factors. *Cancer Res.* **56,** 5513–5521.

Sugawara, A., Sanno, N., Takahashi, N., Osamura, R., and Abe, K. (1997). Retinoid X receptors in the kidney: Their protein expression and functional significance. *Endocrinology* **138,** 3175–3180.

Takeda, T., Nishiguchi, S., Kuroki, T., Kobayashi, K., Hasuma, T., Matsui-Yuasa, I., and Otani, S. (1991). Reduction by interferon-alpha of levels of c-myc protein and DNA synthesis in a human hepatoma cell line mediated by inhibition of putrescine synthesis. *Biochem. Biophys. Res. Commun.* **178,** 378–384.

Tanaka, E. M., Gann, A. A., Gates, P. B., and Brockes, J. P. (1997). New myotubes reenter the cell cycle by phosphorylation of the retinoblastoma protein. *J. Cell Biol.* **136,** 155–165.

Tang, X. M., Strocchi, P., and Cambi, F. (1998). Changes in the activity of cdk2 and cdk5 accompany differentiation of rat primary oligodendrocytes. *J. Cell. Biochem.* **68,** 128–137.

Taylor, C., Kim, Y., Childress-Fields, K., and Yeoman, L. (1992). Sensitivity of nuclear c-myc levels and induction to differentiation-inducing agents in human colon cancer cell lines. *Cancer Lett.* **62,** 95–105.

Tchou, W. W., Rom, W. N., and Tchou-Wong, K. M. (1996). Novel form of p21(WAF1/CIP1/SDI1) protein in phorbol ester-induced G2/M arrest. *J. Biol. Chem.* **271,** 29556–29560.

Tedesco, D., Caruso, M., Fischer-Fantuzzi, L., and Vesco, C. (1995). The inhibition of cultured myoblast differentiation by the simian virus 40 large T antigen occurs after myogenin expression and Rb up-regulation and is not exerted by transformation-competent cytoplasmic mutants. *J. Virol.* **69,** 6947–6957.

Thomas, M., Tebbutt, S., and Williamson, R. (1992). Vitamin D and its metabolites inhibit cell proliferation in human rectal mucosa and a colon cancer cell line. *Gut* **33,** 1660–1663.

Thomas, R. C., Edwards, M. J., and Marks, R. (1996). Translocation of the retinoblastoma gene product during mitosis. *Exp. Cell Res.* **223,** 227–232.

Thomas, X., Anglaret, B., Thiegaut, A., Belhabri, A., Treille-Ritoouet, D., Fiere, D., and Archimbaud, E. (1997). Improvement of prognosis in refractory and relapsed acute promyelocytic leukemia over recent years: The role of all-trans retinoic acid therapy. *Ann. Hematol.* **75,** 195–200.

Tiainen, M., Pajalunga, D., Ferrantelli, F., Soddu, S., Salvatori, G., Sacchi, A., and Crescenzi, M. (1996a). Terminally differentiated skeletal myotubes are not confined to G0 but can enter G1 upon growth factor stimulation. *Cell Growth Differ.* **7,** 1039–1050.

Tiainen, M., Spitkovsky, D., Jansen, D. P., Sacchi, A., and Crescenzi, M. (1996b). Expression of E1A in terminally differentiated muscle cells reactivates the cell cycle and suppresses tissue-specific genes by separable mechanisms. *Mol. Cell. Biol.* **16,** 5302–5312.

Tikoo, R., Casaccia, B. P., Chao, M. V., and Koff, A. (1997). Changes in cyclin-dependent kinase 2 and p27Kip1 accompany glial cell differentiation of central glia-4 cells. *J. Biol. Chem.* **272,** 442–447.

Urashima, M., DeCaprio, J. A., Chauhan, D., Teoh, G., Ogata, A., Treon, S. P., Hoshi, Y., and Anderson, K. C. (1997). p16INK4A promotes differentiation and inhibits apoptosis of JKB acute lymphoblastic leukemia cells. *Blood* **90,** 4106–4115.

Uren, A., Jakus, J., deMora, J. F., Yeudall, A., Santos, E., Gutkind, S., and Heidaran, M. A. (1997). Carboxyl-terminal domain of p27Kip1 activates Cdc2. *J. Biol. Chem.* **272,** 21669–21672.

Uskokovic, M. R., Studzinski, G. P., Gardner, J. P., Reddy, S. G., Campbell, M. J., and Koeffler, P. H. (1997). The 16-ene vitamin D analogs. *Curr. Pharmacol. Design* **3,** 99–123.

Van den Bemd, G., Pols, H., Birkenhäger, J., Kleinkoort, W., and van Leeuwen, J. (1995). Differential effects of 1,25 dihydroxyvitamin D_3-analogs on osteoblast-like cells and on in vitro bone resorption. *J. Steroid Biochem. Mol. Biol.* **55,** 337–346.

Van Grunsven, L. A., Billion, N., Savatier, P., Thomas, A., Urdiales, J. L., and Rudkin, B. B. (1996). Effect of nerve growth factor on the expression of cell cycle regulatory proteins in PC12 cells: Dissection of the neurotrophic response from the anti-mitogenic response. *Oncogene* **12,** 1347–1356.

Van Hal, P. Th., Wijkhuijs, J. M., Mulder, P. G. H., and Hoogseden, H. C. (1995). Proliferation of mature and immature subpopulations of bronchoalveolar monocytes/macrophages and peripheral blood monocytes. *Cell Prolif.* **28,** 533–543.

Van Wijnen, A. J., vanGurp, M. F., deRidder, M. C., Tufarelli, C., Last, T. J., Birnbaum, M., Vaughan, P. S., Giordano, A., Krek, W., Neufeld, E. J., Stein, J. L., and Stein, G. S. (1996). CDP/cut is the DNA-binding subunit of histone gene transcription factor HiNF-D: A mechanism for gene regulation at the G1/S phase cell cycle transition point independent of transcription factor E2F. *Proc. Natl. Acad. Sci. USA* **93,** 11516–11521.

Vincent, I., Jicha, G., Rosado, M., and Dickson, D. W. (1997). Aberrant expression of mitotic cdc2/cyclin B1 kinase in degenerating neurons of Alzheimer's disease brain. *J. Neurosci.* **17,** 3588–3598.

Vogt, M., Haggblom, C., Yeargin, J., Christiansen-Weber, T., and Haas, M. (1998). Independent induction of senescence by p16INK4a and p21CIP1 in spontaneously immortalized human fibroblasts. *Cell Growth Differ.* **9,** 139–146.

Waga, S., Hannon, G., Beach, D., and Stillman, B. (1994). The p21 cyclin dependent kinase inhibitor directly controls DNA replication via interaction with PCNA. *Nature* **369,** 574–578.

Waldman, T., Lenguer, C., Kinzler, K. W., and Volgelstein, B. (1996). Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. *Nature* **381,** 713–716.

Wali, R., Bissonette, M., Khare, S., Hart, J., Sitrin, M., and Brasitus, T. (1995). 1α, 25-Dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol, a noncalcemic analogue of 1α,25 dihydroxyvitamin D_3, inhibits azoxymethane-induced colonic tumorigenesis. *Cancer Res.* **55,** 3050–3054.

Wang, J., and Nadal-Ginard, B. (1995). Regulation of cyclins and p34CDC2 expression during terminal differentiation of C2C12 myocytes. *Biochem. Biophys. Res. Commun.* **206,** 82–88.

Wang, J., Guo, K., Willis, K. N., and Walsh, K. (1997). Rb functions to inhibit apoptosis during myocyte differentiation. *Cancer Res.* **57,** 351–354.

Wang, Q. M., Jones, J. B., and Studzinski, G. P. (1996). Cyclin-dependent kinase inhibitor p27 as a mediator of the G1–S phase block induced by 1,25-dihydroxyvitamin D_3 in HL60 cells. *Cancer Res.* **56,** 264–267.

Wang, Q. M., Luo, X., and Studzinski, G. P. (1997). Cyclin-dependent kinase 6 is the principal target of p27/kip1 regulation of the G1-phase trasverse in 1,25-dihydroxyvitamin D3-treated HL60 cells. *Cancer Res.* **57,** 2851–2855.

Wang, Q. M., Chen, F., Luo, X., Moore, D. C., Flanagan, M., and Studzinski, G. P. (1998). Lowering of p27Kip1 levels by its antisense or by development of resistance to 1,25-dihydroxyvitamin D_3 reverses the G1 block but not differentiation of HL60 cells. *Leukemia,* in press.

Wang, X., and Studzinski, G. P. (1997). Antiapoptotic action in 1,25-dihydroxyvitamin D3 is associated with increased mitochondrial MCL-1 and RAF-1 proteins and reduced release of cytochrome c. *Exp. Cell Res.* **235,** 210–217.

Wang, X., Gardner, J. P., Kheir, A., Uskokovic, M. R., and Studzinski, G. P. (1997). Synergistic induction of HL60 cell differentiation by ketoconazole and 1-desoxy analogues of vitamin D. *JNCI* **89,** 1199–1206.

Weima, S., Van Rooigen, M., Feijen, A., Mummery, C., Van Zoelen, E., Delaat, S., and Van Den Eijnden-Van raaij, A. (1989). Transforming growth factor-beta and its receptor are differentially regulated in human embryonal carcinoma cells. *Differentiation* **41,** 245–253.

Wisdom, R., and Lee, W. (1990). Translation of c-myc mRNA is required for its post-transcriptional regulation during myogenesis. *J. Biol. Chem.* **265,** 19105–19021.

Witt, O., Albig, W., and Doenecke, D. (1997). Transcriptional regulation of the human replacement histone gene H3.3B. *FEBS Lett.* **408,** 255–260.

Yam, L. T., Li, C. Y., and Crosby, W. (1971). Cytochemical identification of monocytes and granulocytes. *Am. J. Clin. Pathol.* **55,** 283–290.

Yan, G. Z., and Ziff, E. B. (1995). NGF regulates the PC12 cell cycle machinery through specific inhibition of the Cdk kinases and induction of cyclin D1. *J. Neurosci.* **15,** 6200–6212.

Yan, G. Z., and Ziff, E. B. (1997). Nerve growth factor induces transcription of the p21WAF1/CIP1 and cyclin D1 genes in PC12 cells by activating the Sp1 transcription factor. *J. Neurosci.* **17,** 6122–6132.

Yan, Y., Frisen, J., Lee, M. H., Massague, J., and Barbacid, M. (1997). Ablation of the CDK inhibitor p57Kip2 results in increased apoptosis and delayed differentiation during mouse development. *Genes Dev.* **11,** 973–983.

Yan, Z., DeGregori, J., Shohet, R., Leone, G., Stillman, B., Nevins, J. R., and Williams, R. S. (1998). Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells. *Proc. Natl. Acad. Sci. USA* **95,** 3603–3608.

Yen, A., and Varvayanis, S. (1994). Late dephosphorylation of the RB protein in G2 during the process of induced cell differentiation. *Exp. Cell Res.* **214,** 250–257.

Yokoe, H., Masumi-Fukazawa, F. A., Sunohara, M., Tanzawa, H., Sato, K., Sato, T., and Fuse, A. (1997). Induction of polyploidization in the human erythroleukemia cell line (HEL) by protein kinase inhibitor (K252a) and the phorbol-ester TPA. *Leukemia Lymphoma* **25,** 333–343.

Yun, K., and Wold, B. (1996). Skeletal muscle determination and differentiation: Story of a core regulatory network and its context. *Curr. Opin. Cell Biol.* **8,** 877–889.

Zabludoff, S. D., Csete, M., Wagner, R., Yu, X., and Wold, B. J. (1998). p27Kip1 is expressed transiently in developing myotomes and enhances myogenesis. *Cell Growth Differ.* **9,** 1–11.

Zacksenhaus, E., Jiang, Z., Chung, D., Marth, J. D., Phillips, R. A., and Gallie, B. L. (1996). pRb controls proliferation, differentiation, and death of skeletal muscle cells and other lineages during embryogenesis. *Genes Dev.* **23,** 3051–3064.

Zavitz, K. H., and Zipursky, S. L. (1997). Controlling cell proliferation in differentiating tissues: Genetic analysis of negative regulators of G1 → S phase progression. *Curr. Opin. Cell Biol.* **9,** 773–781.

Zelent, A., Krust, A., Petrovich, M., Kastner, P., and Chambon, P. (1989). Cloning of murine alpha and beta retinoic receptors and a novel receptor gamma predominantly expressed in the skin. *Nature* **339,** 714–717.

Zhang, F., Godyn, J. J., Uskokovic, M., Binderup, L., and Studzinski, G. P. (1994). Monocytic differentiation of HL60 cells induced by potent analogs of vitamin D_3 precedes the G1/G0 phase cell cycle block. *Cell Prolif.* **27,** 643–654.

Zhang, F., Rathod, B., Jones, J. B., Wang, Q. M., Bernhard, E., Godyn, J. J., and Studzinski, G. P. (1996). Increased stringency of the 1,25-dihydroxyvitamin D_3-induced G1 to S phase block in polyploid HL60 cells. *J. Cell Physiol.* **168,** 18–25.

Zhang, Q., Ahuja, H. S., Zakeri, Z. F., and Wolgemuth, D. J. (1997). Cyclin-dependent kinase 5 is associated with apoptotic cell death during development and tissue remodeling. *Dev. Biol.* **183,** 222–233.

Zhang, W., Grasso, L., McClain, A. M., Cha, Y., Travali, S., Deisseroth, A. B., and Mercer, W. E. (1995). p53-independent induction of WAF1/CIP1 in human leukemia cells is correlated with growth arrest accompaning monocyte/macrophage differentiation. *Cancer Res.* **55,** 668–674.

Zhang, Y., and Chellapan, S. (1996). Transcriptional activation and expression of DP-transcription factors during cell cycle and TPA-induced U937 differentiation. *Mol. Cell. Differ.* **4,** 297–316.

Zhang, Y., Xiong, Y., and Yarbarough, W. G. (1998). ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* **92,** 725–734.

Zheng, Z., Polakowaka, R., Johnson, A., and Goldsmith, L. (1992). Transcriptional control of epidermal growth factor receptor by retinoic acid. *Cell Growth Differ.* **3,** 225–232.

Zukerberg, L. R., Benedict, W. F., Arnold, A., Syson, N., Harlow, E., and Harris, N. L. (1996). Expression of the retinoblastoma protein in low-grade B-cell lymphoma: Relationship to cyclin D1. *Blood* **88,** 268–276.

Zwijsen, R. M., Wientjens, E., Klompmaker, R., van der Sman, J., Bernards, R., and Michalides, R. J. (1997). CDK-independent activation of estrogen receptor by cyclin D1. *Cell* **88,** 405–417.

Nitric Oxide and Endothelin-1 in Coronary and Pulmonary Circulation

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Since the discovery of the vasorelaxant properties of nitric oxide and the vasoconstrictor effect of endothelin-1, there have been many studies of the distribution and functional significance of these agents in various vascular beds. In the coronary and pulmonary circulation nitric oxide and endothelin-1 actions have been largely investigated in terms of an imbalance between the opposing effects of these vasoactive agents leading to pathophysiological conditions. This article reviews functional and immunocytochemical studies with emphasis on the ultrastructural localization of nitric oxide synthase and endothelin-1 in the coronary and pulmonary vascular beds. Localization of nitric oxide synthase (type III or I or II) has been shown in endothelial cells, smooth muscle, and perivascular nerves of the coronary and pulmonary vascular beds and in the neurons, nerve fibers, and the small granule-containing cells within cardiac ganglia. Endothelin-1 was mainly localized in subpopulations of coronary and pulmonary endothelial cells. These immunocytochemical studies provide information about the sources of nitric oxide and endothelin-1 that contribute to the vasomotor control of cardiac and pulmonary circulation under normal and pathophysiological conditions.

KEY WORDS: Coronary vessels, Pulmonary vessels, Nitric oxide, Nitric oxide synthase, Endothelin, Hypertension, Ischemia, Aging.

I. Introduction

For many years studies on the physiology of the cardiovascular system were mainly concerned with the action of circulating catecholamines and the effects of noradrenaline (NA) and acetylcholine (ACh) released from the autonomic nerves within the innervated tissues. However, during the past

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20 years the identification of many new molecules as autonomic neurotransmitters, including neuropeptides, purines, monoamines, and, recently, nitric oxide (NO), and of their roles as cotransmitters has significantly reshaped our knowledge of the mechanisms of autonomic regulation of vascular tone (Burnstock, 1990a,b; Burnstock and Ralevic, 1996).

A crucial contribution to the current understanding of the physiology of the cardiovascular system derived from the seminal discovery by Furchgott and Zawadzki (1980) that the endothelium has a key role in mediating the vasodilator action of ACh and other vasoactive substances via release of an endothelium-derived relaxing factor (EDRF). In 1986 Furchgott and Ignarro advanced independently the hypothesis that EDRF is pharmacologically identical to NO [Fourth Symposium on Mechanisms of Vasodilation, Rochester, Minnesota, July 1986—later published as Furchgott (1988) and Ignarro *et al.* (1988); see also Ignarro *et al.,* 1986] and subsequently EDRF was demonstrated to be chemically identical to NO or a labile nitroso precursor to NO (Furchgott *et al.,* 1987; Ignarro *et al.,* 1987a,b; Palmer *et al.,* 1987, 1988). Several other vasoactive substances, including the potent vasoconstrictor endothelin-1 (ET-1; Yanagisawa *et al.,* 1988), are stored in and released from endothelial cells, under physiological and pathophysiological conditions, and accumulating evidence in this respect has contributed to the current view that the endothelium is an active regulator of vascular tone. Therefore, within the vessel wall, the spatial localization of the contractile smooth muscle layer between autonomic and sensory innervation at the external side and endothelium on the inner surface allows a dual control of the vascular tone by vasoactive substances of both neuronal and endothelial origin (Burnstock and Ralevic, 1994).

Following a general introduction to the neuronal and endothelial mechanisms of regulation of vascular tone, this article will focus on the immunocytochemical localization of nitric oxide synthase (NOS), the enzyme responsible for NO synthesis, used as a marker of NO production and release, and ET-1 in both neuronal and endothelial elements of the vascular wall in the coronary and pulmonary circulation. The influence of intramural ganglia on the control of coronary and pulmonary vascular tone will also be discussed. Evidence will be provided for the vascular effects and functional interactions of NO and ET-1 within coronary and pulmonary circulation. Finally, the plasticity of expression and function of NO and ET-1 in pathophysiological conditions, including coronary ischemia and pulmonary hypertension, will be considered.

A. Neuronal Control of Vascular Tone

The concept of cotransmission, first proposed by Burnstock (1976) and based on evidence that more than one transmitter can be stored and released

from autonomic nerves, is now widely recognized together with the proposed mechanisms of pre- and postjunctional neuromodulation. These involve inhibition or enhancement of the neurotransmitter released and modulation of the transmitter effects at the postjunctional level (Cuello, 1982; Burnstock, 1990a; Mione *et al.,* 1990). It is documented that NA, ATP, and neuropeptide Y (NPY) coexist in perivascular sympathetic nerves, the proportion of the release being dependent on the tissue, species, and the parameters of stimulation (Burnstock, 1986, 1995). Many studies have provided functional evidence for cotransmission involving NA and ATP in large vessels and isolated vascular beds, including the rabbit pulmonary artery (Katsuragi and Su, 1982; Burnstock, 1990b,c, 1995; Burnstock and Ralevic, 1994). In many vessels NPY has little postjunctional direct effects on the vasculature, its major role being that of a prejunctional neuromodulator of NA and ATP release and postjunctional enhancer of the contractile action of NA and ATP (Stjarne, 1989; Saville *et al.,* 1990). Following the initial finding that the brain rather than the endothelium is the major source of NO in the body, a role as autonomic neurotransmitter was suggested for NO (Bult *et al.,* 1990; Bredt and Snyder, 1992; Rand, 1992). "Nitrergic transmission" has been shown in both the central and peripheral nervous system, with NO being involved in the central and peripheral autonomic control of blood flow and pressure (Rand, 1992; Moncada and Higgs, 1993). Immunoreactivity for NOS has been shown in preganglionic sympathetic neurons in the rat (Anderson *et al.,* 1993), together with positive staining for the redox cofactor nicotinamide adenine dinucleotide hydrogen phosphate diaphorase (NADPH-d; Morris *et al.,* 1993).

Information on the parasympathetic cotransmission mainly derives from studies of vessels of the cat salivary gland, in which parasympathetic nerves costore and corelease vasoactive intestinal polypeptide (VIP) with ACh. The picture of parasympathetic cotransmission in the cardiovascular system is more complex because the peptide histidine isoleucine (derived from the same precursor molecule as VIP; namely, prepro-VIP), calcitonin gene-related peptide (CGRP), and NPY have each been separately colocalized with VIP and/or choline acetyltransferase in parasympathetic perivascular nerves (Lundberg, 1981). Vasodilator activity of all these transmitters has been shown in several blood vessels, including the vasodilator effects of VIP in the pulmonary vasculature (Ignarro *et al.,* 1987c; Edvinsson, 1991). Several studies demonstrate that parasympathetic perivascular nerves may produce and release NO as a cotransmitter with ACh and VIP (Snyder, 1992).

Beside sympathetic and parasympathetic neurotransmission, perivascular sensory motor neurotransmission via peripheral release of vasoactive sensory neuropeptides (namely, CGRP, substance P, and related neurokinins) from capsaicin-sensitive sensory fibers has been documented in several

blood vessels, including guinea-pig pulmonary and coronary arteries (Liu *et al.,* 1992). Perivascular sensory-motor neurotransmission has vasodilator actions that are mediated by CGRP and can contribute to the local regulation of vascular tone in several pathophysiological conditions, including ischemia and hypoxia (Rubino and Burnstock, 1996). A role for NO as a sensory neurotransmitter has been suggested (Rand, 1992; Moncada and Higgs, 1993). Furthermore, functional evidence has been provided showing that NO is a nonadrenergic, noncholinergic (NANC) perivascular neurotransmitter in cerebral mesenteric and penile circulation (Burnett *et al.,* 1992; Toda *et al.,* 1993). The contribution of NO and ATP was also shown in perivascular NANC neurotransmission of the rabbit portal vein (Brizzolara *et al.,* 1993).

The contribution of intrinsic neurons of the heart to the physiology of the coronary circulation and cardiac contractility is still little known because of the difficulties in investigating their activity *in situ.* Studies in culture of intrinsic neurons isolated from the atria of newborn guinea pigs have shown immunoreactivity for NPY and/or serotonin. Nitric oxide synthase has also been shown in a subpopulation of intrinsic cardiac neurons (Hassall *et al.,* 1992, 1993; see Section II,A,2). Projections of these neurons *in situ* form perivascular plexuses in small resistance coronary vessels. Few studies have been performed on the projections of intrinsic neurons to the blood vessels supplying other organs, including the lung.

B. Endothelial Control of Vascular Tone

The importance of endothelium as a mediator of vasodilatation stemmed from the pioneering discovery of Furchgott and Zawadzki in 1980 that endothelium is essential to the vasodilator action of ACh and other substances via release of EDRF. Following an important breakthrough in the study of EDRF, when it was proposed that EDRF was NO, it is a general consensus today that EDRF is NO or a labile compound released from L-arginine (Furchgott *et al.,* 1987; Ignarro *et al.,* 1986, 1987a,b; Palmer *et al.,* 1987). Because endothelium-dependent vasodilatation involves hyperpolarization of the smooth muscle cell membrane, which in several blood vessels, including coronary arteries, is not mediated by NO, it was suggested that endothelium can release unidentified endothelium-derived hyperpolarizing factors (EDHF; Garland *et al.,* 1995; Mombouli and Vanhoutte, 1997). Endothelium-mediated vasodilator mechanisms also include the synthesis and release of prostacyclin, which mediate part of the endothelium-dependent hyperpolarization and associated vasodilatation in several blood vessels, including coronary arteries (Corriu *et al.,* 1996).

Endothelial cells are also sources of endothelium-derived vasoconstrictor factors (EDCFs) released following stimulation of endothelial cells by a variety of chemical and mechanical stimuli (Rubanyi and Vanhoutte, 1985; Katusic *et al.,* 1987). Among EDCFs, endothelin-1, originally identified in the culture supernatant of porcine aortic endothelial cells (Yanagisawa *et al.,* 1988), has attracted considerable attention because it is the most potent vasoconstrictor known. Release of endothelin has been shown from isolated aortic endothelial cells exposed to increased flow (Milner *et al.,* 1990), which suggests the contribution of this peptide to vasoconstriction and increased vascular resistance in pathophysiological conditions such as hypoxic vasoconstriction and hypertension, in which shear stress is increased. Beside having direct vasoactive effects, endothelin has mitogenic actions and is implicated in the proliferation of vascular smooth muscle in vascular injury and pathological events during atherogenesis (Yanagisawa *et al.,* 1988; Hirata *et al.,* 1989; Hahn *et al.,* 1993). A comitogen effect of ET-1, potentiating the proliferative effects of other factors has also been shown (Weissberg *et al.,* 1990). Furthermore, ET-1 is an autocrine growth factor for endothelial cells (Eguchi *et al.,* 1995), thus contributing to long-term maintenance of vascular tone and/or structure.

Synthesis and release from the endothelium of substances with opposite vascular effects, such as the vasodilator NO and the vasoconstrictor ET-1, indicate a fine control of the vascular tone within the vascular wall, to which these two agents appear to give the most significant contribution. Several studies indicate a direct interaction between NO and ET-1 at the level of mechanisms of synthesis and release as well as at a functional level, as detailed in the following sections.

C. NO and NOS Isoforms

Nitric oxide is produced via oxidation of L-arginine to NO and L-citruline, with NOS being the catalyzing enzyme in this conversion (Palmer *et al.,* 1987, 1988; Moncada *et al.,* 1991; Knowles and Moncada, 1994). Because the half-life of NO in biological tissues is of the order of milliseconds, its localization is technically difficult. Therefore, the enzyme responsible for its synthesis, NOS, and its redox-active cofactor NADPH have been used as markers for NO localization. The enzymatic reaction which leads to cytosolic production of NO is also the target of functional studies. Most investigations on the biological actions of NO were made possible because one of the most useful features of NO synthesis is that a number of L but not D-arginine analogs (e.g., L-NAME and L-NMMA) can be taken up by cells and competitively inhibit NO synthesis.

Following the initial localization of NOS in the endothelium, data obtained in different tissues have led to the current view that a family of NOS isoenzymes catalyzes NO production, including the "constitutive" isoenzymes NOS-III [endothelial NOS (eNOS)] and NOS-I [brain or neuronal NOS (bNOS or nNOS)] and the "inducible" isoenzyme NOS-II [iNOS; macrophage NOS (macNOS)] (Bredt and Snyder, 1990; Förstermann *et al.,* 1991; Moncada *et al.,* 1991; Knowles and Moncada, 1994). At least two putative variants of NOS-I have been described; namely, $NOS\text{-}I_{144}$ and $NOS\text{-}I_{155}$, the expression of which (in the brain) may not be constitutive but locally induced (Ogilvie *et al.,* 1995). Although NOS-I is generally thought to be the isoform for the generation of NO in neurons (Bredt *et al.,* 1990), subpopulations of endothelial cells in various vascular beds also seem to contain NOS-I (Loesch and Burnstock, 1996a).

D. Endothelins

Endothelin-1 is a representative member of structurally related peptides of the endothelin family, which includes ET-1, endothelin-2 (ET-2), and endothelin-3 (ET-3). The human gene encoding for ET-1 has been localized on chromosome 6 (Inoue *et al.,* 1989; Tasaka and Kitazumi, 1994). All three endothelins are cleaved from the endothelin precursors. A 38-amino acid intermediate, big ET-1, becomes mature ET-1 after proteolytic processing catalyzed by ET-converting enzyme (Yanagisawa *et al.,* 1988). Each endothelin consists of 21 amino acid residues, although each of these has a different pattern of tissue distribution and all possess different biological activities (Tasaka and Kitazumi, 1994). Only ET-1 has been localized in vascular endothelial cells, whereas ET-2 and ET-3 are also expressed in other nonvascular cells within the brain, lung, and kidney.

E. Mechanisms of Action of NO and ET-1

Following its synthesis in endothelial or neuronal cells, NO reaches the extracellular fluid by passive diffusion through the plasma membrane. The principal physiological actions of either endothelial or neuronal NO on the vascular smooth muscle are associated with the activation of cytosolic (soluble) guanylate cyclase (GC), consequent formation of cGMP, and muscle relaxation (Lincoln, 1989; Moncada *et al.,* 1991). Although GC is the NO "receptor" on the target cell, it has also been reported that some NO effects are not cGMP dependent, suggesting the involvement of other second messenger systems (Garg and Hassid, 1991).

Once released from the endothelium, ET-1 exerts its biological actions via activation of specific receptors. To date, two subtypes of endothelin receptors have been cloned, sequenced, and pharmacologically characterized: the ET_A receptor, selective for ET-1 over ET-3 and mediating vasoconstriction, and the ET_B receptor, evoking vasodilatation when present on the vascular endothelium or constriction when present on vascular smooth muscle (Arai *et al.*, 1990; Masaki *et al.*, 1991). A subdivision of the ET_B receptor into ET_{B1} and ET_{B2} is based mainly on pharmacological data. However, evidence indicates that there are subtypes of ET_B receptors that are influenced differently by receptor antagonists and by the opposing vasodilator effect of NO (Inoue *et al.*, 1989; Masaki *et al.*, 1991; Hay and Luttman, 1997). Following activation of these specific receptors, functional responses to ET-1 occur via the cyclooxygenase pathway, release of NO, and activation of ATP-sensitive potassium channels (Rubanyi and Polokoff, 1996). The contribution of ET_A and ET_B receptor subtypes to the vasoconstrictor effect of ET-1 differs in different species and blood vessels and their expression may be modified in pathophysiological conditions, as detailed in the following sections.

II. Coronary Circulation

A. Localization of NOS and ET-1

1. Endothelium

Light microscopy combined with the immunohistochemistry of polyclonal antibodies to NOS-I from rat brain demonstrated for the first time immunoreactivity for NOS localized in the endothelium of the rat coronary blood vessels (Bredt *et al.*, 1990). A subsequent study with a rabbit polyclonal anti-NOS-I antibody (raised to soluble NOS-I purified from rat cerebellum; Schmidt *et al.*, 1992) combined with the preembedding peroxidase–antiperoxidase (PAP) immunocytochemistry for electron microscopy revealed labeling for NOS within the cytoplasm of endothelial cells and vascular smooth muscle of coronary arteries of the newborn rat (Fig. 1). PAP immunocytochemistry of normotensive and hypertensive rats localized immunoreactivity for NOS-III (by monoclonal antibody) within a subpopulation of coronary endothelial cells (Shochina *et al.*, 1997). Postembedding immunogold techniques for electron microscopy showed association of gold particles marking antigenic sites for NOS-I in the cytoplasmic matrix of the endothelial cells of the Wistar rat coronary arteries (Dikranian *et al.*, 1994). Light microscopic examination of the human hearts revealed immu-

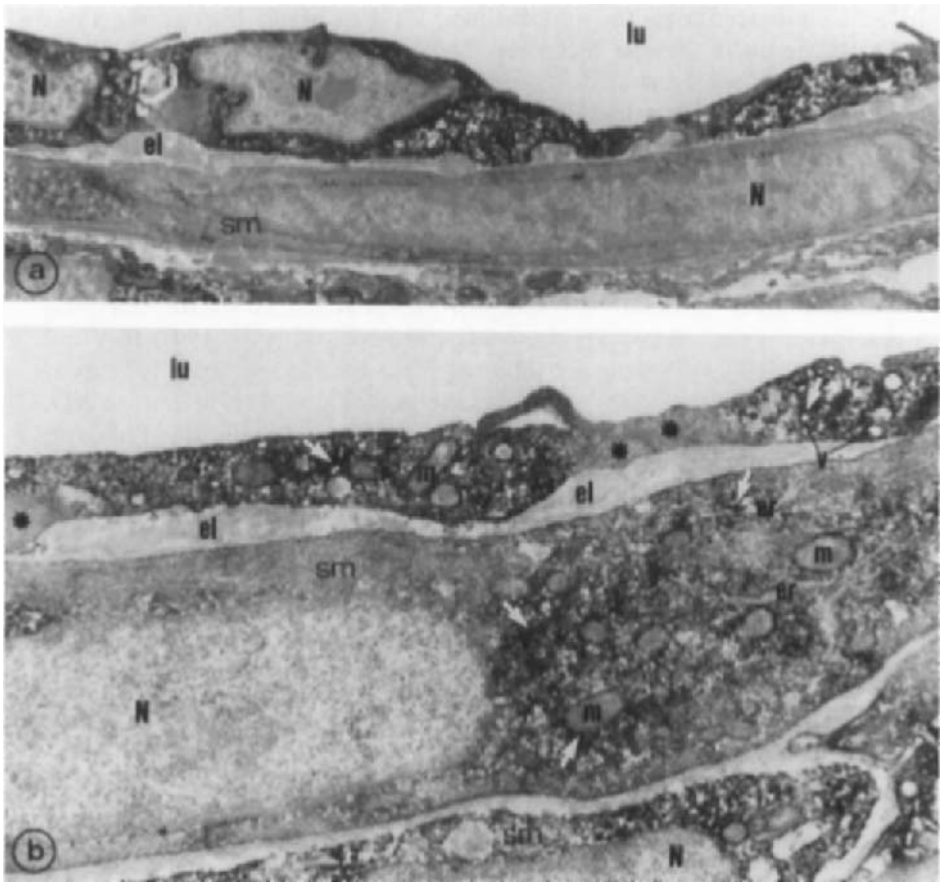

FIG. 1 Electron micrographs of newborn rat coronary artery labeled for NOS. (a) Note a group of NOS-positive endothelial cells (showing "black" cytoplasmic labeling). N, nucleus; el, elastic lamina; sm, smooth muscle; lu, lumen. Magnification, ×9,000. (b) Higher magnification example of NOS-positive endothelium and NOS-positive vascular smooth muscle; unlabeled endothelial cell profiles are also seen (asterisks). Note particles of immunoprecipitate (arrows) in the cellular matrix and in association with membrane of mitochondria (m), endoplasmic reticulum (er), and cytoplasmic vesicles (v). Magnification, ×22,000 (reproduced with permission from Loesch and Burnstock, 1995).

noreactivity for "constitutive" NOS (cNOS) in the endothelium of the intermyocardial and endocardial vasculature, including arteries, veins, arterioles, and venules (Wildhirt *et al.*, 1995a).

Using light microscopy, Wharton and colleagues (1991) revealed the presence of ET-1 in a heterogeneous population of vascular endothelial cells in human fetal heart. Ultrastructural studies of the rat coronary

vascular bed demonstrated cytoplasmic localization of ET-1 in subpopulations of intact endothelial cells (Fig. 2; Loesch and Burnstock, 1995; Shochina *et al.,* 1997).

Postembedding colloidal gold double-immunolabeling techniques for electron microscopy directly revealed the colocalization of NOS-I with ET-1, substance P, and arginine–vasopressin in intact endothelial cells of rat coronary artery (Dikranian *et al.,* 1994). PAP ultrastructural studies combined with the semiquantitative assessment of the distribution of NOS-I- and ET-1-positive endothelial cells in the coronary arteries of newborn rats indirectly indicated that NOS/NO and ET-1 are colocalized in some of the coronary endothelial cells (Loesch and Burnstock, 1995; see Section IV).

2. Perivascular Nerves—Intramural Ganglia

Light microscopic studies have revealed in rat and guinea-pig heart that NOS-I immunoreactivity and NADPH-diaphorase activity are localized in the cardiac ganglion cells and in nerve fibers innervating the sinoatrial and atrioventricular nodes, the myocardium, local neurons, and coronary arteries (Hassall *et al.,* 1992; Klimaschewski *et al.,* 1992; Schmidt *et al.,* 1992; Pollock *et al.,* 1993; Tanaka *et al.,* 1993a,b). These studies, therefore, provided immunocytochemical evidence for a role of NO in the local control of the heart by intrinsic neurons. Electron immunocytochemistry of the rat and guinea-pig heart (Sosunov *et al.,* 1995) revealed many NOS-I-labeled nerve fibers in the walls of the main branches of the coronary arteries and in arterioles, capillaries, and postcapillary venules (Figs. 3 and 4); NOS-I-positive fibers were also present in nerve bundles located near blood vessels or in connective tissue of the endocardium and myocardium. Varicosities of NOS-I-positive nerve fibers associated with the adventitial layer of the coronary blood vessels (rat and guinea pig) contain predominantly small agranular synaptic vesicles but some larger agranular vesicles may also be present (Sosunov *et al.,* 1995). It is suggested that these fibers may represent the processes of intracardiac neurons and/or sensory neurons of extrinsic origin (Sosunov *et al.,* 1995, 1996). Ultrastructural studies of the rat and guinea-pig hearts show that a subpopulation of intracardiac neurons display NOS-I immunoreactivity throughout the neuronal cell bodies and their processes; these neurons receive synapses made by NOS-I-negative nerve fibers (Sosunov *et al.,* 1996). These studies also revealed many NOS-I-positive nerve fibers that made synaptic contacts with NOS-I-negative intracardiac neurons. Finally, a subpopulation of small granule-containing cells (SIF cells) in rat and guinea-pig hearts displays immunoreactivity for NOS-I and contacts with the NOS-I-negative nerve fibers; NOS-positive nerve fibers were associated with the NOS-I-negative SIF cells (Sosunov *et al.,*

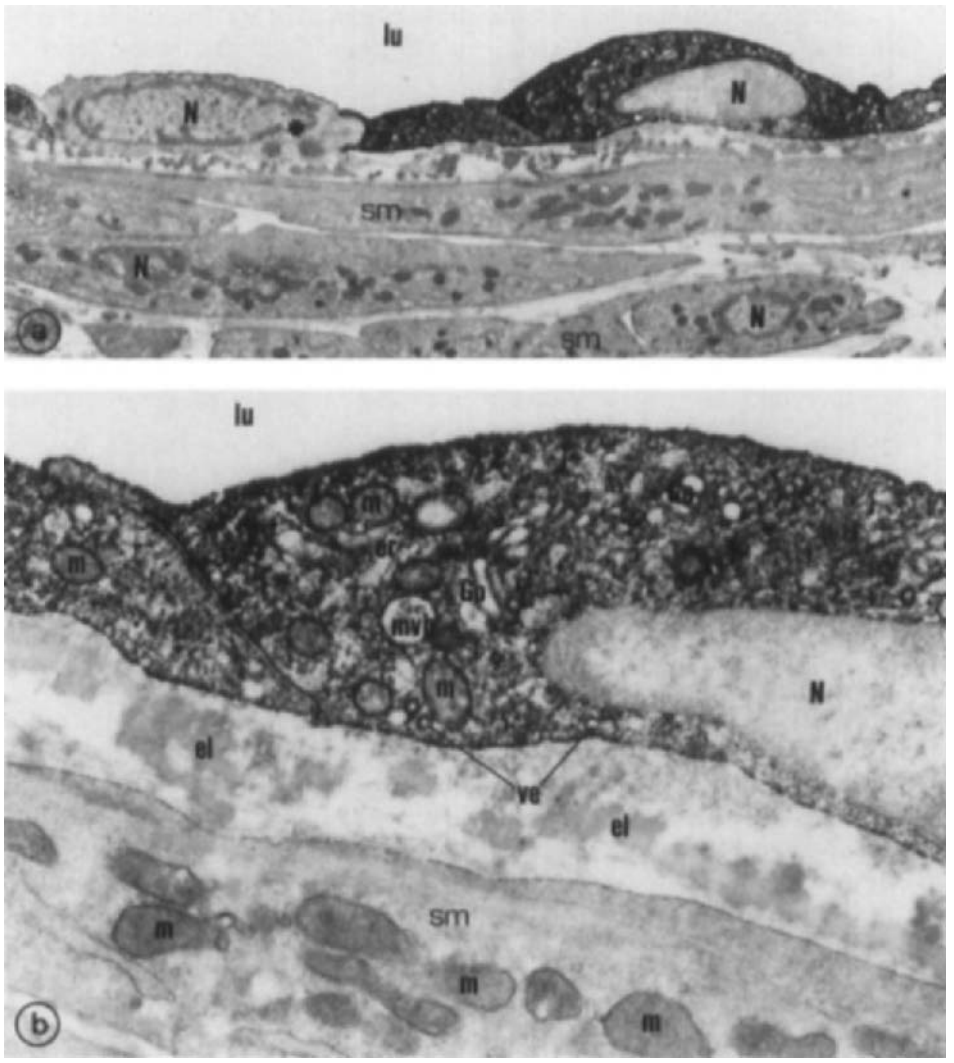

FIG. 2 Electron micrographs of newborn rat coronary artery labeled for ET-1. (a) Neighboring ET-1-positive endothelial cell profiles (showing "black" cytoplasmic labeling) and one ET-1-negative endothelial cell (asterisk) are seen. Note unlabeled vascular smooth muscle (sm). N, nucleus; lu, lumen. Magnification, ×7,300. (b) Higher magnification example of ET-1-positive endothelium showing intense immunoprecipitate throughout the cytoplasm and in association with the membranes of mitochondria (m), endoplasmic reticulum (er), Golgi complex (Go), multivesicular body (mvb), and cytoplasmic vesicles (ve); lumina of cytoplasmic vesicles are free of labeling. el, elastic lamina. Magnification, ×27,400 (reproduced with permission from Loesch and Burnstock, 1995).

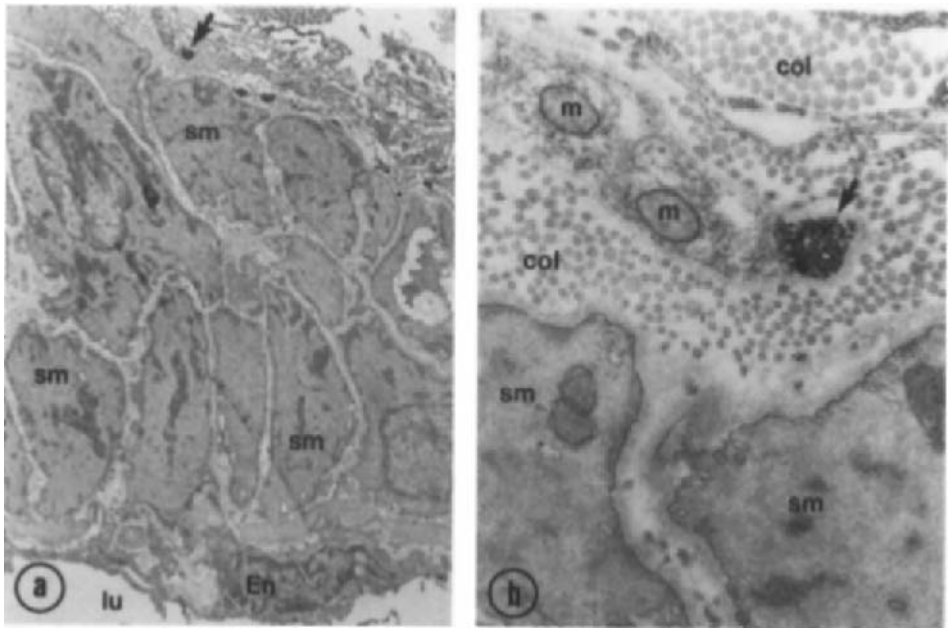

FIG. 3 Electron micrographs of main branches of adult rat coronary arteries labeled for NOS. (a) NOS-positive perivascular nerve fibers (arrow) in small nerve bundles are seen in the vicinity of the outer layer of vascular smooth muscle (sm). En, endothelium; lu, lumen. Magnification, ×5,600. (b) Magnified portion of the artery illustrated in (a) shows the intense NOS-labeling of the axon profile (arrow). m, mitochondria; col, collagen fibers. Magnification, ×39,000 (reproduced with permission from Sosunov *et al.*, 1995).

1996). It has also been demonstrated that the stellate ganglion, the major sympathetic ganglion supplying nerve fibers to the heart (rat), contains NOS-I-positive preganglionic fibers and postganglionic neurons (Schwarz *et al.*, 1995).

There is little information on the precise localization of ET-1 in neuronal components of the coronary bed. However, immunocytochemical studies suggest that the ET-1 immunoreactivity in the cardiopulmonary tissues (guinea pig) is associated with autonomic neural supply (Franco-Cereceda *et al.*, 1990).

B. Vascular Effects of NO and ET-1

Both the endogenous vasodilator NO and the potent vasoconstrictor ET-1 play a significant role in the regulation of coronary vascular tone, and

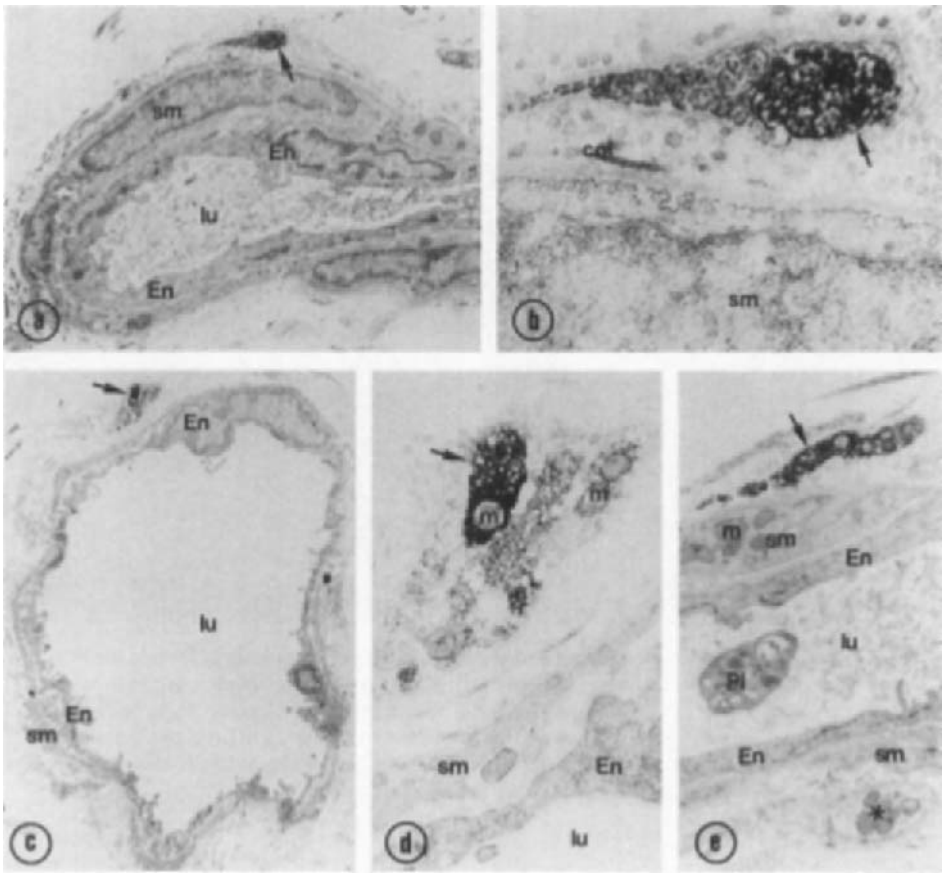

FIG. 4 Electron micrographs of arterioles and postcapillary venules in adult rat and guinea-pig atria labeled for NOS. (a) Intensely labeled NOS-positive perivascular nerve fibers (arrow) in arterioles of rat atria. (b) A labeled axonal varicosity containing small clear vesicles is shown at higher magnification. (c) A nerve bundle containing a NOS-positive nerve fiber (arrow) close to a postcapillary venule in guinea pig atria. (d) Magnified region of the venule illustrated in c showing the intense NOS-labeling of the axon profile (arrow). (e) Note the presence of NOS-positive (arrow) and NOS-negative (asterisk) axon profiles on opposite sides of the venule. En, endothelium; sm, smooth muscle; lu, lumen; col, collagen fibers; m, mitochondrion; Pl, platelet. Magnification: a, ×6,000; b, ×28,300; c, ×3,500; d, ×13,300; e, ×8,900 (reproduced with permission from Sosunov *et al.*, 1995).

functional reciprocal interactions have been established between these opposing agents.

When applied exogenously NO evokes very fast and potent coronary vasodilator responses (Fig. 5) associated with an increased release of cGMP

in the coronary perfusate (Kelm and Schrader, 1990). The L-arginine pathway within endothelial cells in the blood vessel wall appears to be the primary source of production of the endogenous NO within the coronary circulation (Ursell and Mayes, 1993; Simonsen *et al.*, 1997). However, NOS-positive nerve fibers originating from the intrinsic cardiac ganglia are mainly related to the coronary vasculature (Hassall *et al.*, 1992; Tanaka *et al.*, 1993a,b; Sosunov *et al.*, 1995), which suggests a contribution of neuronal NO to the regulation of coronary vascular tone. Release of NO from guinea-pig heart into the coronary effluent perfusate, associated with cGMP release, was demonstrated in basal conditions in a quantity sufficient to be a major factor in setting the resting tone of coronary resistance vessels (Kelm and Schrader, 1990). Furthermore, vasodilators such as bradykinin (Fig. 6), serotonin, ACh, and partially ATP evoke release of NO and cGMP

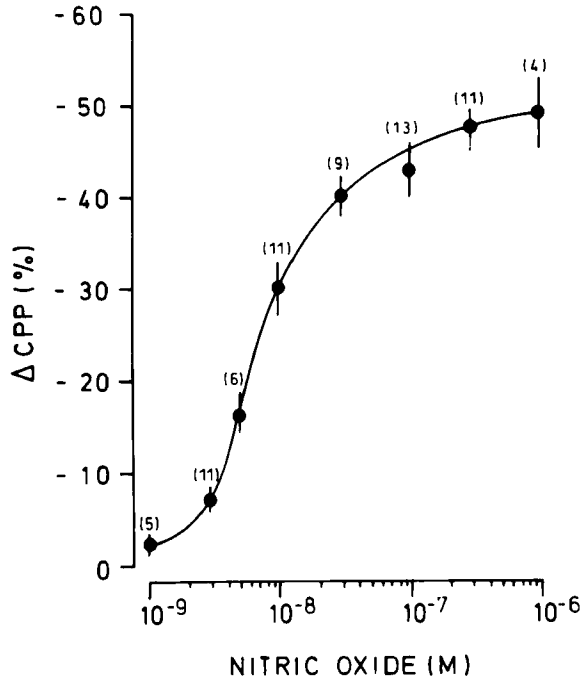

FIG. 5 Vasodilator effect of NO in the isolated guinea-pig coronary vascular bed. NO applied at increasing concentrations evokes a decrease of the coronary perfusion pressure (ΔCPP), expressed as a percentage of baseline value (70 ± 1 mmHg). The isolated guinea-pig atria was perfused according to the Langendorff techniques at constant flow (10 ml/min). Numbers in parentheses indicate number of experimental observations; means ± SEM (reproduced with permission from Kelm and Schrader, 1990).

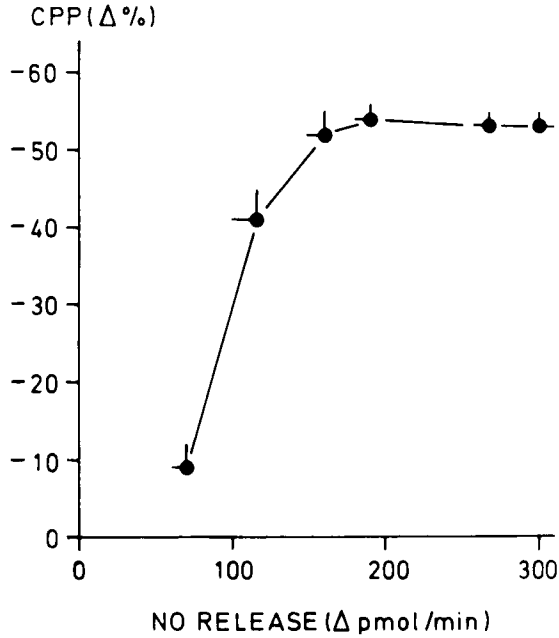

FIG. 6 Relationship between changes in coronary perfusion pressure and release of NO induced by bradykinin in the isolated guinea-pig heart. Nanomolar concentrations of bradykinin evoke a decrease of coronary perfusion pressure (CPP) which correlates to an increase in the NO release, indicating that the vasodilator action of bradykinin is mediated by production of NO (reproduced with permission from Kelm and Schrader, 1988).

in the coronary vascular bed in cause–effect relation to their vasodilator actions (Amezcua *et al.*, 1988, 1989; Kelm and Schrader, 1988, 1990). Mechanical stimuli, such as vessel wall deformation and shear stress, also evoke release of NO in the coronary vascular bed (Lamontagne *et al.*, 1992). Beside its direct vasodilator effects on the vascular smooth muscle, NO can modulate the vascular actions of other neurotransmitters and vasoactive agents. Inhibition of NO synthesis enhances centrally induced sympathetic coronary vasoconstriction (Goodson *et al.*, 1994) and both endogenous and exogenous NO inhibits noradrenaline outflow from sympathetic nerves in the isolated perfused rat heart (Schwarz *et al.*, 1995). Furthermore, it was recently demonstrated that endothelial NO can modulate neurogenic vasodilatation of lamb resistance coronary arteries, mediated by release of ATP (Simonsen *et al.*, 1997). Other biological actions of NO include inhibition of leukocyte adhesion to the endothelium, platelet aggregation, and protein extravasation, all of which contribute to the maintenance of an appropriate coronary flow (Moncada

et al., 1991). Inhibition of endogenous NO synthesis leads to an increase in protein extravasation and potentiates the permeability effects of ET-1 in the coronary circulation (Filep *et al.,* 1993).

Bolus injections of ET-1 into the coronary vascular bed produce transient vasodilator and prolonged vasoconstrictor actions as the net effect of the stimulation of both ET_A and ET_B receptors (Baydon *et al.,* 1989; Pernow and Modin, 1993; Wang *et al.,* 1994). However, low concentrations of ET-1 which mimic pathophysiological concentrations result in coronary vasoconstriction via ET_A receptors, whereas ET_B receptors may mediate a dual vasodilator and vasoconstrictor effect, with ET_B-evoked vasoconstriction being in part counteracted by release of NO (Pernow and Modin, 1993; Cannan *et al.,* 1995). In canine and porcine coronary arteries it has been shown that the vasoconstrictor action of ET-1 is more pronounced in smaller diameter ring preparations (Tippins *et al.,* 1989; Rigel and Lappe, 1993) and is inhibited by NO (Miller *et al.,* 1989). Pharmacological evidence indicates that in the pig coronary artery ET-1-evoked vasoconstriction involves at least two receptors—one with the pharmacological profile of the ET_A type and the other being different from the ET_B receptor (Harrison *et al.,* 1992). A potent vasoconstrictor action of ET-1 has been shown in the human coronary artery (Franco-Cereceda, 1989) in which specific binding sites for ET-1 have been demonstrated, mainly localized on the tunica media (Power *et al.,* 1989). Both ET_A and ET_B receptor subtypes have also recently been localized on perivascular nerves and in the region of neovascularization of human coronary artery, thus suggesting the involvement of ET-1 in the growth and maintenance of structure and innervation of the coronary vascular bed (Dashwood *et al.,* 1996). Furthermore, ET-1 may contribute to the regulation of coronary vascular tone via its neuromodulatory effects on perivascular innervation. In canine coronary arteries it has been demonstrated that high concentrations of ET-1 reduce electrically evoked release of NA from perivascular sympathetic nerves, though the major site of action of ET-1 appears to be at the postjunctional level (Aarnio *et al.,* 1993).

III. Pulmonary Circulation

A. Localization of NOS and ET-1

1. Endothelium

Light and/or electron microscopic studies revealed immunoreactivity for cNOS/NOS-I and/or ET-1 in intact endothelial cells of the rat main pulmo-

nary artery (Tomlinson *et al.,* 1991; Loesch and Burnstock, 1995, 1996b; Milner *et al.,* 1996) and pulmonary vein (Loesch and Burnstock, 1996b); NOS-I was also localized in intact endothelium of the adult guinea-pig pulmonary artery (Klimaschewski *et al.,* 1992). Examination of NADPH-d activity and immunofluorescence of monoclonal anti-NOS antibody (which recognizes NOS-I, NOS-II, and NOS-III) demonstrated NOS-positive endothelium in large pulmonary and small bronchial vessels but not in small pulmonary resistance vessels of the rat (Xue *et al.,* 1994). Light microscopy of the human pulmonary bed/airways showed NOS-positive endothelium (by heterologous antibody) in the blood vessels of trachea and bronchus (Springall *et al.,* 1992). Application of the monoclonal antibodies also revealed NOS-III-positive endothelial cells in the human large pulmonary vein and in arterioles of trachea (Pollock *et al.,* 1993). Giaid and coworkers (1991) demonstrated immunoreactivity to ET-1 and the presence of ET-1 mRNAs in the developing and adult human lung. Ultrastructural studies have shown predominantly cytoplasmic localization of NOS and ET-1 immunoreactivity in intact pulmonary endothelial cells (Figs. 7 and 8). According to Nakamura *et al.,* (1990) and Naruse *et al.* (1990) the immunoreactive ET-1 (labeled by the immunogold method) and mRNAs to ET-1 are associated with a number of intracellular organelles and structures, including the endoplasmic reticulum, Golgi complex, lysosome-like bodies, and cytoplasmic matrix as observed in cultured endothelial cells from bovine pulmonary artery. However, the presence of ET-1 mRNA in the Golgi complex and lysosomes does not agree with the mRNA trafficking within cells.

Semiquantitative assessment of the distribution of NOS-I- and ET-1-positive endothelial cells in the main pulmonary artery of newborn rats provided indirect evidence that NOS/NO and ET-1 are colocalized in some of the pulmonary endothelial cells (Loesch and Burnstock, 1995; see Section IV,A).

2. Perivascular Nerves—Intramural Ganglia

Immunocytochemical evidence clearly suggests the presence of NOS-containing neuronal components (neurons and nerve fibers) in the mammalian pulmonary vascular bed. NADPH-d/NOS-I-positive nerve fibers innervating pulmonary vessels were localized for the first time at the light microscopic level in the rat and guinea pig (Klimaschewski *et al.,* 1992). NADPH-d activity was subsequently found in the guinea-pig paratracheal neurons (Hassall *et al.,* 1993). A histochemical/immunohistochemical study of the guinea-pig lower airways demonstrated that the number of NADPH-d/NOS-I-containing nerve fibers was increased progressively from the cervi-

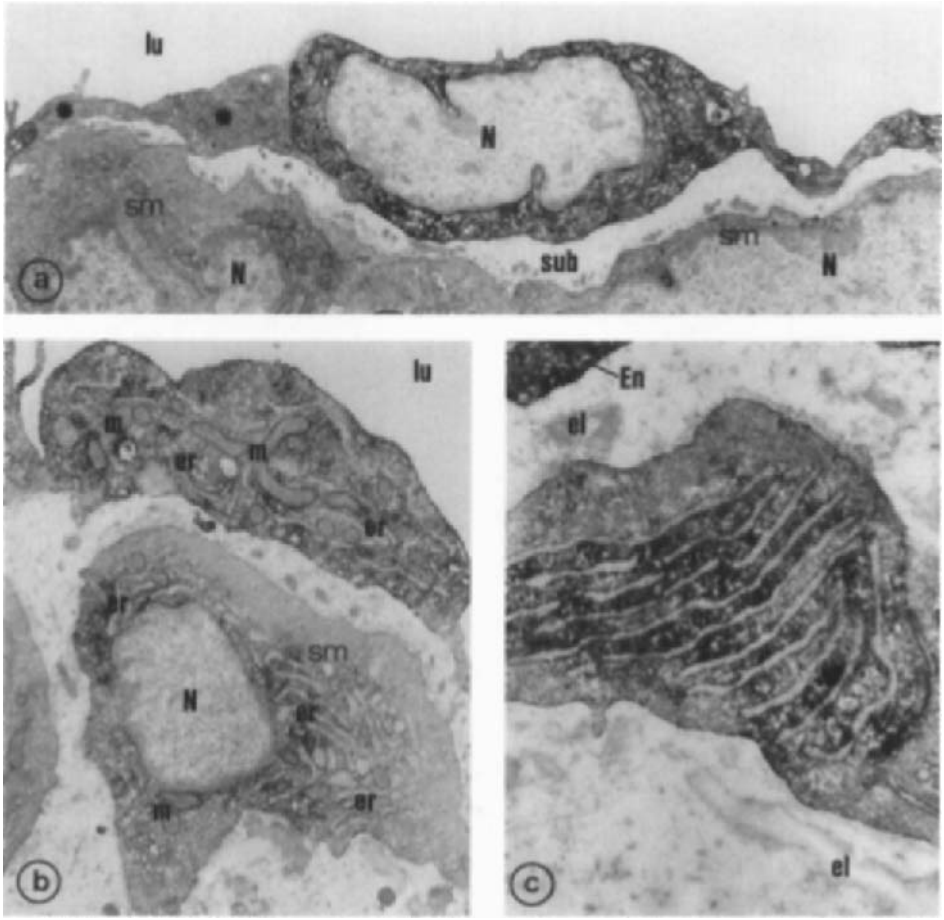

FIG. 7 Electron micrographs of newborn rat pulmonary artery labeled for NOS. (a) One NOS-I-positive (showing "black" cytoplasmic stain) and two NOS-I-negative (asterisks) endothelial cells are seen. N, nucleus; sub, subendothelial zone; sm, smooth muscle; lu, lumen. Magnification, ×8,300. (b) Note that both NOS-positive endothelium and NOS-positive smooth muscle are rich in endoplasmic reticulum (er) and mitochondria (m). Magnification, ×10,500. (c) Higher magnification example of NOS-positive smooth muscle showing heavy labeling of the cytoplasm associated with a rich system of parallel-arranged cisternae of endoplasmic reticulum. Note that the periphery of this muscle is free of labeling. el, elastic lamina; En, fragment of NOS-positive endothelium. Magnification, ×23,500 (reproduced with permission from Loesch and Burnstock, 1995).

cal trachea toward principal bronchi and then decreased to complete absence in bronchioli, and that networks of NOS-positive nerve fibers innervated tracheal arteries and pulmonary arteries and veins (Fischer *et al.*, 1993).

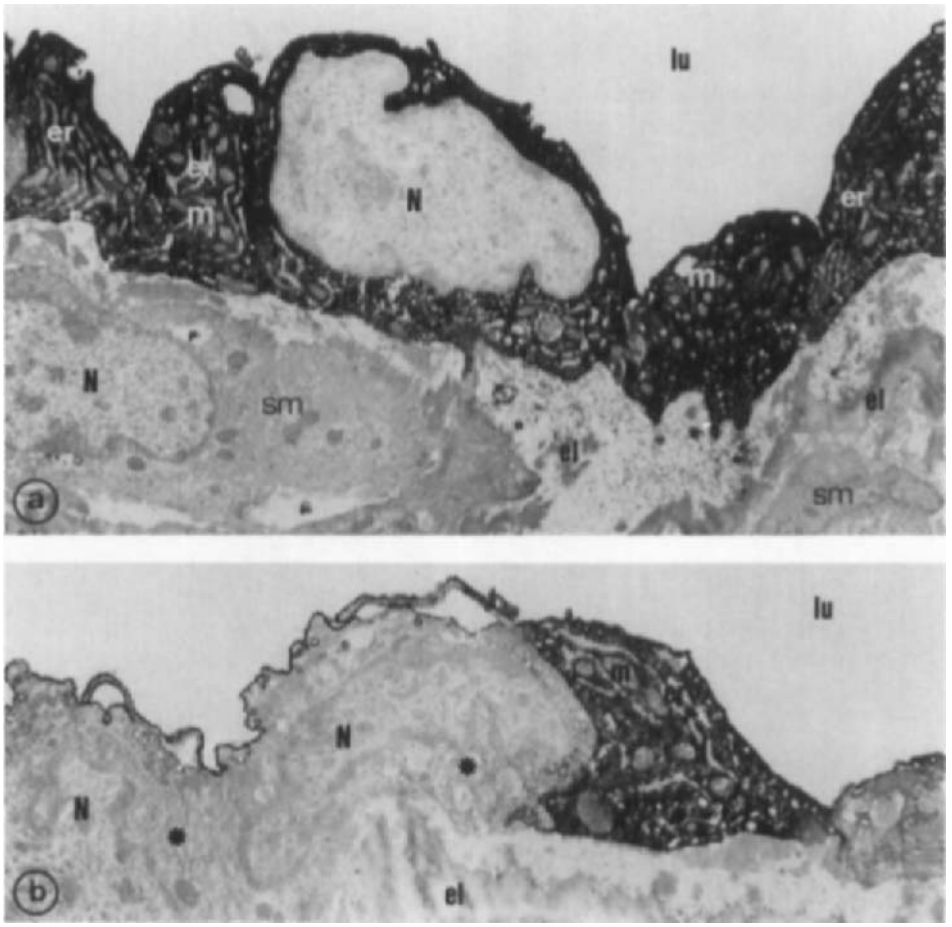

FIG. 8 Electron micrographs of newborn rat pulmonary artery labeled for ET-1. (a) Note intense ET-1 labeling of endothelial cells. Labeled cells are rich in endoplasmic reticulum (er) and mitochondria (m). Smooth muscle (sm) is ET-1 negative. N, nucleus; el, elastic lamina; lu, lumen. Magnification, ×9,500. (b) ET-1-positive process of an endothelial cell; two NOS-negative cells (asterisks) are also seen. Magnification, ×14,200 (reproduced with permission from Loesch and Burnstock, 1995).

There is some immunocytochemical evidence for neuronal ET-1 supply to the pulmonary vascular bed. For example, studies by Franco-Cereceda and colleagues (1990) suggest the presence of ET-1-containing autonomic nerves in the guinea-pig pulmonary bed. *In vitro* microautoradiography studies of the porcine lung suggest the existence of endothelin receptors (ET_B) in pulmonary plexuses and perivascular nerves, probably of parasym-

pathetic nature since they also show acetylcholinesterase activity (Kobayashi *et al.*, 1993).

B. Vascular Effects of NO and ET-1

In contrast to most systemic vascular beds, including the coronary vasculature, the pulmonary circulation is a low-resistance and high-compliance vascular bed. The mechanisms involved in the maintenance of a low pulmonary vascular tone are not entirely understood and much attention has been given to the possible role of NO in this contest. On the other hand, ET-1 has been regarded as a possible factor contributing to the phenomenon of hypoxic vasoconstriction and pulmonary hypertension.

Endothelium-dependent neurogenic relaxation was demonstrated in the pulmonary artery of rabbit, cats, and monkeys (Frank and Bevan, 1983) soon after the discovery of EDRF. The involvement of the L-arginine–NO–cGMP pathway in the vasodilator responses to electrical stimulation of perivascular nerves has been subsequently demonstrated in bovine and guinea-pig pulmonary arteries (Buga and Ignarro, 1992; Liu *et al.*, 1992). Furthermore, endothelium-derived NO inhibits neurogenic vasoconstriction of the pulmonary vasculature both *in vitro* and *in vivo* (Liu *et al.*, 1991; Tabrizchi and Triggle, 1991). Vasodilator responses of the pulmonary vessels to ACh, NA, bradykinin, VIP, substance P, ATP, arachidonic acid, and endothelins have been shown to be mediated by the evoked release of NO from the endothelium (Barnes and Liu, 1995). However, while basal release of NO plays a key role in the regulation of the systemic blood pressure, the contribution of tonic release of NO to maintenance of a low pulmonary vascular tone varies between species. Inhibition of NOS increases pulmonary resistance in guinea pigs, rabbits, and lambs, but not dogs (Wiklund *et al.*, 1990; Gordon and Tod, 1993; Barnard *et al.*, 1993). Basal release of NO also contributes to the regulation of pulmonary vascular tone in humans (Celermajer *et al.*, 1993). There is evidence that in the pulmonary vascular bed endothelial release of NO increases when vascular resistance is higher (e.g., in pulmonary hypertension; see Section IV,D), thus providing a balancing antagonism on the increased pulmonary blood pressure.

The lung is a rich source of ET-1 produced by endothelial cells and activating specific receptors on intrapulmonary vessels and airways. Molecular biology and binding studies have shown the presence of both ET_A and ET_B receptor subtypes on pulmonary vascular smooth muscle and endothelial cells, respectively (Hosada *et al.*, 1991; Ogawa *et al.*, 1991). Exceptionally, in the rabbit pulmonary artery the two subtypes coexist on

the smooth muscle (LaDouceur *et al.*, 1993). In isolated pulmonary artery and vein as well as in the isolated perfused pulmonary vascular bed, vasomotor responses to ET-1 depend on the vascular tone. At baseline conditions ET-1 evokes vasoconstriction in many species, including lamb (Fig. 9), rat, rabbit, guinea pig, and humans (Barnard *et al.*, 1991; Mann *et al.*, 1991; Hay *et al.*, 1993; Wang *et al.*, 1995). When the vascular tone is experimentally increased (by vasoconstriction or hypoxia), ET-1 evokes pulmonary vasodilatation, involving activation of ET_B receptors and consequent production of NO (Lippton *et al.*, 1993; Pinheiro and Malik, 1993). Contractile responses are generally mediated by ET_A receptors (Hay *et al.*, 1993; Lippton *et al.*, 1993; Wang *et al.*, 1995; Fig. 9). However, regional differences in the distribution of the receptor subtypes have been shown within the pulmonary vascular tree. In the rat and human pulmonary vasculature, ET_A receptors predominantly mediate vasoconstriction of large arteries, whereas ET_{B2}-like receptors are responsible for vasoconstrictor responses to ET-1 in small resistance intrapulmonary arteries (MacLean and McCulloch, 1998). The vasomotor activity of ET-1 in the pulmonary circulation in the intact animal varies and appears to be species dependent. In the cat, rabbit, and dog, bolus injection of ET-1 evokes vasoconstriction (Barman and Pauly, 1995), whereas in the rat and piglet vasodilatation is shown (Hasunuma *et al.*, 1990; Pinheiro and Malik, 1993).

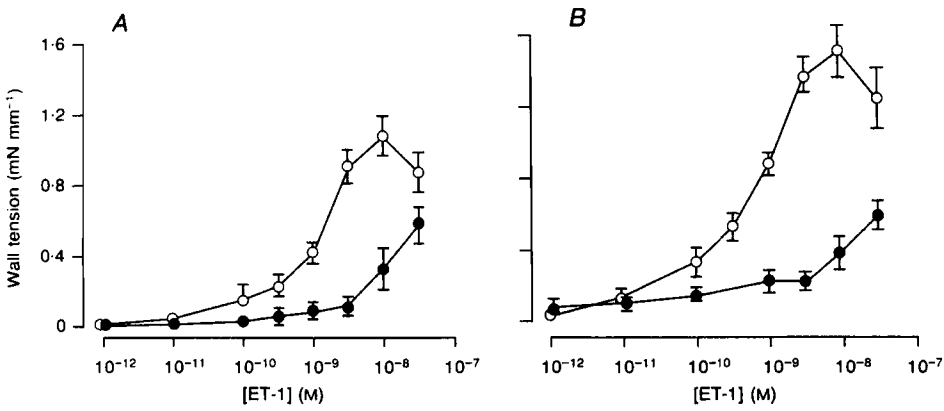

FIG. 9 Vasoconstrictor effect of ET-1 via ET_A receptors in isolated resistance vessel of fetal lamb. Concentration-dependent vasoconstrictor responses to ET-1 in isolated resistance pulmonary artery (A) and vein (B) of fetal lamb at fetal P_{O2} before (○) and after (●) treatment with the ET_A receptor antagonist BQ123 (1 μM). Differences between control and treated curves are significant ($p < 0.001$), indicating that ET_A receptors mediate the vasoconstrictor effect of ET-1 (reproduced with permission from Wang *et al.*, 1995).

The potent vasoconstrictor effect of ET-1 in the pulmonary blood vessel of several species, including humans, has led to investigations of the possible contribution of this peptide to the phenomenon of hypoxic vasoconstriction, as detailed in the following section.

IV. Plasticity of Expression of NOS and ET-1

Consistent with the potent vascular actions of NO and ET-1 in the coronary and pulmonary circulation, an unbalanced interaction between their opposing effects may lead to pathophysiological conditions or contribute to the onset of a pathological state not only in the coronary and pulmonary circulation but also overall in the cardiovascular system. Together with functional evidence (though often controversial) that indicates the involvement of the L-arginine–NO pathway and ET-1 in the aging process of cardiovascular function, ischemia/reperfusion damage and heart failure, and systemic and pulmonary hypertension, light and electron-immunocytochemical studies of coronary and pulmonary vascular beds in different pathophysiological conditions have shown the plasticity (quantitative and qualitative changes in immunoreactivity) of NOS- and ET-1-positive endothelial cells, as detailed in the following sections.

A. Development and Aging

Aging is associated with a reduced endothelium-dependent vascular responsiveness due to reduced synthesis and/or release of NO. In contrast, release of ET-1 increases with aging while vascular responses to the vasoconstrictor are attenuated (Dohi *et al.,* 1995). However, in the rat coronary arteries, increased responsiveness to the contractile effect of ET-1 was shown, with no variation in the vascular responses via the L-arginine–NO pathway (Tschudi and Luscher, 1995). The hemodynamic effects of ET-1 on the pulmonary circulation have also been proved to depend on postnatal age (Wang *et al.,* 1994). Functional variations may correlate to changes in the proportions of endothelial cells containing cNOS/NOS-I and ET-1 observed during developmental and aging. In the newborn rat (Wistar), endothelial cells of the coronary and pulmonary arteries are rich in both NOS and ET-1 (80 and 55% of endothelial cells of the coronary artery immunolabeled for NOS and ET-1, respectively, and 77 and 60% of endothelial cells of the pulmonary artery contain NOS and ET-1, respectively). This suggests an important role for endothelial NO and ET-1 in these vessels during the early stages of postnatal development (Loesch and Burnstock, 1995). An

immunocytochemical study on the pulmonary vasculature suggests that NO and ET-1 in endothelial cells may play a role in the local control of vascular tone throughout the life span of rats and even in older animals when there is some damage to the intima (Figs. 10 and 11). However, fewer immunopositive cells for NOS-I and ET-1 are seen in the pulmonary artery at 1, 6, and 12 months than in the neonate (Loesch and Burnstock, 1996b). Using a mouse anti-NOS-III antibody in immunocytochemical studies of the lungs from fetal, neonatal, and adult sheep, Halbower *et al.* (1994) showed that pulmonary endothelial cells immunoreactive for NOS-III are present very early in fetal life, e.g., in the developing capillaries coursing through fetal mesenchyme, but they appear to decrease postnatally. Histochemical and immunohistochemical studies of fetal Fischer rat hearts

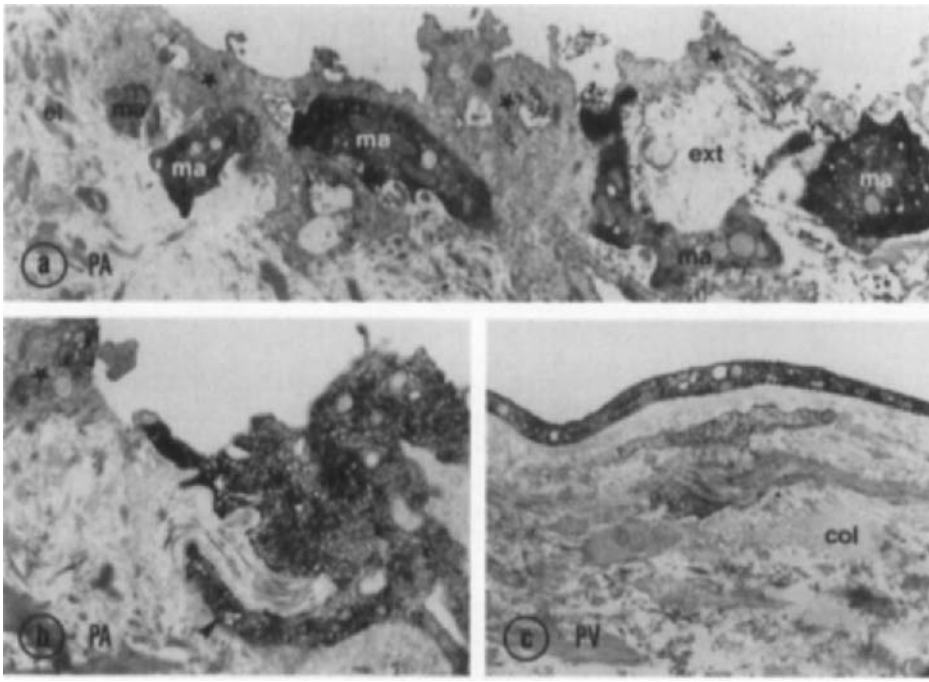

FIG. 10 Electron micrographs of pulmonary artery (PA) (a and b) and pulmonary vein (PV) (c) of 12-month-old rat labeled for NOS. (a) Note that NOS-negative endothelial cells (stars) are irregularly shaped. In subendothelial zone, note numerous macrophage-like cells (ma) displaying NOS immunoreactivity. el, elastic lamina; ext, extracellular matrix. Magnification, ×7,900. (b) Example of NOS-positive endothelial cell with a process (arrowhead) penetrating into subendothelial matrix. Magnification, ×10,300. (c) Note NOS-positive endothelium and substantially enlarged subendothelial zone. col, bundles of collagen fibers. Magnification, ×9,300 (reproduced with permission from Loesch and Burnstock, 1996b).

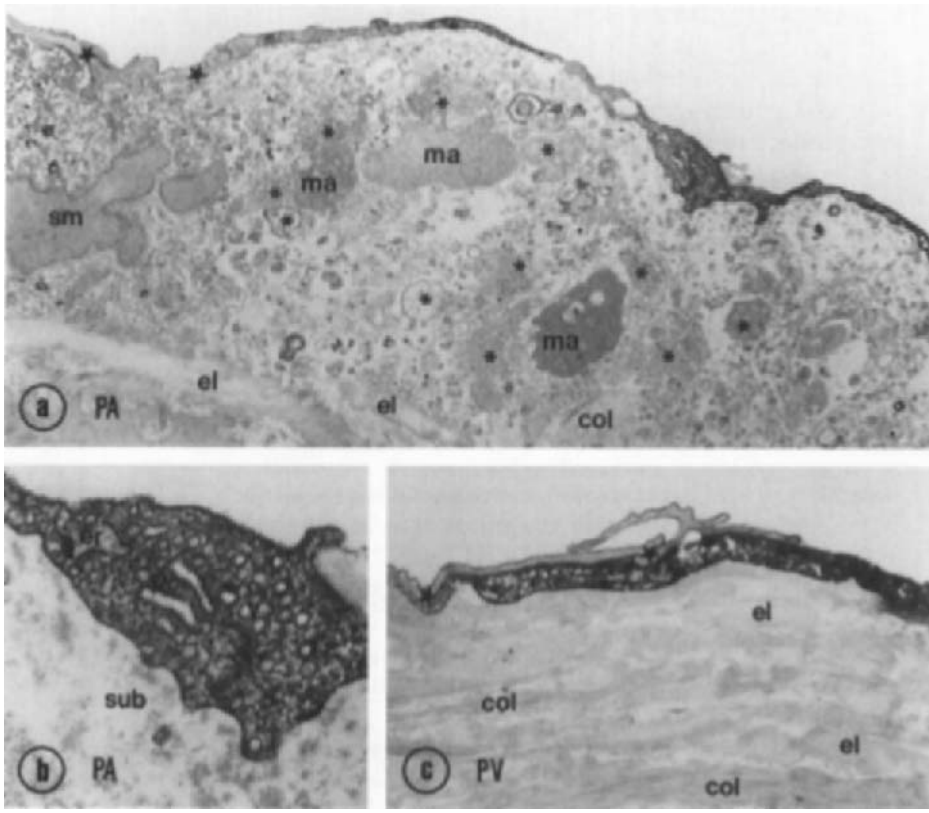

FIG. 11 Electron micrographs of pulmonary artery (PA) (a and b) and pulmonary vein (PV) (c) of 24-month-old rat labeled for ET-1. (a) A fragment of PA shows ET-1-negative (stars) and ET-1-positive endothelial cells on the luminal aspect of substantially enlarged intima. In intima, note various forms of nonimmunoreactive degenerating macrophages (ma; asterisks). sm, smooth muscle; el, elastic lamina; col, collagen fibers. Magnification, ×7,700. (b) Magnified portion of PA illustrated in a showing numerous cytoplasmic vesicles in ET-1-positive endothelium. sub, subendothelial matrix. Magnification, ×24,500. (c) Fragment of PV displays ET-1-positive and ET-1-negative endothelial cells. Note that intima is substantially enlarged and contains elastic lamina and bundles of collagen fibers. Magnification, ×13,400 (reproduced with permission from Loesch and Burnstock, 1996b).

showed that NOS-III is initially restricted to the developing endocardium (14 days gestation), but when the myocardium becomes compact (18 days gestation) NOS-III is also present in numerous small vessels throughout the compact myocardium (Ursell and Mayes, 1996). At birth, NOS-III immunoreactivity is distributed in myocardial arteries and veins and numerous capillaries.

B. Systemic Hypertension

Despite the modifications of vascular responses to NO and ET-1 associated with systemic hypertension, the role of NO and ET-1 in the onset and maintenance of the disease is still controversial (Nava and Luscher, 1995). Synthesis and release of NO are both modified during hypertension, depending on the degree of variation of systemic blood pressure and on the animal model studied. In spontaneously hypertensive rats (SHRs), production of NO appears to be increased, whereas in salt-induced hypertensive rats it is impaired. Inhibition of NOS in the SHR is associated with malignant hypertension and enhanced expression of the ET-1 gene in some blood vessels with severe vascular hypertrophy, whereas normally SHRs do not overexpress the ET-1 gene and exhibit only limited vascular hypertrophy (Sventek *et al.,* 1996). In endothelial cells of DOCA/salt hypertensive rats ET-1 production is upregulated (Takada *et al.,* 1996). However, in both animal models of hypertension, the vasodilatatory endothelial ET_B receptor is predominant, whereas in the normotensive rat the prevailing role of ET_B receptors is vasoconstriction (Clozel and Breu, 1996). In humans, impairment of NO-mediated vasodilatation during systemic hypertension has been documented (Nava and Luscher, 1995; Burnett, 1997). While plasma levels of ET-1 are normal in most hypertensive patients, vascular responsiveness to this vasoconstrictor may be increased (Neild, 1994; Nava and Luscher, 1995).

Ultrastructural studies of renal hypertensive rats (one kidney/one clip model) showed a decrease of NOS-III-positive endothelial cells in coronary artery (by monoclonal anti-NOS-III antibody), but the percentage of endothelial cells immunostaining for ET-1 did not differ from that of normotensive controls (Shochina *et al.,* 1997). Consequently, the ratio of NOS-III to ET-1 (percentage of NOS-III-positive endothelial cells:percentage of ET-1positive endothelial cells) was reduced in coronary artery during renal hypertension. Studies on the coronary arteries of SHRs have shown decreased vasoconstriction to ET-1 with no changes of NO-mediated vascular responses (Tschudi and Luscher, 1995).

C. Cardiac Ischemia/Reperfusion and Heart Failure

The interactions between NO and ET-1 systems, largely documented at the level of gene expression, synthesis, and/or release and opposing vascular effects, may be reinforced or antagonized by changes occurring as a result of cardiac ischemia/reperfusion. Basal release of NO has been reported to be either reduced or impaired after ischemia and reperfusion (Maulik *et al.,* 1995; Brunner, 1997). Consequently, endothelium-dependent respon-

siveness of coronary arteries may be impaired after experimental ischemia. In contrast, during reperfusion, but not ischemia, there is an increase of the ET-1 overflow, which can be suppressed by NO donors and potentiated by inhibition of NO synthesis (Wang *et al.,* 1995; Brunner, 1997). Interestingly, deterioration of cardiac function during ischemia/reperfusion correlates with higher tissue levels, and improvement with lower tissue levels, of ET-1 (Brunner, 1997). Consistent with these observations is the increase of ET-1 production and density of myocardial endothelin receptors shown in experimental heart failure (Sakai *et al.,* 1996). However, investigation of biopsies of myocardium from porcine heart demonstrated that in the ischemic and nonischemic regions, there was no difference in the intensity of ET-1 immunoreactivity; in both conditions, little ET-1 immunoreactivity was detected in vascular endothelium or vascular smooth muscle (Tonnessen *et al.,* 1995). In experimental myocardial infarction of rabbits, macrophages are a major source of NOS-II immunoreactivity in the infarct (Wildhirt *et al.,* 1995b,c).

D. Hypoxic Pulmonary Vasoconstriction and Pulmonary Hypertension

Vascular tone in the pulmonary circulation is under the influence of respiratory gases, with alveolar hypoxia being the principal stimulus of hypoxic pulmonary vasoconstriction (HPV). Fifty years after its initial discovery (Von Euler and Liljestrand, 1947), the mechanisms underlying HPV remain to be elucidated. The contribution of autonomic innervation appears to be irrelevant because HPV can be evoked in *in vitro* lung preparations, after sympathetic and sensory denervation, and in the presence of cholinergic blockers. Despite intense investigation in the past two decades, the role of endothelium in the phenomenon of HPV remains controversial because HPV can be evoked in pulmonary arterial rings deprived of endothelium. However, functional evidence is available showing the modulatory role of endothelium in HPV and pulmonary hypertension, with NO and ET-1 being the major mediators of such a modulation. Inhibition of NOS exacerbates HPV, whereas administration of exogenous NO inhibits the condition (Roberts *et al.,* 1993). Whether suppression of NOS activity mediates HPV is uncertain. Hypoxia reduces the constitutive NOS transcript in isolated endothelial cells (Phelan and Faller, 1996) and impaired endothelium-dependent vasodilatation is seen during hypoxia (Rodman *et al.,* 1990). Both reduced and increased dilator responses mediated by NO have been observed in pulmonary hypertension following chronic hypoxia (Adnot *et al.,* 1991; Russ and Walker, 1993), whereas basal release of NO is maintained in human lungs with pulmonary hypertension (Cremona *et al.,* 1994). Local-

ization studies on NADPH-d activity and NOS immunoreactivity in lungs of normoxic rats showed that the endothelial cells of small pulmonary resistance vessels do not display NOS, whereas a prominent presence of NOS-positive endothelial cells is noted in these vessels in rats exposed to 2–4 weeks of chronic hypoxia (Xue *et al.,* 1994). Furthermore, it has been shown that chronic hypoxia upregulates endothelial and i-NOS gene and protein expression in the rat lung (LeCras *et al.,* 1996).

Pharmacological evidence indicates a possible contribution of ET-1 to HPV because vasoconstrictor responses of pulmonary artery and vein to hypoxia are inhibited by the ET_A receptor antagonist BQ123 (Fig. 12; Wang *et al.,* 1995). Furthermore, ET-1 could be involved in the development of chronic hypoxic pulmonary hypertension. In rats exposed to chronic hypoxia and that developed pulmonary hypertension, ET-1-evoked pulmonary vasodilatation via ET_B endothelial receptors and production of NO was lost, although no changes were observed in ET_A or ET_B receptor binding (Eddahibi *et al.,* 1993). It has been demonstrated in patients with pulmonary hypertension that ET-1 immunoreactivity and ET-1 mRNA expression are increased in pulmonary vascular endothelial cells (Giaid *et al.,* 1993). Oral administration of the mixed antagonist of ET_A and ET_B receptors, bosantan, prevents pulmonary hypertension due to chronic exposure to hypoxia (Eddahibi *et al.,* 1995).

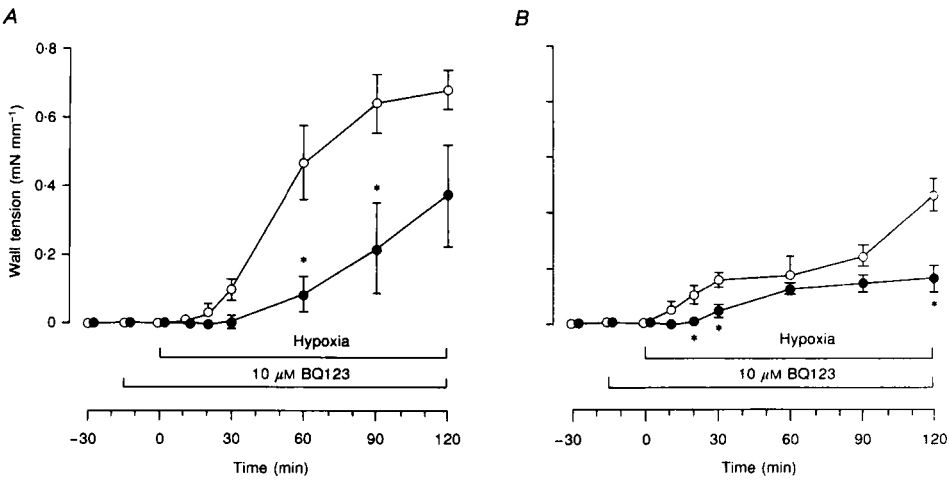

FIG. 12 Effect of ET-1 receptor blockade on the hypoxic vasoconstriction of lamb pulmonary artery and vein. In isolated resistance pulmonary artery (A) and vein (B), contractile responses to hypoxia (○) are significantly (*, $p < 0.05$) reduced in the presence (●) of the ET_A receptor antagonist BQ123 (reproduced with permission from Wang *et al.,* 1995).

V. Conclusions

In the past few years much information has accumulated about the role of the endothelium and perivascular nerves in the regulation of vascular tone. Two revolutionary discoveries in this respect are represented by the findings of EDRF/NO and ET-1 generation from the endothelium. In two vascular regions, such as the coronary and pulmonary circulations, the opposing effects of the NO and ET-1 systems have been particularly investigated. The experimental evidence described in this article demonstrates that (i) the normal/resting coronary and pulmonary endothelial cells immunoreact with antibodies to NOS-I, NOS-III, and ET-1 and that (ii) certain stimuli lead to the expression of NOS-II in these cells. In contrast, coronary and pulmonary perivascular nerves immunoreact mainly with anti-NOS-I antibody, at least in normal/resting conditions. Subpopulations of endothelial cells contain NOS and ET-1, but NOS and ET-1 are also localized within the same endothelial cells. It is established that the endothelial ET_B receptor is functionally coupled to NOS and coordinates the generation of NO via tyrosine kinase (Tsukahara *et al.,* 1994). On the other hand, in cultured vascular smooth muscle NO regulates ET_A receptors through a cGMP-dependent mechanism (Redmond *et al.,* 1996). Furthermore, the modification in the ratio ET-1: NOS observed in animal models of pathophysiological conditions herein described suggests that a balanced activity of NO and ET-1 is essential to the homeostasis of coronary and pulmonary circulation.

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References

Aarnio, P., McGregor, C. G. A., and Miller, V. (1993). Autonomic modulation of contraction to endothelin-1 in canine coronary arteries. *Hypertension* **21,** 680–686.

Adnot, S., Raffestin, B., Eddahibi, S., Braquet, P., and Chabrier, P. E. (1991). Loss of endothelium-dependent relaxant activity in the pulmonary circulation of rats exposed to chronic hypoxia. *J. Clin. Invest.* **87,** 155–162.

Amezcua, J. L., Dusting, G. P., Palmer, R. M. J., and Moncada, S. (1988). Acetylcholine induces vasodilatation in the rabbit isolated heart through the release of nitric oxide, the endogenous vasodilator. *Br. J. Pharmacol.* **95,** 830–834.

Amezcua, J. L., Palmer, R. M., de-Souza, B. M., and Moncada, S. (1989). Nitric oxide synthesized from L-arginine regulates vascular tone in the coronary circulation of the rabbit. *Br. J. Pharmacol.* **97,** 1119–1124.

Anderson, C. R., Edwards, S. L., Furness, J. B., Bredt, D. S., and Snyder, S. H. (1993). The distribution of nitric oxide synthase-containing autonomic preganglionic terminals in the rat. *Brain Res.* **614,** 78–85.

Arai, I. H., Hori, S., Aramori, I., Ohkubo, H., and Nakinishi, S. (1990). Cloning and expression of cDNA encoding an endothelin receptor. *Nature* **348,** 731–732.

Barman, S. A., and Pauly, J. R. (1995). Mechanism of action of endothelin-1 in the canine pulmonary circulation. *J. Appl. Physiol.* **79,** 2014–2020.

Barnard, J. W., Barman, S. A., Adkins, W. K., Longenecker, G. L., and Taylor, A. E. (1991). Sustained effects of endothelin-1 on rabbit, dog, and rat pulmonary circulation. *Am. J. Physiol.* **261,** H479–H486.

Barnard, J. W., Wilson, P. S., Moore, T. M., Thompson, J. W., and Taylor, A. E. (1993). Effect of nitric oxide and cyclooxygenase products on vascular resistance in dog and rat lungs. *J. Appl. Physiol.* **74,** 2940–2948.

Barnes, P. J., and Liu, S. F. (1995). Regulation of pulmonary vascular tone. *Pharmacol. Rev.* **47,** 87–131.

Baydon, A. R., Peers, S. H., Cirino, G., and Woodward, B. (1989). Effects of endothelin-1 on the rat isolated heart. *J. Cardiovasc. Pharmacol.* **13**(Suppl. 5), 193–196.

Bredt, D. S., and Snyder, S. H. (1990). Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. USA* **87,** 682–685.

Bredt, D. S., and Snyder, S. H. (1992). Nitric oxide: A novel neuronal messenger. *Neuron* **8,** 3–11.

Bredt, D. S., Hwang, P. M., and Snyder, S. H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* **347,** 768–770.

Brizzolara, T., Crowe, R., and Burnstock, G. (1993). Evidence for the involvement of both ATP and nitric oxide in non-adrenergic, non-cholinergic inhibitory neurotransmission in the rabbit portal vein. *Br. J. Pharmacol.* **109,** 606–608.

Brunner, F. (1997). Interaction of nitric oxide and endothelin-1 in ischemia/reperfusion injury of rat heart. *J. Mol. Cell. Cardiol.* **29,** 2363–2374.

Buga, G. M., and Ignarro, L. J. (1992). Electrical field stimulation causes endothelium-dependent and nitric oxide-mediated relaxatin of pulmonary artery. *Am. J. Physiol.* **262,** H973–H979.

Bult, H., Boeckxstaens, G. E., Pelckmans, P. A., Jordaens, F. H., Van Maercke, Y. M., and Herman, A. G. (1990). Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature* **345,** 346–368.

Burnett, A. L., Lowenstein, C. J., Brendt, D. S., Chang, T. S. K., and Snyder, S. H. (1992). Nitric oxide: A physiologic mediator of penile erection. *Science* **257,** 401–403.

Burnett, J. C. (1997). Coronary endothelial disfunction in the hypertensive patient: From myocardial ischemia to heart failure. *J. Hum. Hypertension* **11,** 45–49.

Burnstock, G. (1976). Do some nerve cells release more than one transmitter? *Neuroscience* **1,** 239–248.

Burnstock, G. (1986). The changing face of autonomic neurotransmission (The First von Euler Lecture in Physiology). *Acta Physiol. Scand.* **126,** 67–91.

Burnstock, G. (1990a). Co-transmission. The Fifth Heymans Lecture. *Arch. Int. Pharmacodyn. Ther.* **304,** 7–33.

Burnstock, G. (1990b). Local mechanisms of blood flow control by perivascular nerves and endothelium. *J. Hypertension* **8,** S95–S106.

Burnstock, G. (1990c). Noradrenaline and ATP as cotransmitters in sympathetic nerves. *Neurochem. Int.* **17,** 357–368.

Burnstock, G. (1995). Noradrenaline and ATP: Cotransmitters and neuromodulators. *J. Physiol. Pharmacol.* **46,** 365–384.

Burnstock, G., and Ralevic, V. (1994). New insights into the local regulation of blood flow by perivascular nerves and endothelium. *Br. J. Plastic Surg.* **47,** 527–543.

Burnstock, G., and Ralevic, V. (1996). Cotransmission. *In* "The Pharmacology of Vascular Smooth Muscle" (J. Garland and J. A. Angus, Eds.), pp. 210–232. Oxford Univ. Press, Oxford.

Cannan, C. R., Burnett, J. C., Brandt, R. R., and Lerman, A. (1995). Endothelin at pathophysiological concentrations mediates coronary vasoconstriction via the endothelin-A receptor. *Circulation* **92,** 3312–3317.

Celermajer, D. S., Cullen, S., and Deanfield, J. E. (1993). Impairement of endothelium-dependent pulmonary artery relaxation in children with congenital heart disease and abnormal pulmonary hemodynamics. *Circulation* **87,** 440–446.

Clozel, M., and Breu, V. (1996). The role of ET_B receptors in normotensive and hypertensive rats as revealed by the non-peptide selective antagonist Ro 46-8443. *FEBS Lett.* **383,** 42–45.

Corriu, C., Feletou, M., Canet, E., and Vanhoutte, P. M. (1996). Endothelium-derived factors and hyperpolarization of the carotid artery of the guinea-pig. *Br. J. Pharmacol.* **119,** 959–964.

Cremona, G., Wood, A. M., Hall, L. W., Bower, E. A., and Higenbottam, T. (1994). Effect of inhibitors of nitric oxide release and action on vascular tone in isolated lungs of pig, sheep, dog and man. *J. Physiol. (London)* **481,** 185–195.

Cuello, A. C. (Ed.) (1982). "Co-transmission." Macmillan, London.

Dashwood, M. R., Timm, M., Kaski, J. C., Murday, A. J., and Madden, B. P. (1996). [^{125}I]-ET-1 binding to perivascular nerves of human epicardial coronary arteries. *Endothelium* **4,** 231–234.

Dikranian, K., Loesch, A., and Burnstock, G. (1994). Localisation of nitric oxide synthase and its colocalisation with vasoactive peptides in coronary and femoral arteries. An electron microscope study. *J. Anat.* **184,** 583–590.

Dohi, Y., Kojima, M., Sato, K., and Luscher, T. M. (1995). Age-related changes in vascular smooth muscle and endothelium. *Drugs Aging* **7,** 278–291.

Eddahibi, S., Springall, D., Mannon, M., Courlle, C., Chabrier, P. E., Levame, M., Raffestin, B., Polak, J., and Adnot, S. (1993). Dilator effects of endothelins in pulmonary circulation: Changes associated with chronic hypoxia. *Am. J. Physiol.* **265,** L571–L580.

Eddahibi, S., Raffestin, B., Clozel, M., Levame, M., and Adnot, S. (1995). Protection from pulmonary hypertension with an orally active endothelin receptor antagonist in hypoxic rats. *Am. J. Physiol.* **268,** H828–H835.

Edvinsson, L. (1991). Innervation and effects of dilatory neuropeptides on cerebral vessels. *Blood Vessel* **28,** 34–45.

Eguchi, S., Hirata, Y., Imai, T., and Marumo, F. (1995). Endothelin-1 as an autocrine growth factor for endothelial cells. *J. Cardiovasc. Pharmacol.* **26**(Suppl. 3), 279–283.

Filep, J. G., Foldes-Filep, E., and Sirois, P. (1993). Nitric oxide modulates vascular permeability in the rat coronary artery circulation. *Br. J. Pharmacol.* **108,** 323–326.

Fischer, A., Mundel, P., Mayer, B., Preissler, U., Philippin, B., and Kummer, W. (1993). Nitric oxide synthase in guinea-pig lower airway innervation. *Neurosci. Lett.* **149,** 157–160.

Förstermann, U., Schmidt, H. H. H. W., Pollock, J. S., Sheng, H., Mitchell, J. A., Warner, T. D., Nakane, M., and Murad, F. (1991). Isoforms of nitric oxide synthase. Characterization and purification from different cell types. *Biochem. Pharmacol.* **42,** 1849–1857.

Franco-Cereceda, A. (1989). Endothelin- and neuropeptide Y-induced vasoconstriction of human epicardial coronary arteries. *Br. J. Pharmacol.* **97,** 968–972.

Franco-Cereceda, A., Matran, R., Lou, Y. P., and Lundberg, J. M. (1990). Occurrence and effects of endothelin in guinea-pig cardiopulmonary tissue. *Acta Physiol. Scand.* **138,** 539–547.

Frank, G. W., and Bevan, J. A. (1983). Electrical stimulation causes endothelium-dependent relaxation in lung vessels. *Am. J. Physiol.* **244,** H793–H798.

Furchgott, R. F. (1988). Studies on relaxation of rabbit aorta by sodium nitrite: Basis for the proposal that the acid-activated inhibitory factor from bovine retractor penis is inorganic

nitrite and endothelium-derived relaxing factor is nitric oxide. *In* "Mechanisms of Vasodilatation" (P. M. Vanhoutte, Ed.), pp. 401–414. Raven Press, New York.

Furchgott, R. F., and Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288,** 373–376.

Furchgott, R. F., Khan, M. T., and Jothianandan, D. (1987). Evidence supporting the proposal that endothelium-derived relaxing factor is nitric oxide. *Thromb. Res.* (Suppl. VII), 5.

Garg, U. G., and Hassid, A. (1991). Nitric oxide decreases cytosolic free calcium in Balc/c 3T3 fibroblasts by a cyclic GMP-independent mechanism. *J. Biol. Chem.* **266,** 9–12.

Garland, C. J., Plane, F., Kemp, B. K., and Cocks, T. M. (1995). Endothelium-dependent hyperpolarisation—A role in the control of vascular tone. *Trends Pharmacol. Sci.* **16,** 23–30.

Giaid, A., Polak, J. M., Gaitonde, V., Hamid, Q. A., Moscoso, G., Legon, S., Uwanogho, D., Roncalli, M., Shinmi, O., Sawamura, T., *et al.* (1991). Distribution of endothelin-like immunoreactivity and mRNA in the developing and adult human lung. *Am. J. Respir. Cell Mol. Biol.* **4,** 50–58.

Giaid, A., Michel, R. P., Stewart, D. J., Sheppard, M., Corrin, B., and Hamid, Q. A. (1993). Expression of endothelin-1 in lungs of patients with cryptogenic fibrosing alveolitis. *Lancet* **341,** 1555–1554.

Goodson, A. R., Leibold, J. M., and Gutterman, D. D. (1994). Inhibition of nitric oxide synthase augments centrally induced sympathetic coronary vasoconstriction in cat. *Am. J. Physiol.* **267,** H1272–H1278.

Gordon, J. B., and Tod, M. L. (1993). Effects of ω-nitro-L-arginine on total and segmental vascular resistances in developing lamb lung. *J. Appl. Physiol.* **75,** 76–85.

Hahn, A. W., Regenass, S., Resink, T. J., Kern, F., and Buhler, F. R. (1993). Morphogenic effects of endothelin-1 on vascular smooth muscle cells. *J. Vasc. Res.* **30,** 192–201.

Halbower, A. C., Tuder, R. M., Franklin, W. A., Pollock, J. S., Förstermann, U., and Abman, S. H. (1994). Maturation-related changes in endothelial nitric oxide synthase immunolocalization in developing ovine lung. *Am. J. Physiol.* **267,** L585–591.

Harrison, V. J., Randriantsoa, A., and Schoeffter, P. (1992). Heterogeneity of endothelin-sarafotoxin receptors mediating contraction of pig coronary artery. *Br. J. Pharmacol.* **105,** 511–513.

Hassall, C. J. S., Saffrey, M. J., Belai, A., Hoyle, C. H. V., Moules, E. W., Moss, J., Schmidt, H. H. H. W., Murad, F., Förstermann, U., and Burnstock, G. (1992). Nitric oxide synthase immunoreactivity and NADPH-diaphorase activity in a subpopulation of intrinsic neurones of the guinea pig heart. *Neurosci. Lett.* **143,** 65–68.

Hassall, C. J. S., Saffrey, M. J., and Burnstock, G. (1993). Expression of NADPH-diaphorase activity by guinea-pig paratracheal neurones. *NeuroReport* **4,** 49–52.

Hasunuma, K., Rodman, D. M., O'Brien, R. F., and McMurty, I. F. (1990). Endothelin-1 causes pulmonary vasodilatation in the rat. *Am. J. Physiol.* **259,** H48–H54.

Hay, D. W. P., and Luttman, M. A. (1997). Nonpeptide endothelin receptor antagonists. Characterization of endothelin receptors in guinea-pig bronchus with SB209670 and other endothelin receptor antagonists. *J. Pharmacol. Exp. Ther.* **280,** 956–965.

Hay, D. W. P., Luttman, M. A., Hubbard, W. C., and Undem, B. J. (1993). Endothelin receptor subtypes in human and guinea-pig pulmonary tissues. *Br. J. Pharmacol.* **110,** 1175–1183.

Hirata, Y., Takagi, Y., Fukuda, Y., and Marumo, F. (1989). Endothelin is a potent mitogen for rat vascular smooth muscle cells. *Atherosclerosis* **78,** 225–228.

Hosada, K., Nakao, K., and Arai, H. (1991). Cloning and expression of human endothelin-1 receptor cDNA. *FEBS Lett.* **287,** 23–26.

Ignarro, L. J., Wood, K. S., and Byrns, R. E. (1986). Pharmacological and biological properties of EDRF: Evidence that EDRF is closely related to nitric oxide (NO) radical. *Circulation* **74** (Suppl. II), II–287.

Ignarro, L. J., Bugas, G. M., Wood, K. S., Byrns, R. E., and Chaudhuri, G. (1987a). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. USA* **84,** 9265–9269.

Ignarro, L. J., Byrns, R. E., Bugas, G. M., and Wood, K. S. (1987b). Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Circ. Res.* **61,** 866–879.

Ignarro, L. J., Byrns, R. E., Bugas, G. M., and Wood, K. S. (1987c). Mechanisms of endothelium-dependent vascular smooth muscle relaxation elicited by bradykinin and VIP. *Am. J. Physiol.* **253,** H1074–H1082.

Ignarro, L. J., Byrne, R. E., and Wood, K. S. (1988). Biochemical and pharmacological properties of endothelium-derived relaxing factor and its similarity to nitric oxide radical. *In* "Mechanisms of Vasodilatation" (P. M. Vanhoutte, Ed.), pp. 427–436. Raven Press, New York.

Inoue, A. Yanagisawa, M., Kimura, S., Kasuya, W., Miyauchi, T., Goto, M., and Masaki, T. (1989). The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separated genes. *Proc. Natl. Acad. Sci. USA* **86,** 2863–2867.

Katsuragi, T., and Su, C. (1982). Augmentation by theophylline of [^{3}H]purine release from vascular adrenergic nerves: Evidence for presynaptic autoinhibition. *J. Pharmacol. Exp. Ther.* **220,** 152–156.

Katusic, Z. S., Sheperd, J. T., and Vanhoutte, P. M. (1987). Endothelium-dependent contractions to stretch in canine basilar artery. *Am. J. Physiol.* **252,** H671–H673.

Kelm, M., and Schrader, J. (1988). Nitric oxide release from the isolated guinea pig heart. *Eur. J. Pharmacol.* **155,** 317–321.

Kelm, M., and Schrader, J. (1990). Control of coronary vascular tone by nitric oxide. *Circ. Res.* **66,** 1561–1575.

Klimaschewski, L., Kummer, W., Mayer, B., Couraud, J. Y., Preissler, U., Philippin, B., and Heym, C. (1992). Nitric oxide synthase in cardiac nerve fibres and neurones of rat and guinea-pig heart. *Circ. Res.* **71,** 1533–1537.

Knowles, R. G., and Moncada, S. (1994). Nitric oxide synthases in mammals. *Biochem. J.* **298,** 149–158.

Kobayashi, M., Ihara, M., Sato, N., Saeki, T., Ozaki, S., Ikemoto, F., and Yano, M. (1993). A novel ligand [^{125}I]BQ-3020, reveals the localization of endothelin ET_B receptors. *Eur. J. Pharmacol.* **235,** 95–100.

LaDouceur, D. M., Flynn, M. A., Keiser, J. A., Reynolds, E., and Haleen, S. J. (1993). ET_A and ET_B receptors coexist on rabbit pulmonary artery vascular smooth muscle mediating contraction. *Biochem. Biophys. Res. Commun.* **196,** 209–215.

Lamontagne, D., Pohl, U., and Busse, R. (1992). Mechanical deformation of vessel wall and shear stress determine the basal release of endothelium-derived relaxing factor in the intact rabbit coronary vascular bed. *Circ. Res.* **70,** 123–130.

LeCras, T. D., Xue, C., Rengasami, A., and Johns, R. A. (1996). Chronic hypoxia upregulates endothelial and inducible NO synthase gene and protein expression in rat lung. *Am. J. Physiol.* **270,** L164–L170.

Lincoln, T. M. (1989). Cyclic GMP and mechanisms of vasodilatation. *Pharmacol. Ther.* **41,** 479–502.

Lippton, H. L., Hauth, T. A., Cohen, G. A., and Hyman, A. L. (1993). Functional evidence for different endothelin receptors in the lung. *J. Appl. Physiol.* **75,** 38–48.

Liu, S. F., Crawley, D. E., Evans, T. W., and Barnes, P. J. (1991). Endogenous nitric oxide modulates adrenergic neural vasoconstriction in guinea-pig pulmonary artery. *Br. J. Pharmacol.* **104,** 565–569.

Liu, S. F., Crawley, D. E., Evans, T. W., and Barnes, P. J. (1992). Endothelium-dependent non-adrenergic non-cholinergic neural relaxation in guinea-pig pulmonary artery. *J. Pharmacol. Exp. Ther.* **260,** 541–548.

Loesch, A., and Burnstock, G. (1995). Ultrastructural localization of nitric oxide synthase and endothelin in coronary and pulmonary arteries of newborn rats. *Cell Tissue Res.* **279,** 475–483.

Loesch, A., and Burnstock, G. (1996a). Immunocytochemistry of vasoactive agents and nitric oxide synthase in vascular endothelial cells with emphasis on the cerebral blood vessels. *Cell Vision* **3,** 346–357.

Loesch, A., and Burnstock, G. (1996b). Ultrastructural localization of nitric oxide synthase and endothelin in rat pulmonary artery and vein during postnatal development and ageing. *Cell Tissue Res.* **283,** 355–365.

Lundberg, J. M. (1981). Evidence for coexistence of vasoactive intestinal polypeptide (VIP) and acetylcholine in neurons of cat exocrine glands. Morphological, biochemical and functional studies. *Acta Physiol. Scand. Suppl.* **496,** 1–57.

MacLean, M. R., and McCulloch, K. M. (1998). Influence of applied tension and nitric oxide on responses to endothelins in rat pulmonary resistence arteries: Effect of chronic hypoxia. *Br. J. Pharmacol.* **123,** 991–999.

Mann, J., Farrukh, I. S., and Michael, J. R. (1991). Mechanisms by which endothelin-1 induces pulmonary vasoconstriction in the rabbit. *J. Appl. Physiol.* **71,** 410–416.

Masaki, J., Kimura, S., Yanagisawa, M., and Goto, K. (1991). Molecular and cellular mechanisms of endothelin regulation. Implications for vascular function. *Circulation* **84,** 1457–1468.

Maulik, N., Engelman, D. T., Watanabe, M., Engelman, R. M., Maulik, G., Cordis, G. A., and Das, D. K. (1995). Nitric oxide signaling in ischemic heart. *Cardiovasc. Res.* **30,** 593–601.

Miller, V. M., Komori, K., Burnett, J. C., Jr., and Vanhoutte, P. M. (1989). Differential sensitivity to endothelin in canine arteries and veins. *Am. J. Physiol.* **257,** H1127–H1131.

Milner, P., Bodin, P., Loesch, A., and Burnstock, G. (1990). Rapid release of endothelin and ATP from isolated aortic endothelial cells exposed to increased flow. *Biochem. Biophys. Res. Commun.* **170,** 649–654.

Milner, P., Loesch, A., and Burnstock, G. (1996). Neonatal sensory denervation affects the expression of endothelial peptides in the adult rat pulmonary artery: More cells contain substance P and less contain endothelin. *Endothelin* **4,** 71–76.

Mione, M. C., Ralevic, V., and Burnstock, G. (1990). Peptides and vasomotor mechanisms. *Pharmacol Ther.* **46,** 429–468.

Mombouli, J.-V., and Vanhoutte, P. M. (1997). Endothelium-derived hyperpolarizing factor(s): Updating and unknown. *Trends Pharmacol. Sci.* **18,** 252–256.

Moncada, S., and Higgs, A. (1993). The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* **329,** 2002–2012.

Moncada, S., Palmer, R. M. J., and Higgs, E. A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43,** 109–142.

Morris, R., Southam, E., Gittins, S. R., and Garthwaite, J. (1993). NADPH-diaphorese staining in autonomic and somatic cranial ganglia of the rat. *NeuroReport* **4,** 62–64.

Nakamura, S., Naruse, M., Naruse, K., Demura, H., and Uemura, H. (1990). Immunohistochemical localization of endothelin in cultured bovine endothelial cells. *Histochemistry* **94,** 475–477.

Naruse, K., Naruse, M., Nakamura, S., Yoshiwara, I., Uemura, H., Koji, T., Kato, Y., and Snyder, S. H. (1990). Immunocytochemical localization of endothelin (ET) and in situ hybridization of mRNA in cultured endothelial cells. *J. Hypertension* **8,** S65. [Abstract]

Nava, E., and Luscher, T. (1995). Endothelium-derived vasoactive factors in hypertension: Nitric oxide and endothelin. *J. Hypertension Suppl.* **13,** 39–48.

Neild, G. H. (1994). Endothelin plasma levels in hypertensive patients with vascular disease. *J. Hypertension Suppl.* **12,** 17–20.

Ogawa, Y., Nakao, K., Arai, H., Nakagawa, O., Hosoda, K., Suga, S., Nakanishi, S., and Imura, H. (1991). Molecular cloning of a non-isopeptide-selective human endothelin receptor. *Biochem. Biophys. Res. Commun.* **178,** 248–255.

Ogilvie, P., Schilling, K., Bellingsley, M. L., and Schmidt, H. H. H. W. (1995). Induction and variants of neuronal nitric oxide synthase type I during synaptogenesis. *FASEB J.* **9,** 799–806.

important constituent of several complex organic compounds was recognized more that 100 years ago but it was not until 1930 that the first inositol-containing lipids were identified. R. J. Anderson discovered that inositol could be isolated from a "phosphatide" fraction from tubercle bacilli, and the presence of inositol in brain phospholipids was described by Folch and Woolley in 1942.

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Shochina, M., Loesch, A., Rubino, S., Miah, S., Mcdonald, G., and Burnstock, G. (1997). Immunoreactivity for nitric oxide synthase and endothelin in the coronary and basilar arteries of renal hypertensive rats. *Cell Tissue Res.* **288,** 509–516.

Simonsen, U., Garcia-Sacristan, A., and Prieto, D. (1997). Involvement of ATP in the non-adrenergic non-cholinergic inhibitory neurotransmission of lamb isolated coronary small arteries. *Br. J. Pharmacol.* **120,** 411–420.

Snyder, S. H. (1992). Nitric oxide and neurones. *Curr. Opin. Neurobiol.* **2,** 323–327.

Sosunov, A. A., Hassall, C. J. S., Loesch, A., Turmaine, M., and Burnstock, G. (1995). Ultrastructural investigation of nitric oxide synthase-immunoreactive nerves associated with coronary blood vessels of rat and guinea-pig. *Cell Tissue Res.* **280,** 575–582.

Sosunov, A. A., Hassall, C. J. S., Loesch, A., Turmaine, M., and Burnstock, G. (1996). Nitric oxide synthase-containing neurones and nerve fibres within cardiac ganglia of rat and guinea-pig: An electron-microscopic immunocytochemical study. *Cell Tissue Res.* **284,** 19–28.

Springall, D. R., Riveros-Moreno, V., Buttery, L. D. K., Suburo, A., Bishop, A. E., Merrett, M., Moncada, S., and Polak, J.M. (1992). Immunological detection of nitric oxide synthase(s) in human tissues using heterologous antibodies suggesting different isoforms. *Histochemistry* **98,** 259–266.

Stjarne, L. (1989). Basic mechanisms and local modulation of nerve-impulse-induced secretion of neurotransmitters from individual sympathetic nerve varicosities. *Rev. Physiol. Biochem. Pharmacol.* **112,** 1–137.

Sventek, P., Li, J. S., Grove, K., Deschepper, C. F., and Schiffrin, E. L. (1996). Vascular structure and expression of endothelin-1 gene in L-NAME-treated spontaneously hypertensive rats. *Hypertension* **27,** 49–55.

Tabrizchi, R., and Triggle, C. R. (1991). Influence of N^{ω}-nitro-L-arginine on pressor responses elicited by sympathetic nerve stimulation in pithed normotensive and hypertensive rats. *Life Sci.* **49,** 1989–1995.

Takada, K., Matsumura, Y., Dohmen, S., Mitsutomi, N., Takaoka, M., and Morimoto, S. (1996). Endothelin-1 secretion from cultured vascular endothelial cells of DOCA-salt hypertensive rats. *Life Sci.* **59,** PL111–PL116.

Tanaka, K., Hassall, C. J. S., and Burnstock, G. (1993a). Distribution of intracardiac neurones and nerve terminals that contain a marker for nitric oxide, NADPH-diaphorase, in the guinea-pig heart. *Cell Tissue Res.* **273,** 293–300.

Tanaka, K., Oshima, H., Esumi, H., and Chiba, T. (1993b). Direct synaptic contacts of nitric oxide synthase-immunoreactive nerve terminals on the neurones of the intracardiac ganglia of the guinea-pig. *Neurosci. Lett.* **158,** 67–70.

Tasaka, K., and Kitazumi, K. (1994). The control of endothelin-1 secretion. *Gen. Pharmacol.* **25,** 1059–1069.

Tippins, J. R., Antoniw, J. W., and Maseri, A. (1989). Endothelin-1 is a potent constrictor in conductive and resistance coronary arteries. *J. Cardiovasc. Pharmacol.* **13**(Suppl. 5), 177–179.

Toda, N., Ayajiki, K., and Okamura, T. (1993). Cerebroarterial relaxations mediated by nitric oxide derived from endothelium and vasodilator nerves. *J. Vasc. Res.* **30,** 61–67.

Tomlinson, A., Van Vlijmen, H., Loesch, A., and Burnstock, G. (1991). An immunohistochemical study of endothelial cell heterogeneity in the rat: Observation in "en face" Häutchen preparations. *Cell Tissue Res.* **263,** 173–181.

Tonnessen, T., Giaid, A., Saleh, D., Naess, P. A., Yanagisawa, M., and Christensen, G. (1995). Increased in vivo expression and production of endothelin-1 by porcine cardiomyocytes subject to ischemia. *Circ. Res.* **76,** 767–772.

Tschudi, M. R., and Luscher, T. F. (1995). Age and hypertension differently affect coronary contractions to endothelin-1, serotonin and angiotensins. *Circulation* **91,** 2415–2422.

Tsukahara, H., Ende, H., Magazine, H. E., Bahou, W. F., and Croligorsky, M. S. (1994). Molecular and functional characterization of the non-isopeptide-selective ET_B receptor in endothelial cells. Receptor coupling to nitric oxide synthase. *J. Biol. Chem.* **269,** 21778–21785.

Ursell, P. C., and Mayes, M. (1993). The majority of nitric oxide synthase in pig heart is vascular and not neuronal. *Cardiovasc. Res.* **27,** 1929–1934.

Ursell, P. C., and Mayes, M. (1996). Endothelial isoform of nitric oxide synthase in rat heart increases during development. *Anat. Rec.* **246,** 465–472.

Von Euler, U. S., and Liljestrand, G. (1947). Observations on the pulmonary arterial blood pressure in the cat. *Acta Physiol. Scand.* **12,** 301–320.

Wang, Q. D., Li, X. S., and Pernow, J. (1994). Characterization of endothelin-1-induced vascular effects in the rat heart by using endothelin receptor antagonists. *Eur. J. Pharmacol.* **271,** 25–30.

Wang, Y., Coe Y., Toyoda, O., and Coceani, F. (1995). Involvement of endothelin-1 in hypoxic pulmonary vasoconstriction in the lamb. *J. Physiol (London).* **482,** 421–434.

Weissberg, P. L., Mitchell, C., Davenport, A. P., Hesketh, T. R., and Metclafe, J. C. (1990). The endothelin peptides ET-1, ET-2, ET-3 and sarafotoxin S6b are co-mitogenic with platelet-derived growth factor for vascular smooth muscle cells. *Atherosclerosis* **85,** 257–262.

Wharton, J., Rutherford, R. A., Gordon, L., Moscoso, G., Schiemberg, I., Gaer, J. A., Taylor, K. M., and Polak, J. M. (1991). Localization of endothelin binding sites and endothelin-like immunoreactivity in human fetal heart. *J. Cardiovasc. Pharmacol.* **17**(Suppl 7), S378–S384.

Wiklund, N. P., Persson, M. G., Gustafsson, L. E., Moncada, S., and Hedqvist, P. (1990). Modulatory role of endogenous nitric oxide in pulmonary circulation in vivo. *Eur. J. Pharmacol.* **185,** 123–124.

Wildhirt, S. M., Dudek, R. R., Suzuki, H., Narayan, K. S., Winder, S., Choe, J., and Bing, R. J. (1995a). Expression of nitric oxide synthase isoforms after myocardial infarction in humans. *Endothelium* **3,** 209–224.

Wildhirt, S. M., Dudek, R. R., Suzuki, H., and Bing, R. J. (1995b). Involvement of inducible nitric oxide synthase in the inflammatory process of myocardial infarction. *Int. J. Cardiol.* **50,** 253–261.

Wildhirt, S. M., Dudek, R. R., Suzuki, H., Pinto, V., Narayan, K. S., and Bing, R. J. (1995c). Immunohistochemistry in the identification of nitric oxide synthase isoenzymes in myocardial infarction. *Cardiovasc. Res.* **29,** 526–531.

Xue, C., Rengasamy, A., Le Cras, T. D., Koberna, P. A., Dailey, G. C., and Johns, R. A. (1994). Distribution of NOS in normoxic vs. hypoxic rat lung: Upregulation of NOS by chronic hypoxia. *Am. J. Physiol.* **267,** L667–L678.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yasaki, Y., Goto, K., and Masaki, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332,** 411–415.

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Phosphoinositide Kinases and the Synthesis of Polyphosphoinositides in Higher Plant Cells

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Phosphoinositides are a family of inositol-containing phospholipids which are present in all eukaryotic cells. Although in most cells these lipids, with the exception of phosphatidylinositol, constitute only a very minor proportion of total cellular lipids, they have received immense attention by researchers in the past 15–20 years. This is due to the discovery that these lipids, rather than just having structural functions, play key roles in a wide range of important cellular processes. Much less is known about the plant phosphoinositides than about their mammalian counterparts. However, it has been established that a functional phosphoinositide system exists in plant cells and it is becoming increasingly clear that inositol-containing lipids are likely to play many important roles throughout the life of a plant. It is not our intention to give an exhaustive overview of all aspects of the field, but rather we focus on the phosphoinositide kinases responsible for the synthesis of all phosphorylated forms of phosphatidylinositol. Also, we mention some of the aspects of current phosphoinositide research which, in our opinion, are most likely to provide a suitable starting point for further research into the role of phosphoinositides in plants.

KEY WORDS: Phosphatidylinositol, Kinases, Inositol, Plants, Biosynthesis, Cell signaling, Phosphatidylinositol transfer proteins, Phospholipids.

I. Introduction

Inositol-containing lipids belong to a large and heterogenous group of lipids, many of which remain to be fully characterized. That inositol is an

important constituent of several complex organic compounds was recognized more that 100 years ago but it was not until 1930 that the first inositol-containing lipids were identified. R. J. Anderson discovered that inositol could be isolated from a "phosphatide" fraction from tubercle bacilli, and the presence of inositol in brain phospholipids was described by Folch and Woolley in 1942.

The next important breakthrough in our understanding of the cellular functions of these enigmatic lipids came from experiments carried out by Lowell and Mabel Hokin at the Montreal General Hospital in Canada. The Hokin's discovered that when cholinergic drugs were added to pancreatic tissue (a stimulus which leads to increased secretion of amylase) the incorporation of ^{32}P into a "phospholipide" fraction was increased five- to nine-fold over the incorporation in control tissues (Hokin and Hokin, 1953). It was later shown that the increased ^{32}P labeling was confined to a subset of the cellular phospholipid pool, namely, the phosphoinosities, and the effect has since then colloquially been referred to as "the PI effect."

In the years following the Hokin discovery phosphoinositide research progressed steadily, albeit at a somewhat sedate pace, and the field was largely kept alive by a small group of highly dedicated "PI-oneers." By the early 1960s it was realized that an increase in the rate of turnover of the monoesterified phosphate group of the two polyphosphoinositides, phosphatidylinositol(4)phosphate [PtdIns(4)P] and phosphatidylinositol(4,5)bisphosphate [PtdIns(4,5)P_2], was an integral feature of the PI effect and in the mid-1970 it became evident that the receptors which gave rise to the PI effect all shared a family resemblance: Their mode of action depended on changes in intracellular Ca^{2+}.

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second messenger production, and intracellular Ca^{2+} release, known as "the phosphoinositide system," is briefly summarized in Fig. 1.

Although work has been carried out for more that a century to characterize the metabolism and function of inositol and inositol-containing compounds in plant cells, the discovery of the mammalian phosphoinositide system gave new impetus to this area of plant science because the question of whether a similar system was involved in cell signaling events in plants needed answering. Around the mid-1980s it was discovered that polyphosphoinositides are indeed present in plant cells (Boss and Massel, 1985) together with the kinases and lipases responsible for their turnover (Sandelius and Sommarin, 1986; Melin *et al.,* 1987); in addition, $Ins(1,4,5)P_3$ was found to be capable of mobilizing Ca^{2+} from intracellular stores (Drøbak and Ferguson, 1985). Since these discoveries, it has become evident that phosphoinositides are ubiquitous in eukaryotic cells and have far wider roles in cell signaling events than merely acting as precursors for second messenger production. The unraveling of the bewildering complexities of phosphoinositide-mediated signaling in both higher and lower eukaryotic cells has in the past 10–15 years captivated the attention of a very large number of signal transduction scolars worldwide. Figure 2 illustrates the known members of the phosphoinositide family in eukaryotic cells, and the likely routes of their interconversion are indicated. That the roster of phosphoinositides may not yet be complete is illustrated by the fact that no less than two novel members of the family have been identified during the time spent writing this review.

In the mid- to late 1980s, another aspect of phosphoinositide metabolism emerged when it was found that immunoprecipitates of $pp60^{v\text{-}src}$, polyoma middle T/$pp60^{c\text{-}src}$ complexes, contained a novel type of phosphoinositide kinase activity capable of phosphorylating phosphatidylinositol and 4-phosphoinositides in the D3 position of the inositol ring (Whitman *et al.,* 1988). The reason for the relatively late discovery of this group of lipids is largely due to the very low chemical levels in most mammalian cells; in most cell types they represent only or 1 or 2% of total phosphoinositides. However, much higher levels of 3-phosphorylated phosphoinositides have been found in other organisms such as yeast. In mammalian cells, the levels of the polyphosphorylated forms of 3-phosphoinositides were found to increase rapidly upon cellular stimulation, and since the 3-phosphoinositides are known not to be substrates for either phospholipase C or D the hypothesis emerged that the 3-phosphorylated lipids themselves could act as signal transmitters. The association of phosphoinositide (PtdIns) 3-kinase activity with receptors known to stimulate cell growth further suggested that a modulation of PtdIns 3-kinase activity somehow was linked to the control of mitogenesis and perhaps could also play an important role in the onset of oncogenesis (Cantley *et al.,* 1991). Numerous studies have addressed

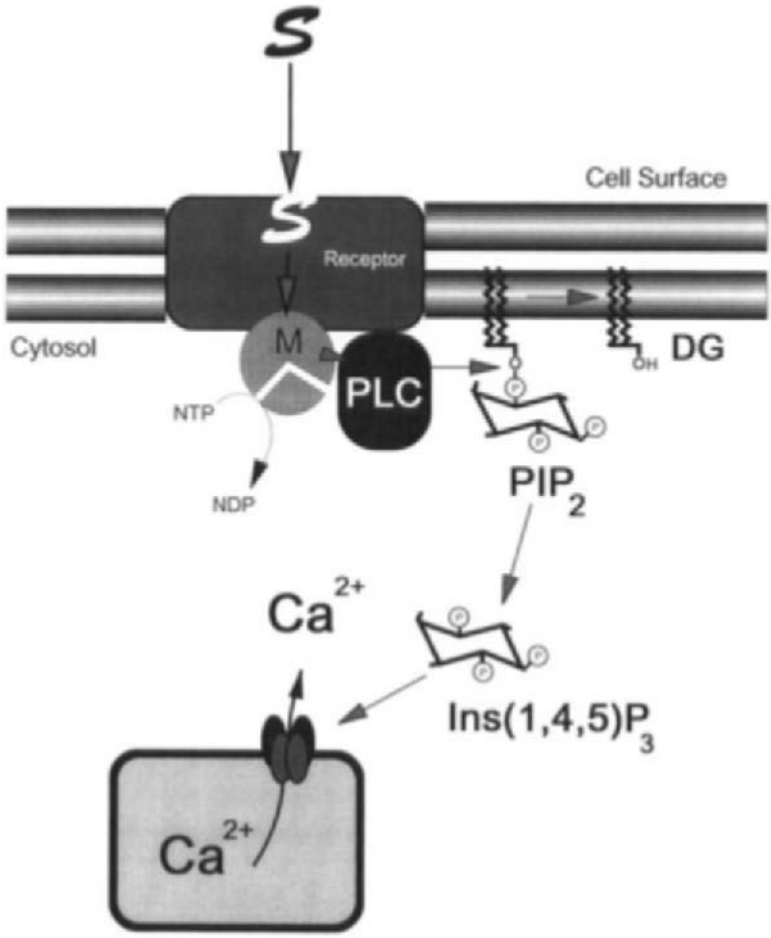

FIG. 1 Generation of the second messenger, inositol(1,4,5)trisphosphate, by phospholipase C-mediated hydrolysis of phosphatidylinositol(4,5)bisphosphate. This figure shows a simplified presentation of some of the key reactions leading from receptor occupancy to inositol(1,4,5)trisphosphate generation and mobilization of intracellular Ca^{2+}. When certain signals (S) arrive at the cell surface and associate with specific cell surface receptors the enzyme phospholipase C (PLC; phosphoinositide-specific phospholipase C also known as phosphoinositidase C) is activated. Several PLC isoforms exist in eukaryotic cells, and the different PIC isoforms have isoform-specific modes of activation. The mammalian PLC-β-isoforms are thus activated through regulatory, heterotrimeric GTP-binding proteins, whereas the γ isoforms depend on receptor-associated tyrosine kinase(s) for activation. Common for these two modes of activation is that a mediator protein(s) (M), as well as ATP/GTP (NTP) hydrolysis, is required. The activated forms of PLC are capable of hydrolyzing phosphatidylinositol(4,5)bisphosphate [PtdIns(4,5)P_2] which results in the liberation of the two second messenger molecules: inositol(-1,4,5)trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG). Ins(1,4,5)P_3 is capable of specifically inducing Ca^{2+} release from intracellular stores, whereas DAG in many cell types modulates the activity of a group of enzymes known as protein kinase(s) C. The concomitant

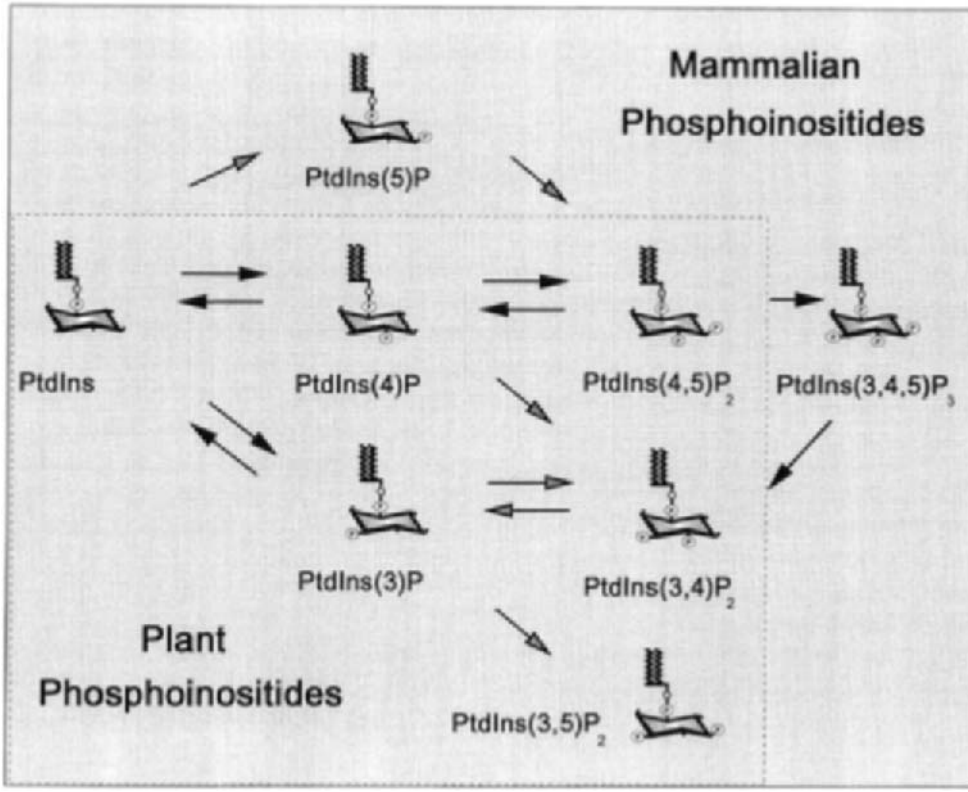

FIG. 2 Metabolic routes for phosphoinositide formation and degradation in mammalian and plant cells. All the currently known members of the eukaryotic phosphoinositide family are shown. These molecules have all been found to be present in mammalian cells. The phosphoinositides which have been identified in plant cells are contained within the stippled box. Metabolic routes which are well established are indicated by solid arrows, whereas suggested metabolic steps which need final confirmation either in plants or in mammalian cells are indicated by open arrows. Further details of the individual reactions can be found in the text.

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increase in cytosolic Ca^{2+} and the switch on of protein kinase activity results in a bifurcated signal and cell activation. When the stimulus ceases to exert its effect, the agonist–receptor complex is dissociated and PLC is converted back into an inactive configuration. When PtdIns(4,5)P_2 is no longer hydrolyzed the cellular levels of Ins(1,4,5)P_3 and DAG decrease and the cytosolic Ca^{2+} levels return to low n*M* courtesy of Ca^{2+} transporting systems removing Ca^{2+} from the cytosol.

these and other possibilities, and although there are many unresolved questions regarding their mode of action, the consensus has been reached that 3-phosphoinositides are ubiquitious in eukaryotic cells and play several vital roles in cellular function. PtdIns 3-kinases have thus been shown to be involved in cellular processes as diverse as mitogenesis, membrane trafficking and ruffling, glucose uptake, oxidative burst responses, chemotaxis, and apoptosis (Carpenter and Cantley, 1996). 3-Phosphoinositides are also present in plant cells, and although comparatively little is known about their function, antisense experiments have revealed that they play an essential role in normal growth and development (see sections II and V,B).

Because of the multitude of important cellular functions it is obvious that the coordinated regulation of the synthesis of phosphoinositides is vital to the proper functioning of eukaryotic cells. Our aim in this review is not to present an exhaustive catalog of all the factors that may influence the synthesis and metabolism of inositol-containing lipids in plants (and other organisms) but rather to focus on the specific kinases responsible for the generation of the individual members of the phosphoinositide family. Also, we will discuss some of the factors which are believed to be important for the presentation of substrates to these kinases. We are fully aware that the phosphoinositide kinases are not the only enzymes responsible for the state of the cellular polyphosphoinositide pools, but since we have relatively limited space we elected to concentrate on the enzymes responsible for the biosynthesis of polyphosphoinositides, i.e., the phosphoinositide kinases.

II. Functions of Polyphosphoinositides in Eukaryotic Cells, with Special Reference to Their Potential Role(s) in Higher Plants

Although many of the recognized functions of the phosphoinositides which we alluded to in the previous section have been reviewed elsewhere, we will briefly summarize some of the recent developments in our understanding of their function before discussing their route of synthesis. Special attention will be given to the aspects which are likely to have most relevance for research on plant systems.

A. PtdIns(4,5)P_2

One of the most prominent physiological roles for PtdIns(4,5)P_2 is unarguably that of being the precursor of the three second messengers: inositol (1,4,5)trisphosphate, 1,2-diacylglycerol, and phosphatidylinositol(3,4,5)tris-

phosphate. The second messenger role of DAG in plant cells is undefined, and currently there is no evidence for the formation of PtdIns(3,4,5)P_3 in lower eukaryotes. In contrast, the role of PtdIns(4,5)P_2 as a precursor for Ins(1,4,5)P_3 production in plant cells is now well established, and Ins(1,4,5)P_3 has been shown to be produced in response to a variety of abiotic and biotic stimuli (Drøbak, 1992, 1996; Coté and Crain, 1993; Munnik *et. al.,* 1998). Being a precursor for no less that three major second messengers seems to be justification for the existence of any molecule, but recent research suggests that PtdIns(4,5)P_2 has several other important functions in cells. One such function of PtdIns(4,5)P_2 is the regulation of cytoskeletal dynamics.

The cytoskeleton in eukaryotic cells consists of three main components—actin, tubulin, and intermediate filaments—and PtdIns(4,5)P_2 has been found to interact intimately with at least actin and tubulin. An important step in the regulation of the cellular actin pool involves the interconversion between monomeric actin (globular actin or G-actin) and the polymerized form of actin (filamentous or F-actin). This interconversion between G- and F-actin is known to be regulated by at least six classes of actin-associated proteins; profilin, villin, fragmin, α-actinin, gelsolin, and depactin.

PtdIns(4,5)P_2 can influence the cytoskeleton by interacting with actin-binding proteins. The interaction between profilin and PtdIns(4,5)P_2 has been particularly well characterized and this interaction has been demonstrated to occur in all eukaryotic cells studied, including plants. Profilin is a small (12–15 kDa) protein which in addition to its actin-binding properties also has specific binding sites for polyphosphoinositides. Profilin thus has a 10-fold higher affinity for PtdIns(4,5)P_2 than for actin, so PtdIns(4,5)P_2 is highly effective in disrupting the formation of profilin–actin (profilactin) complexes and it is thus capable of excerting stringent control over the ability of profilin to interact with actin. However, profilin also controls the availability of PtdIns(4,5)P_2 for second messenger production. In fact, the association of profilin with clusters of PtdIns(4,5)P_2 molecules is so strong that it makes the profilin-bound PtdIns(4,5)P_2 totally refractory to attack by purified mammalian phospholipase-γ (PLC-γ; Goldschmidt-Clermont *et al.,* 1990, 1991). That a very similar scenario also exists in plant cells was demonstrated by Drøbak *et al.* (1994).

PtdIns(4,5)P_2 has also been shown to affect the function of another actin-binding protein, gelsolin. PtdIns(4,5)P_2 is thus known to inhibit the actin-severing activity of gelsolin and also to uncap gelsolin from the fast-growing end of actin filaments *in vivo* thus (indirectly) promoting actin filament growth (Hartwig *et al.,* 1995). In a recent study it was shown that PtdIns(4,5)P_2, in addition to its direct physical–chemical interaction, may also affect gelsolin and other actin-binding proteins in other ways. De Corte *et al.* (1997) demonstrated that PtdIns(4,5)P_2 is capable of dramatically

enhancing the phosphorylation of gelsolin by $pp60^{c\text{-}src}$, a membrane-associated nonreceptor tyrosine kinase equivalent to the transforming protein of Rous sarcoma virus. PtdIns(4,5)P_2 was also able to enhance the $pp60^{c\text{-}src}$ phosphorylation of other actin-binding proteins, such as profilin, fragmin, and CapG. Interactions have also been reported to take place between PtdIns(4,5)P_2 and members of the Rho family of GTP-binding proteins (Rac, Rho, and Cdc42) which are known to mediate extracellular signals to the actin cytoskeleton (see Section V,B).

PtdIns(4,5)P_2 has recently been shown to interact specifically with the other major component of the cytoskeleton, tubulin. In an elegant study, Popova *et al.* (1997) showed how tubulin can enter into complex interactions with the phosphoinositide pathway. First GTP–tubulin at low concentrations was shown to bind the heterotrimeric G protein subunit $G_{\alpha q}$ and activate it by directly transferring GTP. This activation triggers the phosphoinositide signaling cascade which proceeds via PLC-β1-mediated PtdIns(4,5)P_2 hydrolysis. Second, it was found that higher concentrations of dimeric tubulin inhibited PLC-β1 activity. Finally, it was shown that tubulin could interact with PtdIns(4,5)P_2 and prevent PtdIns(4,5)P_2 hydrolysis by PLC-β1. This scenario is very reminiscent of the scenario described previously in which a PtdIns(4,5)P_2–actin-binding protein interaction prevents its hydrolysis by the γ group of PLCs. In summary, very close interactions exist between PtdIns(4,5)P_2 and both of the major components of the eukaryotic cytoskeleton. Current evidence indicates that these interactions are both dynamic and highly complex, and many detailed *in vivo* studies are needed before a genuine understanding of these interactions can be claimed.

PtdIns(4,5)P_2 has also been found to act as a specific activator of several enzymes, including protein kinases (Palmer *et al.,* 1995), GTPase-activating proteins (Liscovitch and Cantley, 1995), and guanine nucleotide exchange factors (Zheng *et al.,* 1996), and PtdIns(4,5)P_2 has also been shown to mediate protein–protein interactions as exemplified, e.g., by the PtdIns(4,5)P_2-driven interaction between ADP-ribosylation factor 1 (Arf) and the Arf–GTPase activating protein (Arf–GAP; Randazzo, 1997). In plant cells, PtdIns(4,5)P_2 has been reported to modulate the activity of a number of enzymes, including vanadate-sensitive plasma membrane H^+-ATPase (Memon *et al.,* 1990), protein kinases (Schaller *et al.,* 1992), and DAG kinase (Lundberg and Sommarin, 1992). The most recent PtdIns(4,5)P_2-regulated enzymes to emerge are the mammalian and plant phospholipase D isozymes (Exton, 1997). Pappan *et al.* (1997a,b) identified a novel member of the plant PLD family in *Arabidopsis thaliana* and this new enzyme, named PLD-β, has been found to be dependent on both PtdIns(4,5)P_2 and submicromolar concentrations of Ca^{2+} for activity. As such, PLD-β appears to be eminently well suited to carry out a role as

a downstream effector enzyme for signals transduced through the plant phosphoinositide pathway.

Another entirely unexpected role for PtdIns(4,5)P_2 has become increasingly evident during the past 2 or 3 years following the discovery that PtdIns(4,5)P_2 is an essential regulatory component in the "priming" of exocytotic vesicles. Vesicle priming is an ATP-dependent process which occurs after vesicle docking but before fusion (Banerjee *et al.*, 1996; Hay and Martin, 1993). Hay *et al.* (1995) found that three cytosolic factors were essential for the successful priming of exocytotic vesicles; two PtdIns transfer proteins (PITPs; see Section IV) and a type I PtdIns(4)P 5-kinase. Together with a vesicular PtdIns 4-kinase, the two PITPs and PtdIns(4)P 5-kinase catalyze the synthesis of PtdIns(4,5)P_2 in the vesicle membrane. Although the precise function of PtdIns(4,5)P_2 in the priming process is still being investigated, it is most likely that PtdIns(4,5)P_2 either functions to recruit PtdIns(4,5)P_2-binding proteins to the vesicle membrane or perhaps activates enzymes within the vesicle membrane. There is no evidence to directly imply a role for PtdIns(4,5)P_2 in vesicle transport in plant cells, although recent experiments by Matsuoka *et al.* (1995) indicate that inhibition of phosphoinositide kinase activity severely affects vesicle transport processes in BY-2 cells (see Section V,B,1,e).

It may soon be possible that summarizing the reactions in which PtdIns(4,5)P_2 either has not been demonstrated or is not suspected to play a role could be an easier task than trying to cover the immense, and varied, spectrum of cellular events in which it does appear to play a role.

B. PtdIns(4)P

Apart from acting as a precursor for PtdIns(4,5)P_2 [and in some cases PtdIns(3,4)P_2], no "independent" physiological role for PtdIns(4)P has been defined in eukaryotic cells, although some early reports indicated that PtdIns(4)P may be involved in the regulation of certain enzymes such as DNA polymerase α (Sylvia *et al.*, 1988). In many cases PtdIns(4)P has been found to share functionality with PtdIns(4,5)P_2, e.g., in its ability to bind to actin-binding proteins, but in all cases PtdIns(4,5)P_2 has been shown to possess the highest affinity. However, it should be remembered that such experiments are often carried out using equimolar concentrations of polyphosphoinositides. Since PtdIns(4)P is by far the dominant polyphosphoinositide in plant cells the possibility should not be discounted that physiologically this lipid may be the important target for a number of *in vivo* processes in plant cells, although PtdIns(4,5)P_2 in *in vitro* experiments appears to be the favored candidate.

C. PtdIns(3)P

Little is known about the function of PtdIns(3)P in plant cells. Both PtdIns(3)P and PtdIns(3,4)P_2 have a turnover rate in plant cells which is greater than that of most structural lipids, indicating that the 3-phosphorylated phosphoinositides, like their 4-phosphorylated counterparts, have the potential to partake in rapid processes such as those encountered during cell signaling events (see Section V,B,1,e).

As will be discussed in more detail in Section V, the most likely role of PtdIns(3)P in plant cells is, as in yeast, in the regulation of vesicle translocation and other processes associated with membrane biogenesis and function. The molecular basis for this function has not been clarified but one possibility is that the phosphorylation of membrane PtdIns by PtdIns 3-kinase leads to local changes in the curvature of membrane bilayers which in turn may lead to the initiation of transport vesicle formation. Another possible function of PtdIns(3)P is as a target for vesicle docking onto target membranes. Perhaps the most likely proposition is that PtdIns(3)P exerts its function by recruiting otherwise soluble proteins to membranes, enabling them to function as downstream effectors of the PtdIns 3-kinase.

D. PtdIns(3,4)P_2

The presence of PtdIns(3,4)P_2 in plant cells appears to be ubiquitous, but no physiological role has been ascribed to this member of the plant phosphoinositide family. Until recently, in mammalian cells PtdIns(3,4)P_2 was viewed simply as the first hydrolysis product of PtdIns(3,4,5)P_3 breakdown. However, this view has changed dramatically and it is now likely that this enigmatic phosphoinositide exerts cellular functions which are equal in importance to those of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. One important target for PtdIns(3,4)P_2 appears to be the *Akt* protooncogene product, Akt. Akt (also referred to as PKB-β or Raca) is a serine–threonine kinase which participates in the activation of the p70 ribosomal protein S6 kinase ($p70^{S6K}$) and also plays a role in proliferative and antiapoptotic cell responses (Franke *et al.*, 1995; Dudek *et al.*, 1997). It is known that Akt activation by growth factors requires PtdIns 3-kinase activity but there are also alternative pathways which can lead to Akt activation. It is thus likely that one (or more) of the phosphoinositide products resulting from 3-kinase activation could be a potential regulator of Akt activity and hence $p70^{S6K}$ activation. Franke *et al.* (1997) investigated the effect of various phosphoinositides on the activity of Akt and found, somewhat surprisingly, that PtdIns(3,4)P_2 was capable of activating Akt both *in vitro* and *in vivo*. The ability to activate Akt was not shared by any of the other phosphoinositides,

indicating that this interaction is of physiological relevance and not just due to the strong negative charges found in PtdInsP_2 molecules for example. An interesting question is whether such a function for PtdIns(3,4)P_2 extends beyond mammalian cells. As mentioned earlier, there is no evidence for the presence of PtdIns(3,4,5)P_3 in plant cells, so the production of PtdIns(3,4)P_2 by a dephosphorylation pathway remains doubtful.

Nevertheless, PtdIns(3,4)P_2 is present in plant cells and has the potential to fulfill physiological roles similar to its mammalian counterpart. There are several poignant examples of signaling molecules fulfilling similar functions in different organisms despite being produced by different synthetic routes. There is no evidence for a homolog of Akt in plant cells but Mizoguchi *et al.* (1995) isolated two cDNA clones (cATPK19 and cATPK6) from *A. thaliana* which have sufficient homology to the mammalian $p70^{S6K}$ to be considered likely functional homolog. It is interesting to note that transcription levels of both the ATPK19 and ATPK6 genes were rapidly and markedly upregulated when plants were subjected to cold or high salt stresses, two environmental stresses that affect components of the plant phosphoinositide pathway. The pattern of PtdIns(3,4)P_2 synthesis and turnover in plant cells and possible responses to environmental stress must be thoroughly investigated in future research.

E. PtdIns(5)P and PtdIns(3,5)P

The inclusion of these phosphoinositides in this article was not envisaged when we started writing this review, but since then these two members have been added to the (seemingly) ever increasing family of phosphoinositides. Rameh *et al.* (1997) surprisingly found that type II PtdInsP kinases phosphorylate PtdIns(5)P in the D4 position rather than phosphorylating PtdIns(4)P in the D5 position as it had been assumed for decades. The authors explained that this previous error in interpretation is due to small (but significant) contaminating amounts of PtdIns(5)P in commercial preparations of PtdIns(4)P. The fact that PtdIns(5)P is present, albeit in small quantities, in fibroblasts strongly suggests that at least a portion of cellular PtdIns(4,5)P_2 is likely to be formed by sequential 5- and 4-phosphorylation of PtdIns.

The presence of PtdIns(3,5)P_2 in mouse fibroblasts was reported by Whiteford *et al.* (1997), and findings by Dove *et al.* (1997) suggest that rapid formation of PtdIns(3,5)P_2, following the activation of a PtdIns(3)P 5-kinase, is a conserved response to osmotic stress in a wide range of cell types. PtdIns(3,5)P_2 has also been identified in plant cells (Dove *et al.*, 1997) and experiments to further investigate this new and enigmatic pathway must be carried out.

III. Biosynthesis and Metabolism of Phosphatidylinositol

De novo biosynthesis of PtdIns in higher plants, as in animals, occurs via the CDP–DAG:inositol 3-phosphatidyltransferase (EC 2.7.8.11) pathway (Moore, 1990). The gene encoding CDP–DAG:inositol-3-phosphatidyltransferase, also called PtdIns synthase, has been cloned in yeast (Nikawa *et al.,* 1987), rats (Tanaka *et al.,* 1996), and humans (Lykidis *et al.,* 1997). Another reaction, initially described in plants, is the PtdIns:Ins exchange reaction in which the inositol head group is exchanged with free inositol in a CMP-dependent manner (Sexton and Moore, 1981; Sandelius and Morré, 1987; Moore, 1990). Using the recombinant enzyme, Lykidis *et al.* (1997) showed that both the CDP–DAG:inositol 3-phosphatidyltransferase and PtdIns:Ins reactions are catalyzed by PtdIns synthase. PtdIns synthase is located primarily on the cytoplasmic face of the endoplasmic reticulum (ER) (Ballas and Bell, 1981).

In addition to being an important component of biological membranes, PtdIns can be metabolized to form inositol sphingolipids in both yeast and plants. In this reaction, the inositol phosphate head group of PtdIns is transferred to a hydroxyl of phytoceramide by inositol phosphorylceramide synthase producing DAG and an inositol phosphorylphytoceramide (Kearns *et al.,* 1997). Inositol phosphorylphytoceramide biosynthesis also takes place in the ER. The regulation of inositol phosphophytoceramide biosynthesis can be a critical factor in membrane biogenesis (Kearns *et al.,* 1997).

There is a paucity of information about plant sphingolipids in general and inositol sphingolipids specifically (Lynch, 1993), although glycosphingolipids can account for as much as 16 mol% of plant plasma membrane lipids (Lynch and Steponkus, 1987). To date, none of the genes of the inositol sphingolipid biosynthetic pathway have been cloned in plants, and the potential involvement of glycosphingolipids in plant development or signaling has been all but ignored. Recently, inositol-containing glycosphingolipids were identified in pea and carrot plasma membranes by using antibodies raised against a guard cell microsomal fraction (Perotto *et al.,* 1995). In addition to localizing inositol sphingolipids within root nodule cells, this work serves as an excellent reminder that antibodies raised against membranes preparations in some cases can recognize lipid and protein antigens. It is hoped that, as the enzymes involved in inositol phosphophytoceramide biosynthesis are identified and the genes cloned, we can engage in more extensive studies of the role of inositol sphingolipids in membrane biogenesis and signal transduction.

PtdIns in the ER is also the substrate for the biosynthesis of the polyphosphorylated inositol phospholipids. Thus, PtdIns is a highly versatile

lipid whose metabolism can have a dramatic effect on the composition and fate of the membranes wherein it resides and ultimately the signal transduction pathways of the cell. For these reasons, it is important to consider the factors which determine whether an individual PtdIns molecule enters the polyphosphoinositide pathway or the sphingolipid pathway or retains its identity as PtdIns.

IV. Role of Phosphatidylinositol Transfer Proteins in the Regulated Synthesis and Turnover of Polyphosphoinositides

Although PtdIns is typically the third most abundant phospholipid (after PtdCho and PtdEth) in nonphotosynthetic plant tissues and may represent up to 20% of the phospholipid content in specific cellular membranes (Harwood, 1980), important questions remain regarding the source of the PtdIns substrate for the inositol lipid kinases responsible for the production of polyphosphoinositides. Are all PtdIns molecules in the vicinity of these kinases equally potential substrates or is this process regulated? Similar questions arise concerning the source of polyphosphoinositides that serve as precursors in cellular signaling events. Evidence of the existence of both agonist-responsive and agonist-nonresponsive pools of polyphosphoinositides (Monaco and Gershengorn, 1992) has led to a search for the factors responsible for determining the utilization, or channeling, of these molecules into signaling pathways.

A. Mammalian PITPs

Progress toward understanding the previously discussed issues was made with the discovery that the mammalian PITP represents an essential component of PLC-β-mediated inositol lipid signaling in myeloid cells (Thomas *et al.*, 1993). Mammalian PITP is an abundant, cytosolic protein that was originally characterized more than 25 years ago because of its ability to facilitate the exchange of PtdIns (and to a lesser extent PtdCho) between distinct lipid bilayers using an *in vitro* assay (Wirtz, 1991). The involvement of PITP in PLC-β signaling represented the first evidence of an *in vivo* function for this protein. In PLC-β-mediated signaling pathways, stimulated cell surface receptors activate specific PLC-β isoforms via heterotrimeric GTP-binding protein intermediates. The other well-characterized pathway of inositol lipid signaling utilizes phospholipases of the PLC family. Regulation of these G protein-independent pathways typically involves the stimula-

tion of tyrosine kinase receptors that activate PLC isoforms by phosphorylation. The demonstration that PITP is also required for stimulated hydrolysis of polyphosphoinositides in PLC pathways (Kauffman-Zeh *et al.,* 1995; Cunningham *et al.,* 1996) suggests that this activity may represent a general requirement in inositol phosphate signaling.

PITP also plays an important role in intracellular vesicular trafficking. Hay and Martin (1993) demonstrated that PITP is one of three cytosolic proteins required to restore Ca^{2+}-activated fusion of secretory granules with the plasma membrane in semi-intact PC12 rat pheochromocytoma cells. Subsequently, a type I PtdIns(4)P 5-kinase was shown to represent another (Hay *et al.,* 1995). PITP also restored Ca^{2+} and GTP-mediated exocytosis in permeabilized myeloid cells that had become refractory to stimulation after the depletion of cytoplasmic proteins (Fensome *et al.,* 1996). Finally, the formation of secretory vesicles from the trans-Golgi network in a neuroendocrine cell-free system was shown to be a PITP-dependent process (Ohashi *et al.,* 1995). Thus, both the formation of secretory vesicles from donor Golgi membranes and the fusion of transport vesicles to the acceptor plasma membrane require PITP.

Although secretory vesicle trafficking and inositol lipid signal transduction are two distinct cellular processes, the specific function of PITP in both cases is believed to be the same, i.e., promoting the synthesis of PtdIns(4)P and PtdIns(4,5)P_2 by presenting the PtdIns substrate to the PtdIns 4-kinase. Studies involving epidermal growth factor (EGF)-stimulated hydrolysis of PtdIns(4,5)P_2 in A431 human epidermoid carcinoma cells showed that the EGF receptor, PtdIns 4-kinase, PLC, and PITP coimmunoprecipitated as an apparent complex in EGF-treated cells (Kauffmann-Zeh *et al.,* 1995). In an elegant series of experiments using permeabilized A431 cells, Kauffman-Zeh *et al.,* demonstrated that even in the presence of an activated EGF receptor and phosphoinositide kinases, promotion of PtdIns(4)P and PtdIns(4,5)P_2 synthesis could occur only after addition of PITP. These results suggest that in the absence of PITP, PtdIns residing in cellular membranes serves as a poor substrate for the PtdIns 4-kinase. Not only is PITP necessary for the stimulated synthesis of polyphosphoinositides but also it apparently represents the rate-limiting step (Thomas *et al.,* 1993; Cunningham *et al.,* 1995). Because PITP has a high affinity for PtdIns(4)P and PtdIns(4,5)P_2 (Van Paridon *et al.,* 1987) and can exist in a complex with PLC, it has been suggested that PITP may also function to present PtdIns(4)P to the PtdIns(4)P 5-kinase and deliver the subsequently derived PtdIns(4,5)P_2 to PLCs to stimulate production of the DAG and Ins(1,4,5)P_3 signaling molecules (Liscovitch and Cantley, 1995).

Evidence that PITP similarly functions to supply PtdIns substrates for polyphosphoinositide synthesis in its role in secretory vesicle trafficking

derives from the previously stated observation that PITP and a PtdIns(4)P 5-kinase represent two of the three cytosolic factors determined to be necessary for restoration of Ca^{2+}-activated exocytosis in semi-intact PC12 cells (Hay *et al.*, 1995). The primary difference distinguishing the involvement of PITP in vesicular trafficking versus signal transduction is reflected in the fates of the resultant polyphosphoinositides. For inositol lipid signaling, associated PLCs hydrolyze the polyphosphoinositides to produce important second messengers that continue signal transmission. For PITP-dependent secretory processes, however, the polyphosphoinositides per se are believed to be the important end product. The role of polyphosphoinositides as regulators of vesicular trafficking has been reviewed by De Camilli *et al.* (1996).

Finally, the proposed model of PITPs functioning to facilitate the regulated delivery of PtdIns to lipid kinases is not strictly limited to the synthesis of D4 polyphosphoinositides. Panaretou *et al.* (1997) demonstrated a threefold enhancement of a human PtdIns 3-kinase activity upon addition of PITP to an *in vitro* assay. In addition, they successfully coimmunoprecipitated PtdIns 3-kinase activity from cell lysates using anti-PITP antisera, providing strong support for the relevance of this interaction as a mechanism for regulating PtdIns 3-kinase activities *in vivo.*

B. Yeast PITP (SEC14p)

Similar to mammalian systems, a yeast cytosolic protein capable of facilitating the *in vitro* exchange of PtdIns and PtdCho between distinct biological or artificial membranes was characterized many years before researchers could establish an *in vivo* function. Although mammalian and yeast PITPs possess nearly identical ligand specificities and are similar in size, they share no primary amino acid sequence homology. The first breakthrough in defining an *in vivo* function for yeast PITP came with the investigation of yeast strains mutated at the *SEC14* locus. SEC14 mutants are defective in secretory vesicle formation from the late Golgi complex (Novick *et al.*, 1980). Bankaitis *et al.* (1990) discovered that yeast PITP is encoded by the *SEC14* gene, suggesting that the transfer protein's function *in vivo* involves the compartment-specific stimulation of membrane vesicle flow. How this is accomplished mechanistically, however, has only recently been elucidated.

Similar to the mammalian PITP, the cellular function of yeast PITP (or SEC14p) appears to have little to do with phospholipid transfer as traditionally defined, i.e., the transfer of PtdIns or PtdCho from one distinct cellular membrane to another. Instead, a growing body of evidence suggests that SEC14p functions as a molecular sensor that monitors and maintains

appropriate levels of PtdIns, PtdCho, and DAG within the Golgi. In the PtdCho-bound state, SEC14p mediates the downregulation of the CDP–choline pathway of PtdCho biosynthesis through the specific inhibition of the enzyme cholinephosphate cytidylyltransferase, the rate-limiting step of the pathway (Skinner *et al.,* 1995). By continually exchanging the PtdIns or PtdCho in its single ligand-binding pocket with Golgi membrane PtdIns and PtdCho, SEC14p is believed to coordinate a sensitive feedback loop that couples Golgi PtdCho levels with cholinephosphate cytidylyltransferase activity. Because the CDP–choline pathway consumes DAG (in contrast to the methylation pathway of PC biosynthesis which does not), SEC14p activity also functions to influence the DAG content of the Golgi. In fact, it is the maintenance of an essential DAG pool in the Golgi (as opposed to its influence on PtdIns or PtdCho levels) that is believed to be the requisite function of SEC14p in facilitating the formation of secretory vesicles (Kearns *et al.,* 1997).

Unlike the mammalian PITP, yeast SEC14p has not been implicated in the stimulation of polyphosphoinositide synthesis. Nevertheless, yeast SEC14p can functionally substitute for mammalian PITP in restoring inositol lipid signaling and secretory vesicle formation in systems lacking the mammalian protein (Ohashi *et al.,* 1995; Cunningham *et al.,* 1996). Conversely, mammalian PITP can rescue yeast strains carrying SEC14 lesions, albeit only when expressed at high levels (Skinner *et al.,* 1993). Because the yeast and mammalian PITPs share no primary amino acid sequence homology, the lipid transfer properties common to both proteins are presumed to be the relevant activities that enable one protein to substitute for the function of the other. This is somewhat enigmatic, however, given that there are no apparent similarities in the mechanisms by which each of these proteins function within their respective native environments.

C. Plant PITP-like Proteins

As described previously, PITP activities in mammals and yeast were recognized many years before *in vivo* functions were elucidated. These early characterizations were facilitated by the fact that these proteins are relatively abundant, cytosolic proteins. In contrast, proteins displaying lipid transfer properties similar to those of mammalian and fungal PITPs have not been described in a higher plant system. Although the occurrence of proteins that apparently display transfer properties specific for a single phospholipid class has been reported (Tanaka and Yamada, 1982), the only well-documented lipid transfer proteins in plants are the class of small molecular weight (9–14 kDa), basic proteins that nondiscriminantly fa-

cilitate transfer of a broad variety of phospholipids and galactolipids (Kader, 1996).

The recent characterization of plant cDNAs that complement SEC14 mutant yeast strains, however, demonstrates that although higher plants do possess PITP-like proteins, they display unique properties in comparison to their mammalian and yeast counterparts. Transformation of the temperature-sensitive yeast strain CTY1-1A (sec14ts) with a soybean cDNA library cloned into a yeast expression vector resulted in the isolation of two distinct classes of cDNAs that complemented the growth and secretory defects of the mutant strain (Kearns *et al.,* 1998). The predicted protein products of these cDNAs, designated SSH1p and SSH2p (*s*oybean *S*EC14 *h*omologs 1 and 2), share approximately 25% primary sequence identity and 50% similarity with yeast SEC14p. Interestingly, the two soybean proteins display no more homology to each other than either shares with the yeast sequence.

An examination of the lipid transfer properties of SSH1p and SSH2p revealed that these proteins are not typical PITPs (Kearns *et al.,* 1998). SSH2p displays a very active *in vitro* PtdIns transfer function but cannot transfer PtdCho. This represents the first example of a PITP from any organism that has been described that does not also recognize PtdCho as a ligand. Even more unexpected were the transfer results of SSH1p; no PtdIns or PtdCho transfer activity could be detected using standard assays.

Although both soybean PITP-like proteins show primary amino acid sequence similarity to yeast SEC14p, three lines of evidence suggest that functionally they may have more in common with the mammalian PITP than does yeast SEC14p (Kearns *et al.,* 1998). First, similar to the mammalian PITP, SSH1p and SSH2p will only complement Sec14 mutants when expressed at high levels. Second, both soybean proteins efficiently bind the polyphosphoinositides PtdIns(4)P and PtdIns(4,5)P_2, a trait shared with mammalian PITP that is not observed with yeast SEC14p (Kearns *et al.,* 1998). Finally, complementation of SEC14 mutants by SSH1p and SSH2p occurs by a mechanism that does not involve the CDP–choline pathway of PC biosynthesis (R. E. Dewey, unpublished results).

The apparent involvement of SSH1p in cellular signaling is also reminiscent of mammalian PITP. SSH1p exists in the plant both in a nonphosphorylated state and as a phosphoprotein (Monks *et al.,* 1998). Under typical growth conditions, nonphosphorylated SSH1p predominates. Upon exposure of the plant to a variety of hyperosmotic stresses, however, SSH1p is rapidly phosphorylated. Surprisingly, the hyperosmotic stimulation of SSH1p phosphorylation is recapitulated in the heterologous yeast system (Kearns *et al.,* 1998). An investigation of the plant enzyme responsible for this phosphorylation led to the discovery that SSH1p is the substrate of an osmoregulated kinase that displays properties characteristic of MAP kinases (Monks *et al.,* 1998). MAP kinase cascades represent important signal-

ing pathways that have been implicated in a number of stress responses, including osmotic stress (Hirt, 1997; Mizoguchi *et al.*, 1997). Although several MAP kinase cascades have been documented in animals and yeast, no complete pathway has been described in a higher plant. However, since all the essential components are known to exist in plants (Mizoguchi *et al.*, 1997), the elucidation of individual pathways should be forthcoming.

The discovery that soybean SSH1p appears to serve as a substrate for an osmoregulated MAP kinase raises interesting questions regarding the downstream consequences of SSH1p phosphorylation and the fate of the polyphosphoinositide ligand. If some functional similarity does exist between SSH1p and mammalian PITP, its role in signal transduction may be to present polyphosphoinositides to other proteins. Candidate proteins include PLCs, leading to increased synthesis of inositol phosphate messengers (as proposed for mammalian PITP), or other proteins that are regulated by polyphosphoinositides (see Section II). Although such suggestions are clearly speculative, further study of SSH1p promises to provide new insights into the role of plant PITP-like proteins and polyphosphoinositides in cellular signaling pathways.

The other soybean PITP-like protein, SSH2p, appears to serve a function very distinct from that of SSH1p. Although SSH2p also exists within the cell as two distinct species (the larger of which possibly represents phosphorylated SSH2p), no alteration in the relative abundance of these species is observed upon exposure of plant materials to a large variety of biotic and abiotic stresses, including hyperosmotic stress (D. E. Monks and R. E. Dewey, unpublished results). The differential localization of the two SSH2p species, however, may provide clues toward the ultimate determination of function. Cellular fractionation experiments have shown the larger SSH2p species to be predominantly cytoskeleton associated, in contrast to the smaller SSH2p, which partitions between both soluble and Triton X-100 extractable membrane fractions (P. D. Courtney and R. E. Dewey, unpublished results). The localization of the larger SSH2p species to the cytoskeleton is particularly intriguing in light of previous studies demonstrating a significant proportion of the plant PtdIns 4-kinase to be cytoskeleton associated (Tan and Boss, 1992; Xu *et al.*, 1992). Because SSH2p is the soybean PITP-like protein that displays *in vitro* PtdIns transfer activity, it is tempting to speculate that, similar to mammalian PITP, one *in vivo* function may be to deliver PtdIns to the PtdIns 4-kinase for stimulation of polyphosphoinositide synthesis. Considering the demonstrated involvement of polyphosphoinositides in the regulation of the actin-based cytoskeleton (Janmey, 1994; Drøbak, 1996), the possibility that cytoskeleton-localized SSH2p and PtdIns 4-kinase may be part of a complex involved in the regulation of actin polymerization/depolymerization warrants further investigation.

V. Phosphoinositide Kinases and the Synthesis of Polyphosphoinositides

A. Synthesis of 4-Phosphoinositides

1. PtdIns 4-Kinases

The seminal work on the phosphorylation of PtdIns to PtdIns(4)P and PtdIns(4,5)P_2 in plant cells was done by Sommarin and Sandelius (1986; Sandelius and Sommarin, 1990). Characteristic of higher plant membranes and in contrast to membranes from animal cells, PtdOH constitutes approximately 70% of the phosphorylated lipid products, whereas PtdIns(4)P and PtdIns(4,5)P_2 typically comprise not more than 20 and 3%, respectively, of the ^{32}P-labeled lipid products (Sandelius and Sommarin, 1990; Gross and Boss, 1992; I. Y. Perera, I. Heilmann, and W. F. Boss, unpublished results). Under similar assay conditions, the specific activity of the PtdIns 4-kinase from rat liver is on the order of 10- to 20-fold greater than that of higher plants (Sandelius and Sommarin, 1990; Heilmann and W. Boss, unpublished results). Understanding the physiological relevance of these differences will lead to new insight into the signaling pathway.

PtdIns 4-kinases are in relatively low abundance and tend to copurify with heat shock proteins. As a result, they eluded researchers for many years and their characterization was limited to biochemical traits of partially purified proteins. For example, PtdIns 4-kinases inhibited by adenosine (K_i, 20–100 μM) and having a low K_m for ATP (30–100 μM) were denoted type II, whereas PtdIns 4-kinases insensitive to 100 μM adenosine (K_i, 1520 μM) and having a high K_m for ATP (150–750 μM) were denoted type III (Carpenter and Cantley, 1990; Pike, 1992). It was not until Flanagan and Thorner (1992) took advantage of the power of yeast genetics by using a heat shock null mutant that the first PtdIns 4-kinase was cloned (Flanagan *et al.*, 1993). Subsequently, PtdIns 4-kinases have been cloned and sequenced from rat (Nakagawa *et al.*, 1996), human (Wong and Cantley, 1994), bovine (Gehrmann *et al.*, 1996), and higher plants (Stevenson *et al.*, 1997).

Based on the predicted amino acid sequences, the PtdIns 4-kinases fall into two major families. One family comprises the large-molecular-weight proteins (~200–230 kDa), which have a C-terminal lipid kinase domain and a lipid kinase unique domain separated by a pleckstrin homology domain (PH domain) (Fig. 3). Members of this family are generally type III, adenosine-insensitive PtdIns 4-kinases (Balla *et al.*, 1997; Nakagawa *et al.*, 1996). The other family contains smaller molecular weight proteins which lack a PH domain. They have the lipid kinase domain at the C terminus, but the unique lipid kinase domain is closer to the N terminus.

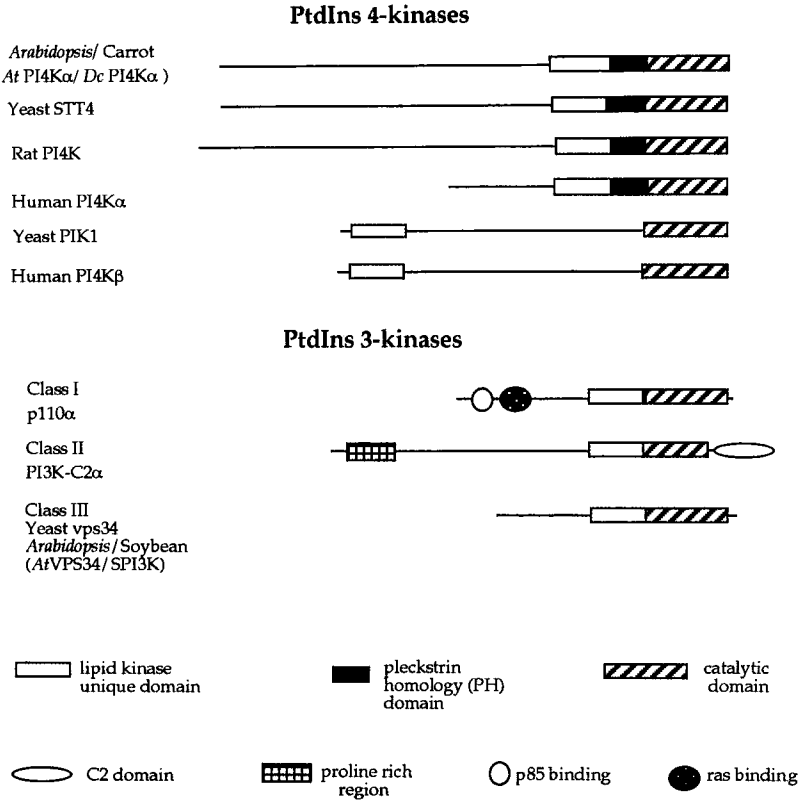

FIG. 3 Structural features of selected PtdIns 4-kinases and PtdIns 3-kinases.

These enzymes are generally type II kinases. Importantly, the sequence data clearly indicate that there are several subclasses of the PtdIns 4-kinases within these two families and distinct isoforms appear to be associated with the ER and Golgi (Wong *et al.*, 1997; Balla *et al.*, 1997). Whether all the isoforms result from unique sequences or some result from alternative splicing remains to be determined (Balla *et al.*, 1997). Based on current data from animal cells, the small molecular weight type II kinases which lack the PH domain are thought to generate the receptor signaling pool of PtdIns(4)P and PtdIns(4,5)P_2 in the plasma membrane, whereas the type III PtdIns 4-kinases with the P domain are loosely associated with endomembranes (Balla *et al.*, 1997).

The carrot plasma membrane PtdIns 4-kinase is a type III kinase based on biochemical studies (Yang and Boss, 1994a). It differs from the plasma membrane 4-kinase of A431 cells (a type II kinase) not only with regard

to adenosine sensitivity but also in its sensitivity to other positively charged compounds (Yang and Boss, 1994a; Vogel and Hoppe, 1986). When solubilized from the plasma membrane, it can be activated four- to eightfold by elongation factor 1α (eEF-1α) (Yang *et al.,* 1993). This activation is dependent on phosphorylation of eEF-1α, but does not involve the GTP hydrolyzing function of this small G protein (Yang and Boss, 1994b) and is thus distinct from the mechanism of activation of PtdIns(4)P 5-kinases by the Rho family of GTP-binding proteins (Hartwig *et al.,* 1995). Most of the membrane-associated PtdIns 4-kinase activity in plants colocalizes with the intact cytoskeleton or a F-actin fraction (Xu *et al.,* 1992; Tan and Boss, 1992). Therefore, regulation by an actin binding and bundling protein such as eEF-1α may be important for the plant enzymes *in vivo.* Only 10% of the total PtdIns 4-kinase is recovered in the soluble fraction and this is a type II enzyme (Okpodu *et al.,* 1995).

The only PtdIns 4-kinases cloned from plants (AtPIKα and DcPIKα) both contain a functional PH domain and have high homology to the large molecular weight, type III enzymes (Stevenson *et al.,* 1998). This is consistent with earlier biochemical data. *In vitro* studies using the *Escherichia coli*-expressed PH domain of AtPIKα showed that it binds PtdIns(4)P but not PtdIns(3)P, suggesting that the binding is biologically relevant (Stevenson *et al.,* 1998). In addition, based on Western blot analysis using antibodies raised to the C terminus containing the conserved lipid kinase domain and the PH domain, AtPIKα was found in the F-actin fraction isolated from microsomes. If the major form of the plant PtdIns 4-kinase is a cytoskeletal-associated type III kinase with a functional PH domain that binds PtdIns(4)P, what can we imply about the regulation?

The PH domain is a region of approximately 100 amino acids which was first identified in pleckstrin, a major protein kinase C substrate, and subsequently found in many proteins involved in signal transduction including PLC (Shaw, 1996). PH domains facilitate the binding of proteins to lipids such as PtdIns(4)P and PtdIns(4,5)P_2, inositol phosphates, and various proteins involved in signaling. One proposed model for the functional role of PH domains is based on studies of PLC in which the PH domain of PLC binds PtdIns(4,5)P_2 and in doing so recruits the enzyme to the plasma membrane. The catalytic domain of PLC then binds the substrate [PtdIns(4,5)P_2] and hydrolyzes it to produce Ins(1,4,5)P_3 (Lemmon *et al.,* 1996). When enough Ins(1,4,5)P_3 is produced, Ins(1,4,5)P_3 will bind the PH domain, dissociating the enzyme from the membrane and thereby decreasing further lipid hydrolysis. While this is a logical model for PLC, what would be the advantage of having the enzyme, PtdIns 4-kinase, bind its product? One explanation is that it sequesters the product. If PtdIns is phosphorylated to PtdIns(4)P on the cytoskeleton *in vivo,* then once the PtdIns(4)P is formed it would be advantageous for the kinase to bind

PtdIns(4)P and protect it from further metabolism. The ultimate fate of the PtdIns(4)P would then depend on competitive binding of the transfer proteins, cytoskeletal binding proteins, lipases, or other kinases with the PH domain. If PtdIns(4)P is in excess or not utilized in other metabolic pathways, then it would remain bound to the PH domain and may feedback regulate the PtdIns 4-kinase.

If the primary PtdIns 4-kinase in plants is the AtPIKα which has a PH domain, this may account for the low levels of PtdIns(4,5)P_2 in that PtdIns(4)P, once formed, is not available for further phosphorylation. This would mean that the soluble, type II PtdIns 4-kinase, which represents only 10% of the total cellular activity, would be the enzyme available for generating or rapidly refilling the PtdIns(4)P and PtdIns(4,5)P_2 pools involved in signal transduction. More biochemical studies will need to be done with purified and recombinant forms of the enzyme to test these hypotheses; however, the biochemical differences in the plant and animal PtdIns 4-kinases and the uniformly low levels of PtdIns(4,5)P_2 compared to the PtdIns(4)P in plants are certainly consistent with a mechanism for tight regulation of PtdIns(4)P biosynthesis.

2. PtdIns 4-Monophosphate 5-Kinases

The PtdIns(4)P 5-kinases form a unique family of lipid kinases which lack sequence homology to the kinase domains of the phosphatidylinositol and inositol phosphate kinases (Boronenkov and Anderson, 1995; Loijens and Anderson, 1996). The absence of sequence homology implies that their catalytic mechanism is distinct, a notion which is consistent with the observation that the PtdIns(4)P 5-kinases can utilize both GTP and ATP as phosphoryl donors. The PtdIns(4)P 5-kinases, like the PtdIns 4-kinases, are classified into two families based on their biochemical characteristics. The activity of type I PtdIns(4)P 5-kinases is stimulated by PtdOH and small G proteins, whereas the type II enzymes are not. Both type I and II are rather promiscuous with regard to substrates (Zhang *et al.*, 1997).

The type I enzyme, in addition to phosphorylating PtdIns(4)P in the D5 position, can phosphorylate PtdIns(3)P and PtdIns(3,4)P_2 in the D4 position and the D5 position, respectively. The type II enzyme will phosphorylate PtdIns(3)P and PtdIns(4)P equally well but does not phosphorylate PtdIns(3,4)P_2. The type II enzyme has also been shown to phosphorylate PtdIns(5)P, a newly identified phospholipid, in the D4 position (Rameh *et al.*, 1997). The general lack of substrate specificity led Zhang *et al.* (1997) to suggest that PtdIns kinases should be renamed PtdInsP 4/5-kinases.

These unique lipid kinases which catalyze the last step in the biosynthesis of PtdIns(4,5)P_2 appear to hold one of the keys to understanding how the Rho family of GTP-binding proteins transduces extracellular signals to the

actin cytoskeleton. When PtdIns(4,5)P_2 binds to actin-capping proteins and monomeric actin-binding proteins, the net result is an increase in filament formation. GTP–Rho binds and activates PtdIns(4)P 5-kinase *in vitro* (Chong *et al.*, 1994; Tolias *et al.*, 1995), and *in vivo* studies of permeabilized and transfected cells indicate that GTP-activated Rac indeed promotes increases in actin filament formation by activating PtdIns(4)P 5-kinase (Hartwig *et al.*, 1995; Shibasaki *et al.*, 1997). Overexpression of PtdIns(4)P 5-kinase alone also results in increases actin filament formation, suggesting that PtdIns(4)P 5-kinase acts downstream of GTP–Rac. In addition, it has been found that not all Rac-related changes in actin polymerization are mediated by PtdIns(4,5)P_2. Importantly, studies of PtdIns(4)P 5-kinases in animal cells indicate that these enzymes have other functions than merely replenishing phosphoinositide pools, and they suggest that the rate of biosynthesis of PtdIns(4,5)P_2 may act as regulator of several cytoskeletal and membrane-associated processes.

Putative PtdIns(4)P 5-kinases have been cloned in *A. thaliana*. Three genomic (accession Nos. U95973, AF007269, and Y12776) and two cDNAs have been reported to date (accession No. AF019380; I. Y. Perera and W. F. Boss, unpublished data). These genes are predicted to encode enzymes ranging in size from 427 to 859 amino acids. These may be tissue-specific isoforms or they may reflect differences in their intracellular locations [plasma membrane (Sommarin and Sandelius, 1988), cytoskeletal (Tan and Boss, 1992), and nuclear (Hendrix *et al.*, 1989)]. At the amino acid level, the *Arabidopsis* PtdIns(4)P 5-kinases are almost equally similar to both the type I and type II isoforms of animal cells. At least two cDNAs are predicted to encode large molecular weight proteins and one has 47% amino acid homology to the yeast Mss4p kinase (Yoshida *et al.*, 1994), which is an essential enzyme that has been classified as a type II PtdIns(4)P 5-kinase (Zhang *et al.*, 1997). Biochemical characterization of the plant PtdIns(4)P 5-kinases is lacking; however, activation by PtdOH is unlikely because there is a large amount of PtdOH produced during the *in vitro* reaction, but the specific activity is at least 10-fold lower than that of most animal cells (Sandelius and Sommarin, 1990). Labeling studies indicate that PtdIns(3,4,5)P_3 is unlikely to be present in plants and that PtdIns(3,4)P_2 most probably is synthesized via phosphorylation of PtdIns(3)P (Brearly and Hanke, 1993; Dove *et al.*, 1994), suggesting that the plant enzyme may also be a PtdInsP 5/4-kinase.

Low PtdIns(4,5)P_2 levels (based on radiolabeling experiments) appear to be a hallmark of higher plants and the upregulation of PtdIns(4) 5-kinase may reflect changes in the physiological status of the cells. Algae, like mammalian cells, have several-fold higher PtdIns(4)P 5-kinase activity when compared to higher plants (Gross and Boss, 1992; I. Heilmann and W. F. Boss, unpublished results). Interestingly, the specific activity of PtdIns(4)P 5-kinase increases in membranes isolated from stationary-phase *Galdieria*

sulphuraria cells (Heilmann and W. Boss, unpublished results) and from senescent petunia flower petals (Borochov *et al.*, 1994). It is hoped that future experiments will determine what factors regulate PtdIns(4)P 5-kinase activity in higher plants and whether the enzyme can be activated to the levels of the animal enzyme or if the plant enzyme is missing factors such as the Rho-activated pathway characteristic of the animal type I kinases.

B. Synthesis of 3-Phosphoinositides

1. PtdIns 3-Kinases

Despite the fact that many of the PtdIns 3-kinases are now well characterized there has until quite recently been considerable controversy regarding the pathway(s) of 3-phosphoinositide synthesis *in vivo*. However, most scientists agree that there are two main routes leading to 3-phosphoinositide formation in higher eukaryotes. The first route involves activation of PtdIns(4,5)P_2 3-kinases and the formation of PtdIns(3,4,5)P_3 followed by sequential dephosphorylation by 5- and 3-phosphatases, resulting in the production of PtdIns(3,4)P_2 and PtdIns(4)P (Stephens *et al.*, 1991). The other route utilizes direct 3-phosphorylation of PtdIns by PtdIns 3-kinases. In some cells, there may be a certain "overlap" between these two pathways, and evidence has been presented which shows that some PtdIns(3,4)P_2 may be formed by 4-phosphorylation of PtdIns(3)P and/or 3-phosphorylation of PtdIns(4)P and that smaller amounts of PtdIns(3)P may also be generated by the action of a 4-phosphatase upon PtdInds(3,4)P_2.

Several groups have searched for 3-phosphorylated phosphoinositides in plant cells and have had varying degree of success. As mentioned previously, the levels of these lipids in mammalian cells are exceedingly low but in yeast and *Neurospora*, as much as 50% of the PtdInsP pool is made up of PtdIns(3)P; however, early studies suggested that if 3-phosphoinositides were present in plant cells they were likely to be only rather minor components of the polyphosphoinositide pool (Irvine *et al.*, 1989). Later work, in which more sensitive methods of detection were used, has shown that PtdIns(3)P and PtdIns(3,4)P_2 are present in plant cells (Brearley and Hanke, 1993; Munnik *et al.*, 1994a,b) and very approximate estimates indicate that PtdIns(3)P in plant cells may constitute 2–20% of the PtdInsP pool, whereas PtdIns(3,4)P_2 in most cases constitutes 5–10% of the PtdInsP_2 pool. However, in a few cases PtdIns(3,4)P_2 may approach the levels of PtdIns(4,5)P_2 but the physiological relevance and frequency of this occurrence remains to be investigated. Despite considerable effort from several different groups, PtdIns(3,4,5)P_3 has eluded detection in plant cells. It should be noted that PtdIns(3,4,5)P_3 has also not been indentified in yeast, slime molds, or fungi.

In mammalian cells PtdIns(3,4,5)P_3 avoided detection for decades, so it is perhaps premature to totally discount the possibility that PtdIns(3,4,5)P_2 can be produced in certain circumstances in plant cells and in cells of other lower eukaryotes. However, the universal presence of PtdIns(3)P and PtdIns(3,4)P_2 and their rapid rate of turnover suggest that these lipids are likely to have several important functions in plant cells.

It has been determined that a number of distinct isoforms of "PtdIns 3-kinase" exist that are capable of selectively phosphorylating PtdIns and/or PtdIns4P/PtdIns(4,5)P_2, the resulting lipids being PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3, respectively. The currently known PtdIns 3-kinases can be divided into four families, each with their preferred substrate(s) and distinct biochemical profile (Domin and Waterfield, 1997).

a. The p110/p85 PI 3-Kinase Family The first group contains type I PtdIns 3-kinases first identified as tyrosine kinase receptor-associated lipid kinases. These enzymes form heterodimeric complexes with an adaptor protein which confers sensitivity to ligand stimulation. The kinase complex is composed of a 110-kDa catalytic subunit (p110) and an 85-kDa regulatory subunit (p85). The p110 subunits contain a domain which interacts with p85, a region thought to be involved in ras interactions, a PI-kinase domain (PIK) shared by all known phosphoinositide kinases, and a C-terminal catalytic domain. The p85 adaptor protein contains two SH2 domains separated by an internal SH2 domain through which the adaptor interacts with the catalytic subunit. In addition, the p85 adaptor protein contains an SH3 domain toward the N terminus which has been suggested to facilitate the interaction with cytoskeletal components. The N terminal also contains a breakpoint cluster region homology domain (BH domain) flanked by two proline-rich regions. Although the role of the BH domain remains contentious, it is possible that it is involved in intramolecular interactions with the SH3 domain.

b. The p110-γ PI 3-Kinase Family The second group of 3-kinases act downstream of receptors which signal via heterotrimeric G proteins. These kinases also exist as heterodimers with a p110 catalytic subunit and a regulatory adaptor subunit called p101. This class of PtdIns 3-kinases is activated by $\beta\gamma$ subunits from heterotrimeric regulatory G proteins. p101 is not related to p85 and its precise mode of action is being investigated.

c. The PI3K-68D/cpk PI 3-Kinase Family The third class of PtdIns 3-kinases includes the largest of the known 3-kinases (170–220 kDa), which all contain a C2 domain (thought to be involved in Ca^{2+}-mediated phospholipid binding) toward the C terminal. The first member of this type of 3-kinase was identified in *Drosophila* as PI3K-68D, an enzyme which is widely

expressed throughout the life cycle of the fly. Other members of this family have been cloned in mouse and humans. A distinct biochemical difference between these enzymes and those of family I and family II described previously is associated with their substrate specificity. Thus, this group of kinases selectively phosphorylate PtdIns and PtdIns(4)P but not PtdIns(4,5)P_2. This finding led to the idea that the structural differences between members of the different PtdIns 3-kinase classes reflect the type of preferred substrate. This idea is still viable, but conclusive evidence is lacking. However, it is possible that the presentation of the substrate to a PtdIns 3-kinase, at least in some cases, may determine the willingness of the enzyme to partake in the reaction.

d. The vps34-Related PI 3-Kinase Family The first member of this family to be identified was the *vps34* gene product from yeast which was identified in a screen of mutants defective in vesicle sorting. Subsequently, a human homolog has also been identified and vps34-related PtdIns 3-kinases have now been found to exist other eukaryotes, including *Dictyostelium, Drosophila,* and plants. Despite a high degree of sequence homology between this family and other PtdIns 3-kinases, the members of the vps34 kinase family have a distinguishing biochemical feature: They only phosphorylate PtdIns both *in vivo* and *in vitro* and cannot use PtdIns(4)P and/or PtdIns(4,5)P_2 as substrate. Relatively little is known about the functional domains of vps34 except that it contains PIK domains, a catalytic lipid kinase domain, and an unidentified motif involved in the interaction with an adaptor protein(s). It is worth mentioning that vps34 also possesses intrinsic protein kinase activity but the physiological relevance of this remains to be determined.

Since this kinase is the only form of PtdIns 3-kinase present in yeast (and probably in higher plants) it may be considered to be the primordial form of PtdIns 3-kinase. However, in a manner similar to the 3-kinases belonging to the group I and group II enzymes, the activity of vps34-related kinases is also dependent on the interaction with an adaptor protein. The partner of vps34 is a ser/thr kinase named vps15. It is believed that vps15 recruits vps34 to membranes and activated its lipid kinase activity. In humans a homolog to vps15 has also been identified and has been named p150. Both vps34 and p150 have N-terminal myristylation sites, a ser/thr kinase domain, a set of HEAT repeats, and WD motifs toward the C terminal.

Experiments in yeast have shown that vps34 and vps15 can form tight complexes *in vivo* and that the formation of such complexes depends on the activation of the vps15 protein kinase activity. It is thus reasonable to assume that vps15 is an upstream regulator of vps34 function. That the level of PtdIns 3-kinase activity is dependent on the vps34–vps15 interaction

has been demonstrated by deletion experiments in which levels of PtdIns(3)P in δvps15 yeast strains (strains in which vps15 has been deleted) were below the level of detection. In addition to the vps15 interaction, it also appears that PI-transfer proteins may play a role in the activation of vps34. Panaretou *et al.* (1997) showed that addition of PITP to human PtdIns 3-kinase–p150 complexes (the equivalent of the yeast vps34–vps15 complex) resulted in a several-fold increase in PtdIns 3-kinase activity, and it was also found that PtdIns 3-kinase activity could be coimmunoprecipitated from human cell lysates using anti-PITP antisera.

e. Plant PtdIns 3-Kinases As mentioned previously vps34-related PtdIns 3-kinases appear to be the only form of PtdIns 3-kinase present in plant cells and as such this presents a situation analogous to that found in yeast. Most of the current structural information regarding plant PtdIns 3-kinases stems from the studies of Hong and Verma (1994) and Welters *et al.* (1994). These groups cloned and sequenced two very similar cDNAs encoding vps34-related enzymes from soybean and *Arabidopsis,* respectively. The structures of these two proteins are shown in Fig. 3 together with other members of the eukaryotic PtdIns 3-kinase and PtdIns 4-kinase family.

The soybean PtdIns 3-kinase gene identified by Hong and Verma (1994) encodes a polypeptide (SPI3K-5) of 814 amino acids and has a predicted molecular mass of 93 kDa; a second PtdIns 3-kinase gene encodes SP13K-1, a polypeptide of 812 amino acids. Both kinases were found to have catalytic PtdIns 3-kinase activity when expressed in *E. coli* and assayed with exogenous PtdIns liposomal substrates. One of the interesting features of the study by Hong and Verma (1994) was the discovery that the two PtdIns 3-kinase genes were differentially expressed during the organogenesis of root nodules induced by *Rhizobium.* Thus, the induction of expression of the *SPI3K-1* gene, encoding the nodule form of PtdIns 3-kinase, was found to repress the expression of SPI3K-5, the root form of PtdIns 3-kinase. Only after nodule maturation was the expression of SPI3K-5 reinduced to prenodulation levels. These induction and repression events were found to be directly correlated with the degree of membrane proliferation in the root nodules. These data lend support to the hypothesis that the plant PtdIns 3-kinase, like its yeast and mammalian counterparts is involved in the trafficking and/or sorting of membrane and in plants plays a central role in membrane biogenesis and the development of endosymbiotic compartments.

The other currently known plant PtdIns 3-kinase gene was identified in *A. thaliana* by Welters *et al.* (1994). AtVPS34 is a polypeptide of 814 amino acids and shares 40% identity with the yeast vps34 and is 25% identical to bovine p110. It is interesting to note that the N-terminal part of AtVPS34 contains a domain with strong homology to the CaLB domain of rabphilin

3α, and Welters *et al.* (1994) suggest that this domain may be involved in the binding of phospholipids. A chimeric gene in which the coding sequence for the C-terminal third of *VPS34* was replaced with the corresponding sequence from the plant gene was able to rescue a yeast-deletion mutant. Perhaps more significant was the finding that the expression of AtVPS34 antisense constructs gave rise to second-generation transformed plants which were severely inhibited in both growth and development, indicating a significant role for PtdIns 3-kinase in these processes.

One of the compounds which has been extensively used in the characterization of mammalian PtdIns 3-kinase enzymes is the fungal metabolite, wortmannin, which has been found to be a very potent inhibitor of certain PtdIns 3-kinase isozymes. Matsuoka *et al.* (1995) used wortmannin to investigate whether PtdIns 3-kinase had the potential to affect vacuolar sorting processes in tobacco BY-2 cells. In these experiments, wortmannin inhibited both PtdIns 3-kinase and PtdIns 4-kinase activity and was highly inhibitory to at least one type of vacuolar sorting. These studies point to a link between PtdIns kinase activity and vesicle trafficking in plant cells, although it must be stressed that it can often be difficult to unequivocally prove causal links from inhibitor studies.

Little is known about the subcellular localization of plant PtdIns 3-kinase(s) but Dove *et al.* (1994) found that the highest specific activity of plant PtdIns 3-kinase was associated with a detergent-resistant nucleocytoskeletal compartment. It is unclear whether this location is a true intracellular site for PtdIns 3-kinase association because the methods for preparing the nucleocytoskeletal fractions involve a number of steps known to dramatically upregulate the plant phosphoinositide signaling system. The targeting and translocation of other signaling enzymes to the cytoskeleton/nucleus in response to cellular stimulation is a well-characterized phenomenon in higher eukaryotic cells. Little is known about the regulation of PtdIns 3-kinase activity in plant cells and a plant adaptor protein corresponding to vps15 and p150 has not been identified.

2. PIK-Related Kinases

In addition to PtdIns 4-kinases and the four families of PtdIns 3-kinases mentioned previously, another enigmatic group of kinases are related quite closely to phosphoinositide kinases. These kinases are the so-called PIK-related kinases (PIK-rk). Despite their similarity to phosphoinositide kinases, their relevant substrates have not been identified and *in vitro* experiments have not been successful in demonstrating that these kinases have the ability to phosphorylate PtdIns. Members of this new kinase family, however, have been shown to fulfill some very important roles in cell physiology.

One member of this family is the *Saccharomycces cerevisiae* gene MEC1/ESR1 (mitosis entry checkpoint mutant), which encodes a 273-kDa PIK-rk. Cells in which MEC1 has been mutated are incapable of blocking entry into mitosis even when DNA replication is incomplete or if cells are exposed to certain genotoxic agents. Thus, MEC1 is essential for the proper function of cellular checkpoint mechanisms monitoring the status of the genome at the G_1/S and G_2/M transition phases. Also, in S phase MEC1 is thought to be involved in the control of correct genomic organization. Functional homologs to MEC1 have been identified in *Drosophila* (mei-41) and in humans (FRP1).

The sensitivity of cells to DNA-damaging agents is also evident in cells with mutations in another PIK-rk, DNA-PKcs. DNA-PKcs is the 465-kDa catalytic subunit of the DNA-dependent protein kinase, and cells containing defect copies of DNA-PKcs become hypersensitive to X-ray radiation and similar mutations can also cause severe dysfunction of the immune system. It is believed that the underlying biochemical causes of these phenotypes are directly related to an inability to rejoin breakages in double-stranded DNA.

A third member of the PIK-rk family is the *S. cerevisiae* gene TEL1, which was first identified in a screen to identify cells with abnormal telomere length. TEL1 encodes a 322-kDa protein which, like the other PIK-rks, plays a central role in the control of mitotic recombination and control of cell cycle checkpoint functions. Since maintenance of telomere length and integrity is generally seen as one of the key functional determinants for longevity, it is highly likely that proper functioning of PIK-rps is also intimately linked to this vital cellular characteristic.

The final member of this family to be mentioned in the current context is the human FKBP-rapamycin associated protein (FRAP). It has been found that FRAP plays a vital role in the successful progression through the G_1 phase of the cell cycle by participating in the activation of p70 S6 kinase which results in an increase in the translation of certain messenger RNAs.

Recent experiments in one of our labs indicate that the relationship between the plant PtdIns 3-kinase, SPI3K-5, and the PIK-rk may be closer than hitherto expected. Using monoclonal antibodies raised to a heterologously expressed truncated form of SPI3K in immunolocalization studies has led to the surprising discovery that a significant proportion of cellular SPI3K-5 activity resides in the nucleolus (T. D. Bunney, P. F. Shaw, and B. K. Drøbak, manuscript in preparation). The intimate association of the plant 3-kinase with this cellular compartment suggests that this enzyme may be involved in the regulation of nucleolar processes; that such processes may be related to those controlled by members of the PIK-rp family is obviously an attractive hypothesis. However, further experimentation is clearly needed to investigate this possibility in more detail.

VI. Summary and Conclusions

We have attempted to summarize some of the current knowledge of phosphoinoside metabolism in plant cells and have presented a few ideas which are currently receiving attention. It is clear that the metabolism and function of phosphoinositide in eukaryotic cells is far more bewildering and complex than it was envisaged just a few years ago. Also, phosphoinositides are far from just being precursor molecules for second messengers; they also themselves play several very important physiological roles. Although there are many apparent similarities between phosphoinsitide metabolism in different eukaryotes, we warn against the oversimplistic view that such similarities necessarily also infer similarity in function. The use of inositol-containing lipids as mediators of many important functions is of early evolutionary origin, but there are many indications of subsequent species-specific adaptation.

We hope that this short overview of some of the current ideas in the plant phosphoinositide field may help to convey some of the complexities and fundamentally important aspects of "inositol lipidology" to both established and young researchers and thus perhaps attract them to the study of this enigmatic and exciting area of modern biology.

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References

Balla, T., Downing, G. J., Jaffe, H., Kim, S., Zólyomi, A., and Catt, K. J. (1997). Isolation and molecular cloning of wortmannin-sensitive bovine type II phosphatidylinositol 4-kinases. *J. Biol. Chem.* **272,** 18358–18366.

Ballas, L., and Bell, R. M. (1981). Topology of glycerolipid synthetic enzymes. Synthesis of phosphatidylserine, phosphatidylinositol and glycerolipid intermediates occurs on the cytoplasmic surface of rat liver microsomal vesicles. *Biochem. Biophys. Acta* **665,** 586–595.

Banerjee, A., Barry, V. A., Dasgupta, B. R., and Martin, T. F. J. (1996). N-ethylmaleimide-sensitive factor acts at a profusion ATP-dependent step in Ca^{2+} activated exocytosis. *J. Biol. Chem.* **271,** 20223–20226.

Bankaitis, V. A., Aitken, J. R., Cleves, A. E., and Dowhan, W. (1990). An essential role for a phospholipid transfer in yeast Golgi function. *Nature* **347,** 561–562.

Borochov, A., Cho, M. H., and Boss, W. F. (1994). Plasma membrane lipid metabolism of petunia petals during senescence. *Physiol. Plant.* **90,** 279–284.

Boronenkov, I. V., and Anderson, R. A. (1995). The sequence of phosphatidylinositol-4-phosphate 5-kinase defines a novel family of lipid kinases. *J. Biol. Chem.* **270,** 2881–2884.

Boss, W. F., and Massel, M. O. (1985). Polyphosphoinositides are present in plant tissue culture cells. *Biochem. Biophys. Res. Commun.* **132,** 1018–1023.

Brearley, C. A., and Hanke, D. E. (1993). The pathway of synthesis of 3,4- and 4,5-phosphorylated phosphatidylinositols in the duckweed Spirodela polyrhiza L. *Biochem. J.* **290,** 145–150.

Cantley, L. C., Auger, K. R., Carpenter, C. L., Duckworth, A., Graziani, R., Kapeller, R., and Soltoff, S. (1991). Oncogenes and signal transduction. *Cell* **64,** 281–302.

Carpenter, C. L., and Cantley, L. C. (1990). Phosphoinositide kinases. *Biochemistry* **29,** 11147–11156.

Carpenter, C. L., and Cantley, L. C. (1996). Phosphoinositide kinases. *Curr. Opin. Cell Biol.* **8,** 153–158.

Chong, L. D., Traynor-Kaplan, A., Bokoch, G. M., and Schwartz, M. A. (1994). The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell* **79,** 507–513.

Cote, G. G., and Crain, R. C. (1993). Biochemistry of phosphoinositides. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44,** 333–356.

Cunningham, E., Thomas, G. M. H., Ball, A., Hiles, I., and Cockcroft, S. (1995). Phosphatidylinositol transfer protein dictates the rate of inositol trisphosphate production by promoting the synthesis of PIP2. *Curr. Biol.* **5,** 775–783.

Cunningham, E., Khoon Tan, S., Swigart, P., Hsuan, J., Bankaitis, V., and Cockcroft, S. (1996). The yeast and mammalian isoforms of phosphatidylinositol transfer protein can restore phospholipase C-mediated inositol lipid signaling in cytosol-depleted RBL-2H3 and HL60 cells. *Proc. Natl. Acad. Sci. USA* **93,** 6589–6593.

De Camilli, P., Emr, S. D., McPherson, P. S., and Novick, P. (1996). Phosphoinositides as regulators in membrane traffic. *Science* **271,** 1533–1539.

De Corte, V., Gettemans, J., and Vanderkerckhove, J. (1997). Phosphatidylinositol 4,5-bisphosphate specifically stimulates pp60c-src catalyzed phosphorylation of gelsolin and related actin-binding proteins. *FEBS Lett.* **401,** 191–196.

Domin, J., and Waterfield, M. D. (1997). Using structure to define the function of phosphoinositide 3-kinase family members. *FEBS Lett.* **410,** 91–95.

Dove, S. K., Lloyd, C. W., and Drøbak, B. K. (1994). Identification of a phosphatidylinositol 3-hydroxy kinase in plant cells: Association with the cytoskeleton. *Biochem. J.* **303,** 347–350.

Dove, S. K., Cooke, F. T., Douglas, M. R., Sayers, L. G., Parker, P. J., and Michell, R. H. (1997). Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis. *Nature* **390,** 187–192.

Drøbak, B. K. (1992). The plant phosphoinositide system. *Biochem. J.* **288,** 697–712.

Drøbak, B. K. (1996). Metabolism of plant phosphoinositides and other inositol-containing lipids. *In* "Membranes: Specialized Functions in Plants" (M. Smallwood, J. P. Knox, and D. J. Bowles, Eds.), pp. 195–214. BIOS Scientific, Oxford, UK.

Drøbak, B. K., and Ferguson, I. B. (1985). Release of Ca^{2+} from plant hypocotyl microsomes by inositol 1,4,5-trisphosphate. *Biochem. Biophys. Res. Commun.* **130,** 1241–1246.

Drøbak, B. K., Watkins, P. A. C., Valenta, R., Dove, S. K., Lloyd, C. W., and Staiger, C. J. (1994). Inhibition of plant plasma membrane phosphoinositide phospholipase C by the actin-binding protein, profilin. *Plant J.* **6,** 389–400.

Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R. J., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997). Regulation of neuronal survival by the serine–threonine protein kinase Akt. *Science* **275,** 661–665.

Exton, J. H. (1997). New developments in phospholipase D. *J. Biol. Chem.* **272,** 15579–15582.

Fensome, A., Cunningham, E., Prosser, S., Khoon Tan, S., Swigert, P., Thomas, G., Hsuan, J., and Cockcroft, S. (1996). ARF and PITP restore GTPγS-stimulated protein secretion from cytosol-depleted HL60 cells by promoting PIP2 synthesis. *Curr. Biol.* **6,** 730–738.

Flanagan, C. A., and Thorner, J. (1992). Purification and characterization of a soluble phosphatidylinositol 4-kinase from the yeast Saccharomyces cerevisiae. *J. Biol. Chem.* **267,** 24117–24125.

Flanagan, C. A., Schnieders, E. A., Emerick, A. W., Kunisawa, R., Admon, A., and Thorner, J. (1993). Phosphatidylinositol 4-kinase: Gene structure and requirement for yeast cell viability. *Science* **262,** 1444–1448.

Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995). The protein kinase encoded by the Akt protooncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81,** 727–736.

Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997). Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* **275,** 665–668.

Gehrmann, T., Verqab, G., Schmidt, M., Klix, D., Meyer, H. E., Varsanyi, M., and Heilmeyer, L. M. G. (1996). Identification of a 200 kDa polypeptide as type-3 phosphatidylinositol 4-kinase from bovine brain by partial protein and cDNA sequencing. *Biochim. Biophys. Acta Mol. Cell Res.* **1311,** 53–63.

Goldschmidt-Clermont, P. J., Machesky, L. M., Baldassare, J. J., and Pollard, T. D. (1990). The actin-binding protein profilin binds to PIP_2 and inhibits its hydrolysis by phospholipase C. *Science* **247,** 1575–1578.

Goldschmidt-Clermont, P. J., Kim, J. W., Machesky, L. M., Rhee, S. G., and Pollard, T. D. (1991). Regulation of phospholipase C-γ1 by profilin and tyrosine phosphorylation. *Science* **251,** 1231–1233.

Gross, W., and Boss, W. F. (1992). Inositol phospholipids and signal transduction. *In* "Control of Plant Gene Expression" (D.P.S. Verma, ed.), pp. 17–32. Telford Press, Caldwell, NJ.

Hartwig, J. H., Bokoch, G. M., Carpenter, C. L., Janmey, P. A., Taylor, L. A., Toker, A., and Stossel, T. P. (1995). Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell* **82,** 643–653.

Harwood, J. L. (1980). Plant acyl lipids: Structure, distribution and analysis. *In* "The Biochemistry of Plants, Vol. 4" (P. K. Stumpf, ed.), pp. 1–55. Academic Press, New York.

Hay, J. C., and Martin, T. F. J. (1993). Phosphatidylinositol transfer protein required for ATP-dependent priming of Ca^{2+}-activated secretion. *Nature* **366,** 572–575.

Hay, J. C., Fisette, P. L., Jenkins, G. H., Fukami, K., Takenawa, T., Anderson, R. A., and Martin, T. F. J. (1995). ATP-dependent inositide phosphorylation required for Ca^{2+}-activated secretion. *Nature* **374,** 173–177.

Hendrix, K. W., Assefa, H., and Boss, W. F. (1989). The polyphosphoinositides, phosphatidylinositol monophosphate and phosphatidylinositol bisphosphate are present in nuclei isolated from carrot protoplasts. *Protoplasma* **151,** 62–72.

Hirt, H. (1997). Multiple roles of MAP kinases in plant signal transduction. *Trends Plant Sci.* **2,** 11–15.

Hokin, M. R., and Hokin, L. E. (1953). Enzyme secretion and the incorporation of 32P into phospholipids of pancreas slices. *J. Biol. Chem.* **203,** 967–977.

Hong, Z. G., and Verma, D. P. S. (1994). A phosphatidylinositol 3-kinase is induced during soybean nodule organogenesis and is associated with membrane proliferation. *Proc. Natl. Acad. Sci. USA* **91,** 9617–9621.

Irvine, R. F., Letcher, A. J., Lander, D. J., Drøbak, B. K., Dawson, A. P., and Musgrave, A. (1989). Phosphatidylinositol(4,5)bisphosphate and phosphatidylinositol(4)phosphate in plant tissues. *Plant Physiol.* **89,** 888–892.

Janmey, P. A. (1994). Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. *Annu. Rev. Physiol.* **56,** 169–191.

Kader, J.-C. (1996). Lipid-transfer proteins in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47,** 627–654.

Kauffmann-Zeh, A., Thomas, G. M. H., Ball, A., Prosser, S., Cunningham, E., Cockcroft, S., and Hsuan, J. J. (1995). Requirement for phosphatidylinositol transfer protein in epidermal growth factor signaling. *Science* **268,** 1188–1190.

Kearns, B. G., McGee, T. P., Mayinger, P., Gedvilaite, A., Phillips, S. E., Kagiwada, S., and Bankaitis, V. A. (1997). Essential role for diacylglycerol in protein transport from the yeast Golgi complex. *Nature* **387,** 101–105.

Kearns, M. A., Monks, D. E., Rivas, M. P., Theibert, A. B., Dewey, R. E., and Bankaitis, V. A. (1998). Expression in yeast of novel phosphoinositide binding proteins from soybean bypasses the requirement for an essential phosphatidylinositol transfer protein in Golgi secretory function. Manuscript in preparation.

Lemmon, M. A., Ferguson, K. M., and Schlessinger, J. (1996). PH domains: Diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell* **85,** 621–624.

Liscovitch, M., and Cantley, L. C. (1995). Signal transduction and membrane traffic: The PITP/phosphoinositide connection. *Cell* **81,** 659–662.

Loijens, J. C., and Anderson, R. A. (1996). Type I phosphatidylinositol-4-phosphate 5-kinases are distinct members of this novel lipid kinase family. *J. Biol. Chem.* **271,** 32937–32943.

Lundberg, G. A., and Sommarin, M. (1992). Diacylglycerol kinase in plasma membranes from wheat. *Biochem. Biophys. Acta* **1123,** 177–183.

Lykidis, A., Jackson, P. B., Rock, C. O., and Jackowski, S. (1997). The role of CDP-diacylglycerol synthase and phosphatidylinositol synthase activity levels in the regulation of cellular phosphatidylinositol content. *J. Biol. Chem.* **272,** 33402–33409.

Lynch, D. V. (1993). Sphingolipids. *In* "Lipid Metabolism in Plants" (T. S. Moore, ed.), pp. 285–308. CRC Press, Boca Raton, FL.

Lynch, D. V., and Steponkus, P. L. (1987). Plasma membrane lipid interactions associated with cold acclimation of winter rye seedlings (Secale cereale L. cv Puma). *Plant Physiol.* **83,** 761–767.

Matsuoka, K., Bassham, D. C., Raikhel, N. V., and Nakamura, K. (1995). Different sensitivity to wortmannin of 2 vacuolar sorting signals indicates the presence of distinct sorting machineries in tobacco cells. *J. Cell Biol.* **130,** 1307–1318.

Melin, P.-M., Sommarin, M., Sandelius, A. S., and Jergil, B. (1987). Identification of Ca2+ stimulated polyphosphoinositide phospholipase C in isolated plasma membranes. *FEBS Lett.* **223,** 87–91.

Memon, A. R., Chen, Q., and Boss, W. F. (1990). Inositol phospholipids activate plasma membrane ATPase in plants. *Biochem. Biophys. Res. Commun.* **162,** 1295–1301.

Michell, R. H. (1975). Inositol phospholipids and cell surface receptor function. *Biochem. Biophys. Acta* **415,** 81–147.

Mizoguchi, T., Hayashida, N., Yamaguchishinozaki, K., Kamada, H., and Shinozaki, K. (1995). 2 genes that encode ribosomal-protein-S6 kinase homologs are induced by cold or salinity stress in Arabidopsis thaliana. *FEBS Lett.* **358,** 199–204.

Mizoguchi, T., Ichimura, K., and Shinozaki, K. (1997). Environmental stress response in plants: The role of mitogen-activated protein kinases. *Trends Biotechnol.* **15,** 15–19.

Monaco, M. E., and Gershengorn, M. C. (1992). Subcellular organization of receptor-mediated phosphoinositide turnover. *Endocr. Rev.* **13,** 707–718.

Monks, D. E., Courtney, P. D., and Dewey, R. E. (1998). A SEC14p-like polyphosphoinositide binding protein from soybean is a substrate of an osmoregulated MAP kinase. Manuscript in preparation.

Moore, T. S., Jr. (1990). Biosynthesis of phosphatidylinositol. *In* "Inositol Metabolism in Plants" (D. J. Morré, W. F. Boss, and F. A. Loewus, eds.), pp. 107–112. Wiley-Liss, New York.

Munnik, T., Irvine, R. F., and Musgrave, A. (1994a). Rapid turnover of phosphatidylinositol 3-phosphate in the green alga Chalmydomonas eugametos—Signs of a phosphatidylinositide 3-kinase signalling pathway in lower plants. *Biochem. J.* **298,** 269–273.

Munnik, T., Musgrave, A., and Devrije, T. (1994b). Rapid turnover of polyphosphoinositides in carnation flower petals. *Planta* **193,** 89–98.

Munnik, T., Irvine, R. F., and Musgrave, A. (1998). Phospholipid signalling in plants. *Biochim. Biophys. Acta* **1389,** 222–272.

Nakagawa, T., Goto, K., and Kondo, H. (1996). Cloning, expression, and localization of 230-kDa phosphatidylinositol 4-kinase. *J. Biol. Chem.* **271,** 12088–12094.

Nikawa, J., Kodaki, T., and Yamashita, S. (1987). Primary structure and disruption of the phosphatidylinositol synthase gene of *Saccharomyces cervisiae. J. Biol. Chem.* **262,** 4876–4881.

Nishizuka, Y. (1992). Intracellular signalling by hydrolysis of phospholipids and the activation of protein kinase C. *Science* **258,** 607–614.

Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* **21,** 205–215.

Ohashi, M., Jan de Vries, K., Frank, R., Snoek, G., Bankaitis, V., Wirtz, K., and Huttner, W. B. (1995). A role for phosphatidylinositol transfer protein in secretory vesicle formation. *Nature* **377,** 544–547.

Okpodu, C. M., Gross, W., Burkhart, W., and Boss, W. F. (1995). Purification and characterization of a soluble phosphatidylinositol 4-kinase from carrot suspension culture cells. *Plant Physiol.* **107,** 491–500.

Palmer, R. H., Dekker, L. V., Woscholski, R., Legood, J. A., Gigg, R., and Parker, P. J. (1995). Activation of PRK1 by phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate—A comparison with protein kinase-C isotypes. *J. Biol. Chem.* **270,** 22412–22416.

Panaretou, C., Domin, J., Cockcroft, S., and Waterfield, M. D. (1997). Characterization of p150, an adaptor protein for the human phosphatidylinositol (PtdIns) 5-kinase—Substrate presentation by phosphatedylinositol transfer protein to the p150-PtdIns 3-kinase complex. *J. Biol. Chem.* **272,** 2477–2485.

Pappan, K., Zheng, S., and Wang, X. (1997a). Identification and characterization of a novel plant phospholipase D that requires polyphosphoinositides and submicromolar calcium for activity in Arabidopsis. *J. Biol. Chem.* **272,** 7048–7054.

Pappan, K., Qin, W., Dyer, J. H., Zheng, L., and Wang, X. (1997b). Molecular cloning and functional analysis of polyphosphoinositide-dependent phospholipase D, PLDβ, from Arabidopsis. *J. Biol. Chem.* **272,** 7055–7061.

Perotto, S., Donovan, N., Drøbak, B. K., and Brewin, N. J. (1995). Differential expression of a glycosylated inositol phospholipid antigen on the peribacteroid membrane during pea nodule development. *Mol. Plant–Microbe Interact.* **4,** 560–568.

Pike, L. J. (1992). Phosphatidylinositol 4-kinases and the role of polyphosphoinositides in cellular regulation. *Endocr. Rev.* **13,** 692–706.

Popova, J. S., Garrison, J. C., Rhee, S. G., and Rasenick, M. M. (1997). Tubulin, Gq, and phosphatidylinositol 4,5-bisphosphate interact to regulate phospholipase CB1 signaling. *J. Biol. Chem.* **272,** 6760–6765.

Rameh, E. L., Tolias, K. F., Duckworth, B. C., and Cantley, L. C. (1997). A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. *Nature* **390,** 192–196.

Randazzo, P. A. (1997). Functional interaction of ADP-ribosylation factor 1 with phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* **272,** 7688–7692.

Sandelius, A. S., and Morré, D. J. (1987). Characteristics of a phosphatidylinositol exchange activity of soybean microsomes. *Plant Physiol.* **84,** 1022–1027.

Sandelius, A. S., and Sommarin, M. (1986). Phosphorylation of phosphatidylinositol in isolated plant membranes. *FEBS Lett.* **201,** 282–286.

Sandelius, A. S., and Sommarin, M. (1990). Membrane-localized reactions involved in polyphosphoinositide turnover in plants. *In* "Inositol Metabolism in Plants" (D. J. Morré, W. F. Boss, and F. A. Loewus, eds.), pp. 139–161. Wiley-Liss, New York.

Schaller, G. E., Harmon, A., and Sussman, M. R. (1992). Characterization of a calcium- and lipid-dependent protein kinase associated with the plasma membrane in oat. *Biochemistry* **31,** 1721–1727.

Sexton, J. C., and Moore, T. S. (1981). Phosphatidylinositol synthesis by a Mn^{2+}-dependent exchange enzyme in castor bean endosperm. *Plant Physiol.* **68,** 18–22.

Shaw, G. (1996). The pleckstrin homology domain: An intriguing multifunctional protein module. *Bioessays* **18,** 35–46.

Shibasaki, Y., Ishihara, H., Kizuki, N., Asano, T., Oka, Y., and Yazaki, Y. (1997). Massive actin polymerization induced by phosphatidylinositol-4-phosphate 5-kinase in vivo. *J. Biol. Chem.* **272,** 7578–7581.

Skinner, H. B., Alb, J. G., Whitters, E. A., Helmkamp, G. M., and Bankaitis, V. A. (1993). Phospholipid transfer activity is relevant to but not sufficient for the essential function of the yeast SEC14 gene product. *EMBO J.* **12,** 4775–4784.

Skinner, H. B., McGee, T. P., McMaster, C. R., Fry, M. R., Bell, R. M., and Bankaitis, V. A. (1995). The Saccharomyces cerevisiae phosphatidylinositol-transfer protein effects a ligand-dependent inhibition of choline-phosphate cytidylyltransferase activity. *Proc. Natl. Acad. Sci. USA* **92,** 112–116.

Sommarin, M., and Sandelius, A. S. (1988). Phosphatidylinositol and phosphatidylinositolphosphate kinases in plant plasma-membranes. *Biochim. Biophys. Acta* **958,** 268–278.

Stephens, L. R., Hughes, K. T., and Irvine, R. F. (1991). Pathway of phosphatidylinositol(3,4,5)triphosphate synthesis in activated neutrophils. *Nature* **351,** 33–39.

Stevenson, J. M., Perera, I. Y., and Boss, W. F. (1998). A phosphatidylinositol 4-kinase pleckstrin homology (PH) domain that binds phosphatidylinositol 4-monophosphate. *J. Biol. Chem.* **273,** 22761–22767.

Streb, H., Irvine, R. F., Berridge, M. J., and Schulz, I. (1983). Release of Ca^{2+} from a nonmitochondrial intracelluler store in pancreatic acinar-cells by inositol-1,4,5-trisphosphate. *Nature* **306,** 67–69.

Sylvia, V., Curtin, G., Norman, J., Stec, J., and Busbee, D. (1988). Activation of a low specific activity form of DNA polymerase α by inositol-1,4-bisphosphate. *Cell* **54,** 651–658.

Tan, Z., and Boss, W. F. (1992). Association of phosphatidylinositol kinase, phosphatidylinositol monophosphate kinase and diacylglycerol kinase with the cytoskeleton and F-actin fractions of carrot (Daucus carota L.) cells grown in suspension culture: Response to cell wall degrading enzymes. *Plant Physiol.* **100,** 2116–2120.

Tanaka, S., Nikawa, J., Imai, H., Yamashita, S., and Hosaka, K. (1996). Molecular cloning of rat phosphatidylinositol synthase cDNA by functional complementation of the yeast *Saccharomyces cervisiae* pis. *FEBS Lett.* **393,** 89–92.

Tanaka, T., and Yamada, M. (1982). Properties of phospholipid exchange proteins from germinated castor bean endosperms. *In* "Biochemistry and Metabolism of Plant Lipids" (J. F. G. M. Wintermans and P. J. C. Kuiper, eds.), pp. 99–106. Elsevier, Amsterdam.

Thomas, G. M. H., Cunningham, E., Fensome, A., Ball, A., Totty, N. F., Truong, O., Hsuan, J. J., and Cockcroft, S. (1993). An essential role for phosphatidylinositol transfer protein in phospholipase C-mediated inositol lipid signaling. *Cell* **74,** 919–928.

Tolias, K. F., Cantley, L. C., and Carpenter, C. L. (1995). Rho family GTPases bind to phosphoinositide kinases. *J. Biol. Chem.* **270,** 17656–17659.

Van Paridon, P. A., Somerharju, P., and Wirtz, K. W. A. (1987). Phosphatidylinositol-transfer protein and cellular phosphatidylinositol metabolism. *Biochem. Soc. Trans.* **15,** 321–323.

Vogel, S., and Hoppe, J. (1986). Polyamines stimulate the phosphorylation of phosphatidylinositol in membranes from A431 cells. *Eur. J. Biochem.* **154,** 253–257.

Welters, P., Takegawa, K., Emr, S. D., and Chrispeels, M. J. (1994). ATVPS34, a phosphatidylinositol 3-kinase of Arabidopsis thaliana, is an essential protein with homology to a calcium-dependent lipid-binding domain. *Proc. Natl. Acad. Sci. USA* **91,** 11398–11402.

Whiteford, C. C., Brearley, C. A., and Ulug, E. T. (1997). Phosphatidylinositol 3,5-bisphosphate defines a novel PI 3-kinase pathway in resting mouse fibroblasts. *Biochem. J.* **323,** 597–601.

Whitman, M., Downes, C. P., Keeler, M., Keller, T., and Cantley, L. C. (1988). Type 1 phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol 3-phosphate. *Nature* **332,** 644–646.

Wirtz, K. W. A. (1991). Phospholipid transfer proteins. *Annu. Rev. Biochem.* **60,** 73–99.

Wong, K., and Cantley, L. C. (1994). Cloning and characterization of a human phosphatidylinositol 4-kinase. *J. Biol. Chem.* **269,** 28878–28884.

Wong, K., Meyers, R., and Cantley, L. C. (1997). Subcellular localizations of phosphatidylinositol 4-kinase isoforms. *J. Biol. Chem.* **272,** 13236–13241.

Xu, P., Lloyd, C. W., Staiger, C. J., and Drøbak, B. K. (1992). Association of phosphatidylinositol 4-kinase with the plant cytoskeleton. *Plant Cell* **4,** 941–951.

Yang, W., and Boss, W. F. (1994a). Regulation of the plasma membrane type III phosphatidylinositol 4-kinase by positively charged compounds. *Arch. Biochem. Biophys.* **313,** 112–119.

Yang, W., and Boss, W. F. (1994b). Regulation of the phosphatidylinositol 4-kinase activity by the protein activator PIK-A49. Activation requires protein phosphorylation. *J. Biol. Chem.* **269,** 3852–2857.

Yang, W., Burkhart, W., Cavallius, J., Merrick, W. C., and Boss, W. F. (1993). Purification and characterization of a phosphatidylinositol 4-kinase activator in carrot cells. *J. Biol. Chem.* **268,** 392–398.

Yoshida, S., Ohya, Y., Nakano, A., and Anraku, Y. (1994). Genetic interactions among genes involved in the STT4-PKC1 pathway of Saccharomyces cerevisiae. *Mol. Gen. Genet.* **242,** 631–640.

Zhang, X., Loijens, J. C., Boronenkov, I. V., Parker, G. J., Norris, F. A., Chen, J., Thum, O., Prestwich, G. D., Majerus, P. W., and Anderson, R. A. (1997). Phosphatidylinositol-4-phosphate 5-kinase isozymes catalyze the synthesis of 3-phosphate-containing phosphatidylinositol signaling molecules. *J. Biol. Chem.* **272,** 17756–17761.

Zheng, Y., Glaven, J. A., Wu, W. J., and Cerione, R. A. (1996). Phosphatidylinositol 4,5-bisphosphate provides an alternative to guanine-nucleotide exchange factors by stimulating the dissociation of GDP from CDC42HS. *J. Biol. Chem.* **271,** 23815–23819.

Chlamydomonas Cell Cycle Mutants

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Because cell division cycle genes encode essential proteins, a genetic approach to dissect the molecular mechanisms of the cell cycle has relied on the isolation of conditional mutants. In most cases these are temperature sensitive mutants which grow at a lower (permissive) temperature but arrest at some point in the cell cycle at a higher (restrictive) temperature. The power of this approach is that mutations can be induced in unknown proteins and then studied. Investigations of temperature-conditional cell cycle mutants, especially in lower eukaryotes such as yeasts, have resulted in a wealth of information about genes which control and coordinate the cell cycle in these organisms. This information has also aided the search for conserved cell cycle genes in other organisms. The haploid biflagellate unicellular photosynthetic alga *Chlamydomonas* shares with the yeast model systems many features which make it ideal for the isolation of cell cycle mutants. Furthermore, *Chlamydomonas* is a plant which divides by multiple fission. These features suggest that *Chlamydomonas* will provide unique insight into the control and coordination of the cell cycle. Studies of the cell cycle in wild-type cells are described here as a reference to the cell cycle mutants. A number of conditional temperature-sensitive and constitutive cell cycle mutants are presented. These mutants have been characterized using a range of techniques, including immunofluorescence microscopy of cytoskeletal proteins such as tubulin and centrin. The rapid advances in molecular techniques in *Chlamydomonas* should ensure that cloning and molecular characterization of cell cycle genes will occur in the near future.

KEY WORDS: Cell division cycle, *Chlamydomonas*, Mutagenesis, Mitosis, Mutations, Temperature sensitive.

I. Introduction

The unicellular, biflagellate, eukaryotic green alga *Chlamydomonas* is an excellent model organism for the study of many biological phenomenon,

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including photosynthesis (Harris, 1989; Rochaix, 1995); flagellar structure and regulation (Lefebvre and Rosenbaum, 1986; Huang, 1986; Johnson and Rosenbaum, 1993; Dutcher, 1995a); basal bodies and the associated cytoskeleton, structure, and function (Dutcher and Lux, 1989; Holmes and Dutcher, 1992); gametic differentiation (Beck and Haring, 1996); cell cycle (John, 1984, 1987; Harper *et al.,* 1995b); and others (Harris, 1989). Indeed, *Chlamydomonas* has many features which make it amenable to genetic and molecular analysis (Harris, 1989; Dentler and Witman, 1995). These include a well-defined, relatively short life cycle and a haploid genome which permits isolation of recessive mutations. *Chlamydomonas reinhardtii* is the most intensively characterized species of the genus *Chlamydomonas* (Harris, 1989) and most of the work described here will refer to this species.

A. Overview

This review concerns the isolation and characterization of *Chlamydomonas* mutants which have provided insight into the control and coordination of the cell cycle in this organism. The power of mutational analysis enables the isolation of mutants which are defective in genes of unknown function. Coupled with the rapidly expanding molecular analyses, the genes and their partners can be identified (Dentler and Witman, 1995). First, I provide a background to the mutant studies by briefly describing the cell and cell cycle in wild-type cells (Section II). I will then describe examples of two general mutant classes in *Chlamydomonas*—conditional (Section III,B) and constitutive (Section III,C)—which are being used to explore the workings of the cell cycle. I will also show the range of mutants and discuss some techniques which are currently being used. In addition, I will discuss the future directions of this area of research. One of my objectives is to provide an appreciation of the usefulness of *Chlamydomonas* as a model system to study the cell cycle of a unicellular plant, which divides by multiple fission. These are two obvious features that the other well-studied model systems, such as yeasts, do not provide. *Chlamydomonas* also has a basal body complex, often referred to as a centrosomal homolog (Salisbury *et al.,* 1986), which enables study of the role of this complex in cell division.

B. What Are Conditional and Constitutive Mutants?

1. Conditional

The isolation of cell cycle mutants in other eukaryotes has usually been of a conditional nature (Pringle, 1978, 1981) because mutations in genes essen-

tial for cell division are lethal. Although conditional mutations can take a number of forms, the most widely isolated are temperature conditional (Pringle, 1975, 1981). The genes for these essential proteins are thought to encode products that are unsually thermolabile. Cells can grow and divide at the permissive temperature, which is usually lower, but become blocked at the same point (or stage-specific event) in the cell cycle on shift to the restrictive or nonpermissive temperature, which is usually higher. At the restrictive temperature the gene products become nonfunctional (Pringle 1975, 1981). A crucial indicator in selection of cell cycle mutations is that cells are blocked in a division event, not just growth, and thus continue to increase in size. The continuation of growth and other stage-specific events, not dependent on prior completion of the mutated gene function, results in the arrest of cells with a characteristic terminal phenotype (Pringle, 1978, 1981). The organisms most studied have been the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Forsburg and Nurse, 1991; Hartwell, 1991) and *Aspergillis* (Doonan, 1992). Cell cycle mutants have also been studied in many other organisms (Lloyd *et al.,* 1982), including animal tissue culture cells (Marcus *et al.,* 1985; Norbury and Nurse, 1992) and higher plants (Traas and Laufs, 1998).

Conditional cell cycle mutants have also been isolated in *Chlamydomonas* by a number of researchers. These include the (temperature-sensitive division (TSD) mutants (Wolff, 1971), the cycle-blocked (Cb) mutants (Howell, and Naliboff, 1973; Howell, 1974), and the cell division blocked (Cdb), cyclic-adenosine monophosphate (cAMP)-requiring and metaphase arresting (*met1*) mutants (Harper *et al.,* 1995b; Sakuanrungsirikul *et al.,* 1996; Wu *et al.,* 1997) which shall all be described in Section III,B.

2. Constitutive

Chlamydomonas also affords the study of constitutive mutants (i.e., those mutants that are expressed throughout the cell cycle resulting in some defect in control and coordination). I loosely term these cell cycle mutations because, although the population as a whole can be propagated, the mutations affect some aspect of the cell cycle which can result in a number of aborted divisions. These mutants provide insights into aspects of the *Chlamydomonas* cell cycle. The first reported isolation of mutants of this type was by Lewin (1952), who described a number of mutations in *Chlamydomonas moewusii,* some of which showed defects in cell division. Other interesting constitutive mutants include those that appear to have lost the ability to coordinate the flagellar/basal body cycle with the cell division cycle. The result is a variable flagellar number (*vfl*) phenotype (Holmes and Dutcher, 1992). Another mutant, called *mat3,* produces abnormally small cells indicating a disruption in cell size control (Armbrust *et al.,* 1995).

The *bld2* mutant, which lacks basal bodies and thus flagella (Goodenough and St. Clair, 1975), has recently been shown to result in defective cytokinesis due to misaligned flagellar rootlets (Ehler *et al.*, 1995). Because these mutants have impact on events related to the cell cycle, they will also be described in Section III,C.

II. *Chlamydomonas*, Cytoskeleton, and the Cell Division Cycle

The *Chlamydomonas* cell has been described previously (Trainor and Cain, 1986; Harris, 1989; van den Ende, 1994). Figure 1 depicts a generalized cell and is used to point out salient features of *Chlamydomonas* which make it useful for the study of several phenomena. The *Chlamydomonas* cell cycle is also outlined (Fig. 2) to highlight multiple fission. In addition, Fig. 3 (see color plate) shows the microtubule and centrin cytoskeletons of *Chlamydomonas* because a number of mutants described later have been characterized using immunocytochemistry.

A. Organelles and Cytoskeleton

Chlamydomonas is a member of the volvocine algae which all have a fundamentally similar and distinct subcellular organization. The molecular analysis of *Volvox*, a colonial relative of *Chlamydomonas* (Schmitt *et al.*, 1992), may also reveal genes involved in cell cycle progression in both organisms.

1. General Features

Chlamydomonas cells have an average diameter of 10 μm and contain a cup-shaped chloroplast which occupies 40–50% of the cell volume (Fig. 1) (Schotz *et al.*, 1972; Gaffal *et al.*, 1995). Within the chloroplast, at the posterior end of the cell, is a large conspicuous spherical pyrenoid, almost completely encased in a layer of starch. The pyrenoid clearly acts as a center for starch deposition and is composed of a high concentration of ribulose bisphosphate carboxylase (Harris, 1989). Cells are propelled through liquid by two 10- to 12-μm long flagella located at the anterior end of the cell (Ringo, 1967). The flagella are nucleated from two biochemically complex basal bodies which are analogous to the centrioles of mammalian cells (Cavalier-Smith, 1974; Pickett-Heaps, 1975; Gaffal, 1988). From the

FIG. 1 A semidiagrammatic representation of an interphase *Chlamydomonas* cell. Cell length, 10 μm; BB, Basal bodies that act as nucleating sites for flagella. Basal bodies are connected by the distal striated fiber; Chl, cup-shaped chloroplast occupying 40–50% of cell volume; Cv, contractile vacuole, two alternately pulsing; Cw, glycoprotein cell wall; Er, endoplasmic reticulum; Es, eye spot; F, Flagella (about 10 μm long), at anterior end. A breast stroke action propels cells in liquid medium; G, Golgi apparatus (Dictyosome); L, lipid body; M, mitochondria, 10–15 per cell; N, nucleus; No, nucleolus; P, pyrenoid; r, ribosomes; S, starch grain; v, vacuole (after Ettl, 1976a).

region of the basal bodies emanate a number of distinct microtubular and fibrous roots (Holmes and Dutcher, 1992).

Chlamydomonas has a thin glycoprotein cell wall (Harris, 1989; van den Ende, 1994) and water is taken up continuously across the plasma membrane. Two alternating pulsating contractile vacuoles located just below the basal bodies expel the accumulating water (Fig. 1) (Harris, 1989; Holmes and Dutcher, 1989). A single nucleus, occupying 10% of the cell volume, lies in the anterior third of the cell below the flagellar basal apparatus (Schotz *et al.*, 1972). Cells usually have at least two dictyosomes (Golgi apparatuses) which lie close to the nucleus (Gruber and Rosario, 1979). The 10–15 mitochondria are elongated and branched, winding about the

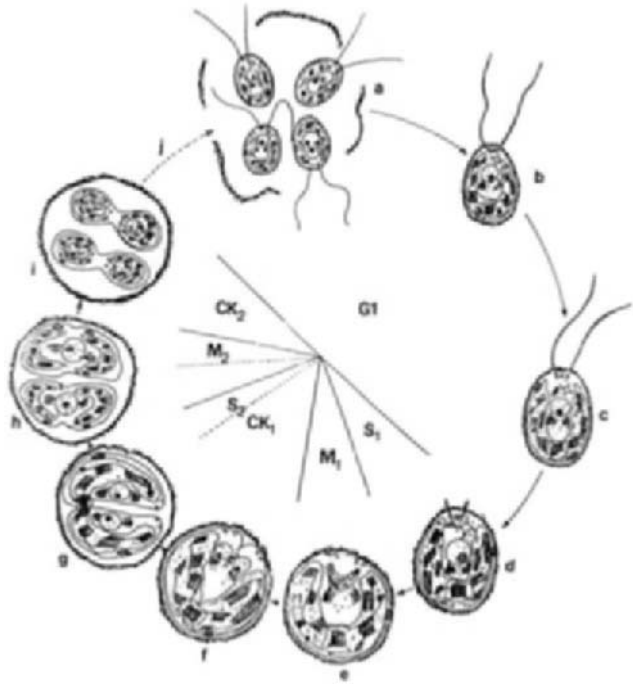


FIG. 2 The *Chlamydomonas* cell cycle observed under light/dark batch culture (Rollins *et al.*, 1983). Autolysin digestion of the mother cell envelope and release of daughter cells (Harris, 1989) marks the beginning of the cell cycle. (a–c) G_1 period during which cells more than double their mass. (d) Flagellar withdrawal marks the beginning of division; this occurs at about the same time as the first DNA synthesis (S_1). (e) First mitosis (M_1); the nucleus becomes cresent-shaped with poles toward the duplicated and separated basal bodies at the cell surface (see Fig. 3C). (f) First cytokinesis (CK_1) begins at the anterior end of the cell with a cleavage furrow progressing circumferentially around the cell between duplicated sets of basal bodies; (g) Chloroplast and pyrenoid are bisected by the cleavage furrow; the second round of DNA synthesis (S_2) may occur at some time during CK_1 and is shown as dotted lines. (h) Second mitosis (M_2) follows S_2 which is then proceeded by the second round of cytokinesis (i) (CK_2) to produce four daughter cells (in this case). (j) Finally, cells regrow flagella and new cell walls before release from the mother cell wall to begin a new life cycle. Basal bodies are shown in most cells as cylinders below the flagellar apparatus (modified with permission from Harper *et al.*, 1995b).

cell. Some are always situated near the flagellar bases (Gaffal, 1987). On the outer surface at one side of the chloroplast is a red-orange pigmented organelle called the eyespot or stigma. This is composed of a stack of lipid droplets which acts as a directional antenna for the rhodopsin-containing photoreceptor in the overlying plasma membrane (Harris, 1989). The positioning of some of these organelles is very precisely determined by the cytoskeleton.

2. Cytoskeleton

Chlamydomonas exhibits a precise cytoskeletal architecture which has as its focus the flagellar basal apparatus (Figs. 3A and 3B, see color plate); this has been reviewed in detail elsewhere (Holmes and Dutcher, 1992). Briefly, there are two sets of acetylated microtubule roots or rootlets (one set contains four and the other contains two microtubules) forming a 4,2,4,2 cruciate pattern extending from the region below the basal bodies. Two sets of acetylated cruciate microtubule roots extend into the cell (Le Dizet and Piperno, 1986; Goodenough and Weiss, 1978; Melkonian, 1984). The roots extend just below the plasma membrane toward the base of the cell (Weiss, 1984) and are believed to be involved in cell shaping. Immunofluorescence using antitubulin labels these rootlets, the flagella, and the secondary microtubules which are nucleated from the rootlets (Fig. 3A). The eyespot is believed to be positioned by the four-membered rootlet which emanates from the *cis* basal body. The other basal body is called the *trans* (Melkonian, 1984; Holmes and Dutcher, 1989). *Chlamydomonas* has an apparent rotational symmetry such that any plane bisecting a cell from the anterior to posterior gives two halves which are approximate 180° rotations. There are two obvious exceptions; the eyespot and mating structure position which produces an asymmetry (Holmes and Dutcher 1989). The basis of this *cis/trans* asymmetry is believed to lie in the fact that the basal body pair consists of a parental/daughter combination which is inherently different (Holmes and Dutcher, 1992; Beech *et al.,* 1991).

The calcium-binding protein centrin (caltractin) has been found to be a major component of calcium-contractile roots in many protists (Koutoulis *et al.,* 1988; Melkonian *et al.,* 1992; Harper *et al.,* 1995a; Levy *et al.,* 1996). Centrin is also a component of centrosomes and is found in the cytoplasm and in association with other noncentrosomal structures in a wide range of cell types (Salisbury, 1995; Schiebel and Bornens, 1995; Paoletti *et al.,* 1996; Levi *et al.,* 1996; Del Vecchio *et al.,* 1997). In *Chlamydomonas,* centrin is a major component of three fiber systems (Wright *et al.,* 1985, 1989; Salisbury *et al.,* 1987, 1988; Huang *et al.,* 1988). First, Centrin forms a distal striated fiber which connects the basal bodies. Second, centrin is a major component of the nucleus basal body connector (NBBC; Fig. 3B). Two fibers extend from the basal bodies to the nucleus, each descending fiber branches several times to produce a number of finger-like projections called fimbria, which embrace the nucleus (Salisbury *et al.,* 1988; Fig. 3B). The NBBC ensures that the nucleus is tethered to the basal bodies and equipartitioning of basal bodies and nuclei (Salisbury *et al.,* 1988; Taillon *et al.,* 1992). Finally, centrin-based fibers are also found in the flagellar transition region and have an essential role in flagellar excision (Sanders and Salisbury, 1989, 1994). These centrin-based fibers form the basis of system II roots in algae

(Lechtreck and Melkonian, 1991). During cell division, the microtubules form a centric mitotic spindle and the centrin-based system contracts, duplicates, and separates to the spindle poles (Fig. 3C) (Doonan and Grief, 1987; Salisbury *et al.,* 1988). More details of the orchestration of cytoskeletal events are given in (Section II,C,2). The microtubule and centrin-based cytoskeletons have been analyzed in the characterization of a number of cell cycle mutants described in Sections III,B,4,5,6 and III,C,2,3a.

Chlamydomonas has another fibrous root system—striated microtubule-associated fibers (SMAFs) or system I fibers (Lechtreck and Melkonian, 1991). A 34-kDa protein, called SF-assemblin, has recently been discovered to be the filament-forming component of these roots. In *Chlamydomonas,* SF-assemblin is localized to a rigid cruciate root below the basal bodies (Lechtreck and Silfow, 1997). This is believed to give the flagellar apparatus stability during flagellar beating. The gene which encodes this protein has recently been cloned from *Chlamydomonas* (Lechtreck and Silfow, 1997).

Immunofluorescence labeling of the actin cytoskeleton shows that it appears to associate with the nucleus during interphase (Harper *et al.,* 1992) and a cruciate rootlet during division. Immunolabeling during division suggests that actin may be involved in basal body positioning and cytokinesis (Harper *et al.,* 1992).

B. Division Control

Chlamydomonas has a cell cycle that lasts approximately 24 h (diurnal), although in constant light cells will complete a cell cycle in 20 h (Donnan and John, 1983; McAteer *et al.,* 1985). Under laboratory conditions of high light intensity and aeration (Rollins *et al.,* 1983) cells have an extended G_1 period (Fig. 1), during which time they will more than double their cell mass. Cells become "committed" to division after a defined amount of time (John, 1987) and this commitment has been likened to the major rate-limiting START event in the yeast cell cycle (John, 1984). START occurs just prior to S phase when cells commit to a single doubling of DNA and cell number without further requirement for growth. Yeast cells divide into two when they have doubled their cell mass. However, because *Chlamydomonas* more than doubles in cell mass before division, other commitments to division ensue. Cell size is compensated for by more than one round of cell division (Fig. 2) producing usually four or eight (2^n) "daughter" cells. This multiple fission mode of division produces a twofold range of cell sizes in the resulting daughter cells (Donnan and John, 1983; Donnan *et al.,* 1985).

How *Chlamydomonas* cell division is controlled has been the subject of a number of studies. In particular, workers have tried to explain how alternating periods of light and dark can synchronize *Chlamydomonas* cell divisions (John, 1987; Goto and Johnson, 1995). Craigie and Cavalier-Smith (1982) proposed that the timing of cell division is determined by a cryptic size control. Fractionation of populations into different size classes revealed that larger cells divided earlier than smaller ones. Spudich and Sager (1980) argued that unlike other photosynthetic protists, *Chlamydomonas* cell division is not under the influence of a circadian oscillator or other timer but is determined by light-dependent and light-independent cell cycle progression. John and colleagues agreed that the *Chlamydomonas* cell cycle did not appear to possess a circadian oscillator controlling the cell cycle and provided evidence that the cell cycle was under both timer and sizer control (Donnan and John, 1983; Donnan *et al.*, 1985; McAteer *et al.*, 1985). They showed that *Chlamydomonas* cells would still divide at about the same time despite shifts from 20 to 30°C, which doubled the cell's growth rates or shifts from 30 to 20°C, which halved growth rates. This mechanism has been called a temperature-compensated or hourglass timer (Donnan and John, 1983). Once divisions begin, a "sizer" mechanism is believed to operate to ensure that cell size does not fall below a certain critical threshold. Further work using CO_2 limiting conditions showed that under very slow growth rates, size determined when cells would divide (John, 1987). In addition, other workers, using photosynthetic inhibitors, have proposed a light/dark switching mechanism which determines the time at which *Chlamydomonas* cells divide (Voigt and Munzner, 1987). Goto and Johnson (1995) presented evidence to support the proposition that *Chlamydomonas* does have a circadian oscillator determining division timing and proposed that previous workers may not have detected the oscillator due to the high light intensities used in their experiments.

It is likely that all the previously discussed experiments have defined various aspects of multiple fission division control in *Chlamydomonas* but the question still remains: What are the molecular mechanisms which determine when cells divide and their size? One way to investigate the molecular mechanism of division control in *Chlamydomonas* is to produce mutations in the genes involved in these controls. Selective procedures for mutations which are of smaller size or divide at a different size could be used. Also, cells which divide at an earlier or later time could be screened for after mutagenesis. These strategies have not been reported. However, some insights may be gained from isolated cells which have been shown to divide at a much smaller cell size than wild-type cells (Armbrust *et al.*, 1995; see Section III,C,4a).

C. Coordination of Division Events

Insights about how *Chlamydomonas* coordinates division events have come from descriptive work using the light and electron microscope to observe wild-type cells grown under differing growth conditions and the effects of various inhibitors on cells. In addition, the use of antibody probes for cytoskeletal proteins has revealed the dynamics of these components during cell division. These studies will be described here because they form a background for the mutant studies.

1. Timing and Order of Division Events

Buffaloe (1958) examined four different species of *Chlamydomonas* under different light intensities and observed that under lower light intensities the pyrenoid appeared to replicate before the nucleus. Jones (1970) describes chloroplast division occurring before nuclear division under the growth conditions he used. Ettl (1976b) showed that chloroplast division in two species of *Chlamydomonas* could occur before nuclear division. It has been observed (Harper and John, 1986) that in the normal cell cycle of *C. reinhardtii,* when cells are grown under relatively high light intensities the nucleus divides before the chloroplast in the first division. The chloroplast is then bisected by the developing cleavage furrow as observed previously (Goodenough, 1970). However, in the second and third divisions that produce four or eight daughter cells the chloroplast appears to divide before the nucleus and incipient cleavage furrows appear before nuclear division (Harper and John, 1986). These variations in the timing of chloroplast and nuclear division, and the initiation of cytokinesis, imply that division timing is not tightly coupled. This is further revealed by inhibitor studies.

Using microspectrophotometry, Coleman (1982) measured the DNA content of individual *Chlamydomonas* cells during vegetative growth and demonstrated that individual nuclei did not become polyploid during multiple fission divisions. It has also been observed that in synchronous cultures chloroplast DNA synthesis can occur early in the cell cycle, long before division of the chloroplast, nucleus, and cell occurs. The implication is that "decisions" about the final cell number may already have been made well before division actually occurs. However, there is also evidence that cells may undergo additional chloroplast DNA synthesis later in the cell cycle (Coleman, 1982). It could be that under certain growth conditions, a cell may later readjust its chloroplast DNA compliment to accommodate better growth conditions in the current cell cycle.

2. Observations of Division Events and Cytoskeletal Components Using Electron- and Immunomicroscopy

A diagrammatic representation of the major features of a *Chlamydomonas* cell cycle showing a cell dividing by multiple fission into four daughter cells is presented in Fig. 2. Under the experimental conditions of light:dark batch culture in autotrophic growth medium (Rollins *et al.,* 1983), the beginning of the cell cycle is marked by the start of the light period, which enables photosynthetic growth (Fig. 2a). Note the extended G_1 period (Figs. 2a–2c) during which cells more than double their mass.

Observations with the electron microscope (Ringo, 1967; Johnson and Porter, 1968; Goodenough, 1970; Triemer and Brown, 1974; Coss, 1974; Cavalier-Smith, 1974; Pickett-Heaps, 1975; Gaffal, 1987, 1988; Gaffal and el-Gammal, 1990; Gaffal *et al.,* 1993) and immunofluorescence localization of tubulin in wild-type cells (Doonan and Grief, 1987; Le Dizet and Piperno, 1986; Holmes and Dutcher, 1989) have revealed the extensive interphase microtubule cytoskeleton which extends from the flagellar basal apparatus. Two sets of acetylated cruciate microtubule roots extend into the cell (Le Dizet and Piperno, 1986; Holmes and Dutcher, 1989, 1992; see Section II,2) and, from these secondary microtubules radiate toward the posterior of the cell and into the cell periphery (Fig. 3A). The secondary microtubules appear to increase in number as the cells enlarge and approach division (Doonan and Grief, 1987). The first sign in a synchronous liquid culture that *Chlamydomonas* cells are about to divide is the gradual shortening of flagella which occurs by disassembly from the flagellar tips (Cavalier-Smith, 1974; Fig. 2d). However, if the pH of the medium has dropped below 6 or cells become palmelloid on solid medium, flagella may not be present (Harris, 1989). The first S phase (Fig. 2, S_1) may occur at the same time as flagellar withdrawal. The G_2 period is believed to be relatively short (Jones, 1970) and it is not represented in Fig. 2. In preprophase, the NBBC contracts, pulling the nucleus close to the basal body region at the cell anterior (Salisbury *et al.,* 1988).

The beginning of mitosis is marked by a condensation of the chromosomes in prophase and an increase in labeling of nuclear nonhistone phosphoproteins with MPM-2 antibody (Harper *et al.,* 1990a). At this time, the basal bodies and associated rootlets have duplicated and begun to separate toward the forming spindle poles (Fig. 3C) (Doonan and Grief, 1987; Gaffal, 1988; Gaffal *et al.,* 1993; Salisbury *et al.,* 1988, Harper *et al.,* 1990b; Holmes and Dutcher, 1989). The interphase microtubule cytoskeleton begins to disassemble in prophase (Doonan and Grief, 1987) and is replaced by the metaphase band of microtubules (Johnson and Porter, 1968). The metaphase band is formed by a rearrangement of the four-membered micro-

tubule rootlets, which indicates the site of the future cleavage plane (Gaffal and el-Gammal, 1990; Gaffal *et al.,* 1993).

Importantly, the band persists during mitosis (Doonan and Grief, 1987; Gaffal and el-Gammel, 1990). The nuclear envelope remains fairly intact, except that large pores (polar fenestrae) develop at the mitotic poles and enable the mitotic spindle microtubules to assemble in the nucleus (Johnson and Porter, 1968; Coss, 1974; see Fig. 2e). During mitosis, as in other organisms, the phosphorylation of many proteins occurs. For instance, the anti-phosphoprotein antibody MPM-2 immunolabels the nuclear periphery and mitotic matrix, reaching a peak at the anaphase/metaphase boundary and decreasing rapidly during telophase (Harper *et al.,* 1990a). After nuclear division, a cleavage furrow develops from the anterior of the cell and progresses circumferentially between the separating daughter nuclei. Cleavage microtubules (the phycoplast or radicoplast; Segaar *et al.,* 1989) lie along the path of the cleavage furrow (Johnson and Porter, 1968; Harper and John, 1986; Doonan and Grief, 1987). Internuclear microtubules previously described between daughter nuclei at right angles to the spindle axis (Johnson and Porter, 1968) are now thought to be part of the phycoplast (Segaar *et al.,* 1989). As the cleavage furrow progresses toward the posterior of the dividing cell, it appears to bisect the chloroplast and pyrenoid, although the chloroplast was seen to constrict before the cleavage furrow reached it (Goodenough, 1970; Fig. 2g). Subsequent multiple fission divisions follow a similar pattern, but there are notable variations in the timing of some events (see Section II,C,1).

The relative timing of division events in *Chlamydomonas* can be estimated by direct observation on living cells (Cavalier-Smith, 1974; Gaffal, 1987). The following estimations of the timing of division events have been made: preprophase, ~3 min; prophase, ~3 min; metaphase, 5–10 min; anaphase, 1 or 2 min; telophase, 5–10 min; and cytokinesis, 7 min. Cells may immediately enter another S phase between mitoses so there is likely to be overlap (see dotted line in Figs. 2g and 2h) in the initiation of a new S phase and the progress of cytokinesis. The *Chlamydomonas* actin cytoskeleton has also been shown by immunofluorescence to undergo dramatic changes in localization during cell division, indicating that it may have a role in basal body segregation and cleavage furrow progression (Harper *et al.,* 1992).

An interesting observation regarding the multiple fission divisions is that for the first division, flagellar withdrawal takes place and flagellar regrowth does not occur until all divisions have taken place. Also, cell walls are not produced until all divisions have ceased. The mature mother cell basal bodies are at least one cell cycle old, whereas the newly mature basal bodies in the remaining daughter cells will be much younger (Gaffal, 1988; Gaffal *et al.,* 1993). Consequently, when a cell divides into four [with each cell

getting a parental and a daughter basal body (Gaffal, 1988)], two daughters will have a mature basal body, which was formed in the previous cell cycle (Beech *et al.,* 1991; Gaffal, 1988).

D. Inhibitor Studies

The changing of the order of cell cycle events, such as chloroplast and nuclear division, depending on the prevailing growth conditions or during multiple fission divisions provides some clues that these events are arranged in separate dependent sequences (Hartwell, 1978; Harper and John, 1986). Using the DNA inhibitors 2′-deoxyadenosine and hydroxyurea, Harper and John (1986) showed that if DNA synthesis is blocked, mitosis does not occur. However, cytokinesis occurred independently of nuclear division because a cleavage furrow was seen to progress until obstructed by the undivided nucleus. Interestingly, some cells attempted a second cleavage furrow at right angles to the first. In inhibited cells, it was also observed that new walls were not assembled around daughter cells which did not complete cytokinesis. This is an indication that the signal to initiate new wall growth is dependent on the completion of cytokinesis.

The previous observations of wild-type *Chlamydomonas* cells provide some insights into how the cell cycle of *Chlamydomonas* is controlled and coordinated. The identification and description of cytoskeletal components during the *Chlamydomonas* cell cycle provides additional background for mutant characterization. In order to determine the molecular mechanisms orchestrating these division events it is essential to generate mutants.

III. Mutant Studies

A. Production of Cell Cycle Mutants

As mentioned previously, I will describe two kinds of mutants: conditional and constitutive (see Sections I,B,1 and I,B,2). In all these mutant studies there were a number of procedures in place which allowed the induction, selection, and characterization of the mutations of interest. Ultraviolet radiation is a commonly used mutagen which has been used by many workers for mutagenesis of *Chlamydomonas* (Lewin, 1952; Howell and Naliboff, 1973). Chemical mutagens, such as nitrosoguanidine (Adams *et al.,* 1982) and ethylmethane sulfonate (EMS; Loppes, 1968; Harper *et al.,* 1995b), have also commonly been employed. Insertional mutagenesis causing gene disruption is increasingly being used to produce mutations in

nonessential genes (Tam and Lefebvre, 1995; see Section IV,C). The power of insertional mutagenesis stems from the development of techniques to efficiently transform *Chlamydomonas* with selectable marker genes that can integrate randomly into the nuclear genome. The marker genes cause gene disruption and enable the subsequent cloning of those disrupted genes (Tam and Lefebvre, 1995). One such gene is the *Chlamydomonas* gene for nitrate reductase, *NIT1* (Fernandez *et al.,* 1989; Kindle *et al.,* 1989). For example, the *C. reinhardtii* strain cc-125 cannot grow on nitrate as a sole nitrogen source (Harris, 1989), but transformation of cells with these genes enables selection on nitrate medium. Integration of the nitrate reductase gene disrupts functional genes which can be found by suitable screens. These genes can then be located using the reductase gene as a marker (Tam and Lefebvre, 1995). Importantly, conditional mutations in essential genes usually result from single base pair substitutions (Pringle, 1981) and not by gene disruption. Therefore, insertional mutagenesis will not produce these mutations. However, insertional mutagenesis has produced some exciting mutants defective in cytokinesis (see Section III,C,3).

I shall describe the procedures that our group has employed in the isolation of conditional cell cycle mutants as a general guide to some of the procedures involved. For specific mutants, the reader can refer to the cited works.

1. Induction

In our studies we choose EMS for the isolation of conditional cell cycle mutants (Harper *et al.,* 1995b; Sakuanrungsirikul *et al.,* 1996; Wu *et al.,* 1997) because it is a mild mutagen which causes a high proportion of base pair substitutions in DNA (Loppes, 1968). A brief outline of the procedure is described (Fig. 4). Sterile synchronized cells, growing photoautotrophically in light/dark batch culture (Rollins *et al.,* 1983), were transferred into subcultures, containing varying amounts of EMS, at 12 or 13 h into the light cycle. This is the time when cells were estimated to be undergoing DNA synthesis. Cultures were shaken for 1 h and cells were washed several times in fresh medium by centrifugation to remove residual EMS. Cells were then transferred to the light to allow completion of divisions in progress. In preliminary experiments, cells were plated onto complete agar growth medium (Harper *et al.,* 1995b) at different densities and compared to control cells to establish the percentage survival after mutagenesis. Cells were grown in continuous light at 21°C, which was chosen as the permissive temperature. A temperature of 33°C was chosen as the restrictive temperature (see Section I,B,1). These temperatures were chosen, after Howell and Naliboff (1973), because wild-type cells could grow and divide at both temperatures and the difference between them was high enough that tem-

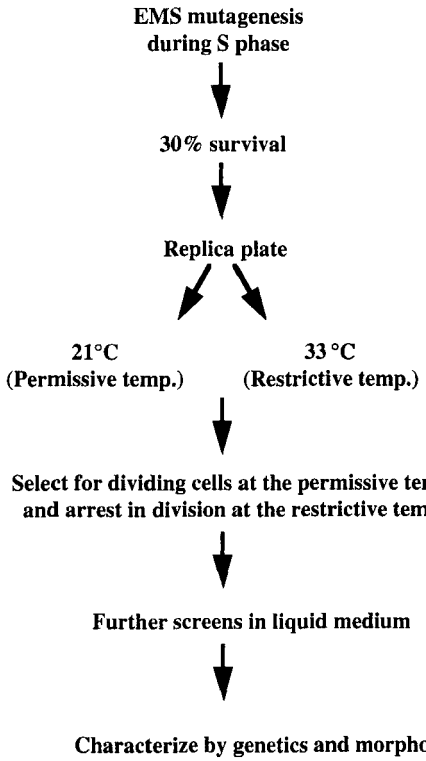


FIG. 4 A flow diagram showing a simple method for the production of conditional temperature-sensitive cell cycle mutants. Cells are mutagenized during S phase using ethylmethane sulfate (EMS) to produce 30% survival. Cells at 100–200 per plate are replica plated and the plates are grown at 21 and 33°C in the light (Harper *et al.*, 1995b). Cells are first screened microscopically on agar plates and later in liquid media.

perature-conditional mutations would be detected. It was established that under the conditions used, 115-mM EMS produced 30% survival, which gave the highest yield of mutations.

2. Selection

After 5 days, when colonies had grown, plates containing between 100 and 200 cells were chosen for replica plating by standard techniques (Harris, 1989) using sterile velveteen. Replica plates were grown under continuous light at 21°C (permissive temperature) and 33°C (restrictive temperature) (Fig. 4). Cells were selected after an additional 5 days growth initially for those that could form colonies at 21°C and not at 33°C. Selected cells were

further screened by replica plating using sterile toothpicks to transfer cells onto agar plates in a grid pattern. These cells were again transferred to the permissive and restrictive temperatures and this time cells were microscopically chosen which enlarged in size at the restrictive temperature (i.e., grew) but could not divide. Further screens involved growing cells in liquid medium (Harper *et al.*, 1995b).

3. Characterization

a. Mendelian Genetic Analysis An important part of the process of characterizing cell cycle mutants is to determine if the mutation is in a single nuclear gene. This is easily done using established tetrad analysis protocols (Harris, 1989). In addition, several back crosses to wild type eliminates secondary mutations which may produce slow growth of cells and interfere with characterization of the putative cell cycle gene (Harper *et al.*, 1995b). Defining the linkage group and position of the gene on the chromosome using linkage tester strains is also important (Harris, 1989).

b. Nuclear DNA Quantitation It is useful when characterizing the arrest phenotype of mutants to measure their nuclear DNA content. Our group devised a protocol to measure DNA in large arrested cells because established protocols were unsuitable for these mutants due to interfering organellar DNA (Sakuanrungsirikul, 1991; Harper *et al.*, 1995b). Briefly, cells are decolorized in a graded ethanol series, resuspended in Tris buffer, and pretreated with RNAse to prevent subsequent reactivity of RNA with propidium iodide. Cells are then resuspended in 50% ethanol and spread on coverslips previously coated with methanol-fixed erythrocytes from 20-day-old chickens, which act as an internal staining control for DNA quantitation. Nuclei are stained with propidium iodide and coverslips washed to remove excess dye, mounted on a slide, and viewed with a confocal microscope. The fluoresence intensities of nuclei from optical sections are summed and calibrated against early G_1 cells (Sakuanrungsirikul, 1991; Harper *et al.*, 1995b).

c. Other Methods There are numerous other methods which can be used to further characterize cell cycle mutants, including immunofluorecence microscopy of cellular components such as microtubules and centrin (Harper *et al.*, 1995b; see Section III,B,4), biochemical assays to measure cell cycle enzyme levels (Wu *et al.*, 1997), temperature-shift experiments to establish the time of cell cycle gene function (Howell and Naliboff, 1973; Howell, 1974; Wu *et al.*, 1997; see Section III,B,6), electron microscopy, induction of suppressor mutations, and gene cloning.

B. Conditional Temperature-Sensitive Mutants

1. TSD Mutants

Wolff (1971) described the development of techniques to produce the first documented conditional cell cycle mutants in *Chlamydomonas.* He called them TSD mutants for *t*emperature-*s*ensitive *d*ivision. Induction of the mutants involved mutagenesis using ultraviolet radiation of synchronized early G_1 liquid cultures (3 or 4 h into the light period) grown at 25°C. The temperatures of 25 and 33°C were chosen as the permissive and restrictive temperatures, respectively. After irradiation cells were left in the dark for 24 h to prevent repair of the DNA damage by photoreactivation. A further selection procedure was then employed by adding the DNA inhibitor hydroxyurea to cultures for 24 h at 33°C to enrich for mutations which were blocked in division processes. It was presumed that cells able to attempt DNA synthesis would be killed by the hydroxyurea. The procedure may have selected for cells blocked in division or growth. Cells were then plated onto agar medium and grown for 5 days at 33°C. There were 5642 colonies which grew on the plates, and their positions on the plates were marked because they were considered not to be putative TSD mutants since they were able to divide at 33°C. The plates were then transferred to 25°C for 5 days. From these plates 116 new colonies grew. Four of these mutants were isolated as putative TSD mutants and characterized by light microscopy as well as growth in microdroplets. The 4 mutants were designated TSLt-1, TSLt-2, TSL, and TSLG. The "Lt" designation indicates an apparent effect of light on the survival of these 2 mutants. The "L" indicates that mutants were killed by the restrictive temperature, whereas the "G" indicates that the mutant produced giant cells. I believe that the screening may have selected against other potential TSD mutants because the plates were left at 33°C for 5 days during the enrichment procedure which may have killed many potential mutants.

2. Cb Mutants

Howell and Naliboff (1973) isolated temperature-sensitive *c*ycle-*b*locked (Cb) mutants from ultraviolet irradiated cells. These mutants were isolated using a similar selection procedure as the one outlined in Section III,A,2). These mutants were described as including, but being more general than, the class of cell cycle mutants described in yeasts. For instance, some may be defective in some aspect of growth. Two types of Cb mutant were described. Both could grow and divide essentially normally at the permissive temperature (21°C) but one set completed no more than one round of cell divisions at the restrictive temperature (33°C). That is, cells produced eight

or less daughter cells at 33°C. The others stopped dividing after an additional round of cell division on shift-up to 33°C. Like the cell division cycle (CDC) mutants described in yeast (Forsburg and Nurse, 1991; Hartwell, 1978, 1991), many of these cells continued to grow on arrest at the restrictive temperature (Fig. 5). Although Howell and Naliboff (1973) mention "parental" plus and minus strains, they do not indicate whether the mutants described were backcrossed or shown to be defective in single nuclear genes. There were 39 mutants isolated (and currently stored in the *Chlamydomonas* culture collection; Harris, 1989). Thirteen were described in three publications (Howell and Naliboff, 1973; Howell, 1974; Howell *et al.,* 1977). Some notable features will be described later. Howell and Naliboff (1973) characterized Cb mutants by light microscopy and their "block point." This is defined as the time in the cell cycle when the temperature conditional gene product completes its function. This time is also called the execution point in yeast (Hartwell, 1971) or the transition point in mammalian cells (Mitchison, 1971; Howell, 1974). In budding yeasts cell cycle stage can easily be determined by the size of the bud (Hartwell, 1978, 1991). However,

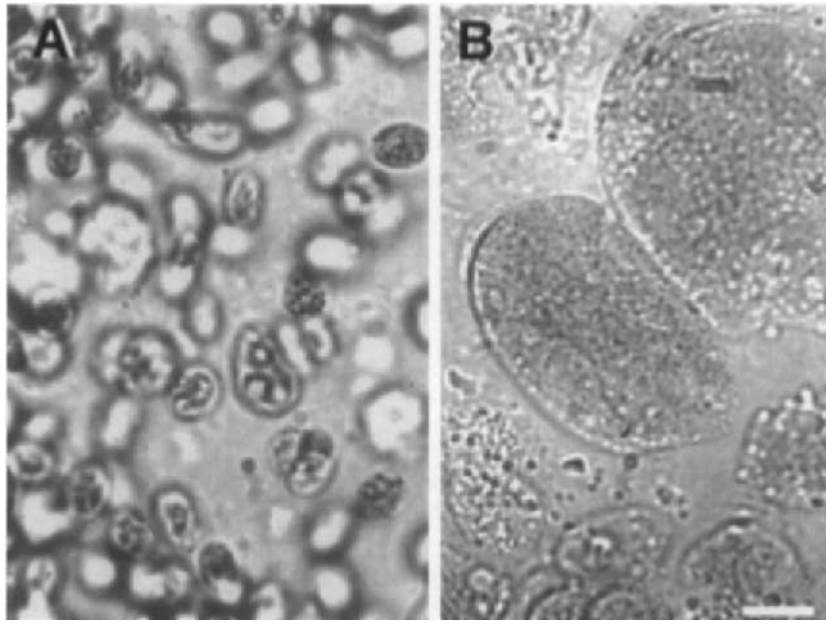


FIG. 5 ts10004 cells (Howell and Naliboff, 1973) on agar plates at the permissive temperature (21°C) divide normally (A). On shift to the restrictive temperature (33°C) cells continued to grow in size (B). Cells shown here (in B) were viewed after 3 days growth at 33°C. Note the large cells and lysed cell debris. Scale Bar = 10 μm.

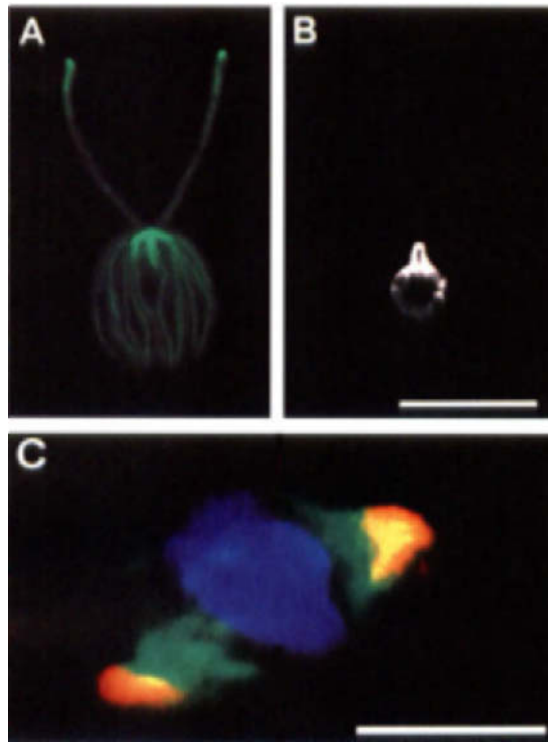
determination of execution or block points in *Chlamydomonas* was more indirect and involved shifting asynchronous mutant cultures up to the restrictive temperature and using the amount of residual cell division after the shift-up to calculate the block point. Some block points were confirmed by shift-up of synchronous cultures at different times. Despite questioning of the accuracy of block point measurements (Harris, 1989), they generally indicate that different gene products complete their functions at unique times during the cell cycle. Howell (1974) reviews the block point analysis and describes results from one mutant, ts10009, which highlight the interdependence of cell cycle events. It was shown that the stepwise increase in two autoregulated enzymes, glutamate dehydrogenase and aspartate transcarbamylase, was not dependent on the completion of the mutated gene product, whereas DNA synthesis and cell separation are dependent on the completion of function of the wild-type ts10009 gene product. Further studies (Howell *et al.*, 1977) using 1-D gel electrophoresis and tritiated arginine labeling of proteins of another mutant, ts10001, showed that the cell cycle program of polypeptide labeling continued largely intact for more than one cell cycle despite cells being arrested in cell division by temperature shift-up. Like the yeast cell cycle mutants, the Cb mutants did not show full reversal of inhibition of cell division when returned to the permissive temperature after exposure to the restrictive temperature (Howell and Naliboff, 1973). In some preliminary investigations in the early 1980s (J. Harper and P. John, unpublished results), we chose two of Howell's mutants (ts10004 and ts10021) for study because they showed the best colony-forming ability on return to the permissive temperature after exposure to the restrictive temperature (Howell and Naliboff, 1973). We believed that after 10 years these mutants may have been the most robust and most likely to have retained their original arrest characteristics. On agar plates, ts10004 became extremely large at the restrictive temperature (Fig. 5), whereas ts10021 appeared to be blocked in growth in our hands and arrested as small single cells. On shift-up of synchronous liquid cultures of these two mutants, ts10021 arrested as small single cells. However, contrary to its arrest on agar, ts10004 arrested as fully divided daughter cells which did not release from the mother cell envelope. That the daughter cells were fully divided was further established by processing cells for electron microscopy and examining both thin conventional electron microscopy sections and thick sections stained with toluidine blue. Cell organelles appeared as for a normal cell and flagella were seen in the unreleased cells. Continued incubation of ts10004 at the restrictive temperature for more than 24 h resulted in the clumping of cells and they came out of suspension. This may have been due to the mother cell envelopes becoming sticky.

At the time, these arrest characteristics seemed at variance with what had been described in the original paper (Howell and Naliboff, 1973) and

we presumed that the vegetative culture of the mutants by mass transfer over a period of 10 years may have resulted in some changes in arrest characteristics. Our own culturing media and conditions may also have caused subtle changes in the arrest phenotypes of these mutants. We therefore decided to begin our own study of cell cycle mutants by generating our own (see Section III,B,4). However, with the benefit of experience in isolating and characterizing *Chlamydomonas* cell cycle mutants, I returned to Howell's mutant collection 10 years later. The collection was kindly sent to me by Dr. Elizabeth H. Harris at the *Chlamydomonas* Genetics Center, Duke University (Durham, NC). The center has a web site (*http://www.botany.duke.edu/DCMB/chlamy.htm*) and is a valuable resource for *Chlamydomonas* biologists. I investigated all 39 Howell mutants for arrest at the restrictive temperature on agar plates and specifically looked for cells which would increase in size (i.e., continue to grow) on arrest. To my surprise, one-third arrested as large cells. Some other cells arrested as small cells, and these were probably defective in growth, which may be how they arrested originally. Because I was not able to further characterize these mutants at that time, I sent the information to the Genetics Center. I understand that some workers have shown renewed interest in Howell's mutants and have requested them. I feel confident that characterization of these using, for instance, DNA quantitation, immunochemistry, and cloning will be very fruitful. However, an important step is to establish whether the defects are in single genes.

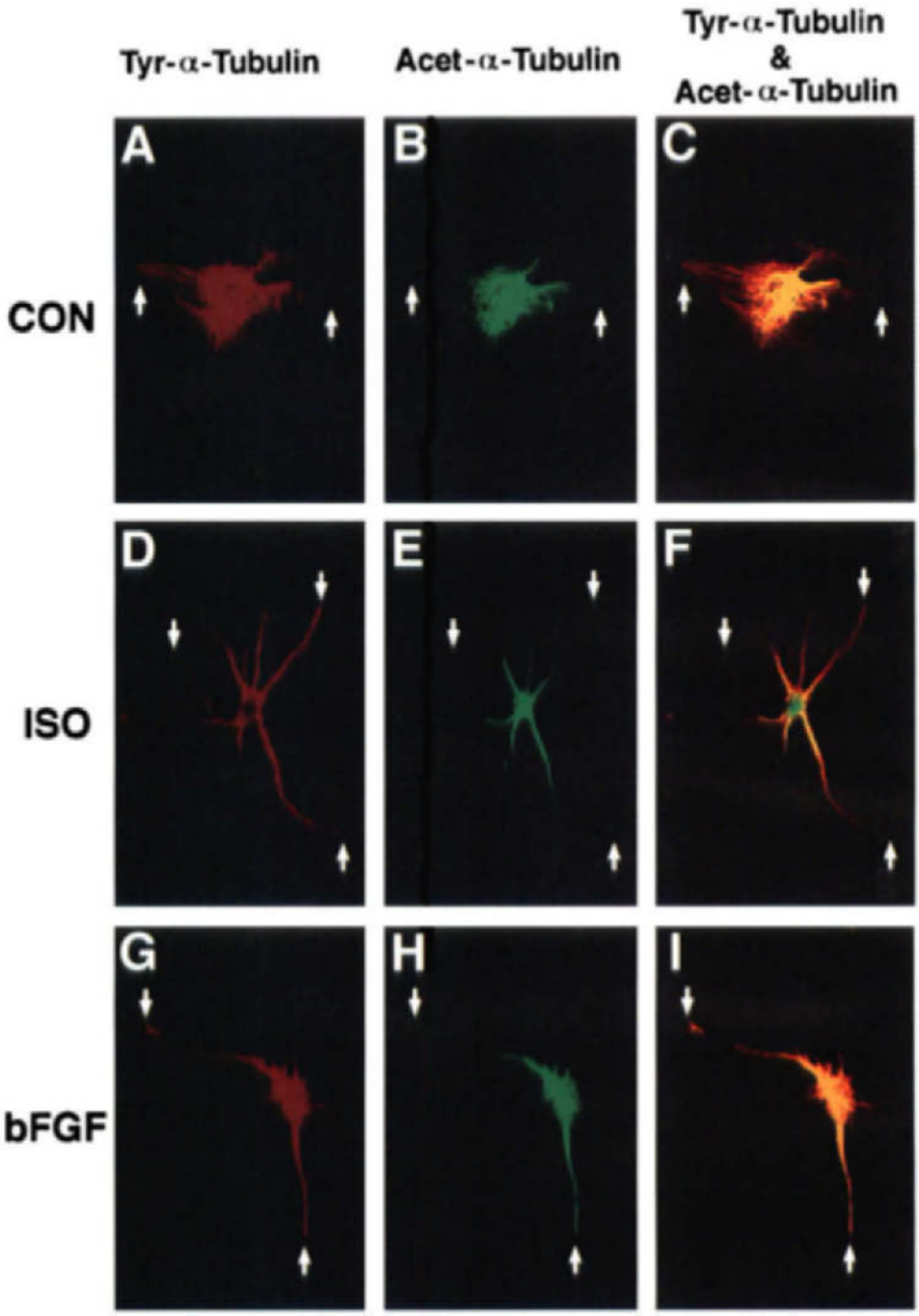
3. Colchicine-Resistant Mutant TS-60

Sato (1976) isolated four temperature-conditional cell cycle mutants from *C. reinhardtii* that could divide at 23°C but not at 34°C. Three of these—TS-1, TS-6, and TS-72;md;were isolated using an enrichment procedure similar to that described by Wolff (1971; see Section III,B,1) except that, after UV mutagenesis, cells were exposed to 5-bromouracil for 48 h. Cells undergoing DNA synthesis at the restrictive temperature incorporated 5-bromouracil into their DNA and were subsequently killed by exposure to light. Mutants exhibited "tight" arrest, stopping division within one cell cycle on temperature shift-up. The mutant TS-60, however, was isolated without 5-bromouracil selection and could not divide at 34 or 15°C. At 34°C, 5-bromouracil killed TS-60, indicating that cells incorporated it into their DNA under division arrest conditions. TS-60 cells also continued to increase in size, indicating that they were blocked in division and not growth. Intriguingly, TS-60 cells were also shown to be resistant to the microtubule depolymerizing drug colchicine at a concentration which inhibited cell division in wild-type cells. Both thermolability and colchicine resistance were shown by Mendelian genetics and reversion analysis to be caused



CH. 4, FIG. 3 Immunofluorescence images of *Chlamydomonas* during interphase (A, B) and metaphase (C); labeled with anti-β-tubulin (A, C) and anticentrin antibodies (B). Microtubules (A) emanate from the flagellar basal apparatus and are a major component of the flagellar axonemes. Microtubules also form the cruciate rootlets which lie just below the plasma membrane and extend toward the posterior of the cell. (B) The centrin-based nucleus–basal body connector (NBBC) extends as two fibers from the base of the basal bodies and bifurgates over the nucleus to form finger-like projections called fimbria (Salisbury *et al.*, 1988). Centrin also labels the striated distal fiber connecting the two basal bodies. (C) A metaphase cell triple labeled to show microtubules in the spindle (green), centrin at the spindle poles (orange/yellow), and metaphase chromosomes (blue). Scale bars: A and B (in B) = 10 μm; C = 2 μm (Fig. 3B reproduced from *The Journal of Cell Biology*, 1988, **107**, pp. 635–641, by copyright permission of The Rockefeller University Press).

CH. 5, FIG. 9 ISO and bFGF induce different distributions of tyrosinated and acetylated α-tubulin in the AS583-8 cell line. Confocal images were generated of AS583-8 cells double labeled for tyr-α-tubulin using a TR-conjugated secondary antibody (red) and acet-α-tubulin using a FITC-conjugated secondary antibody (green) and images merged to compare distributions (C, F, and I). (A–C) Untreated cells exhibit tyr-α-tubulin radiating out from the perinuclear region and extending to the periphery of the cell (A), whereas acet-α-tubulin is localized to the perinuclear region and does not extend to the periphery of the cell (B, arrows delineate cell periphery). Merged confocal images of untreated cells demonstrate that tyr-α-tubulin is more widely distributed throughout the cell than acet-α-tubulin (C). (D–F) ISO-treated cells exhibit tyr-α-tubulin extending out into the distal processes (D, arrows delineate cell periphery), whereas acet-α-tubulin is more highly concentrated in the cell body and proximal processes (E). Merged confocal images of ISO-treated cells demonstrate the difference in tyr-α-tubulin and acet-α-tubulin (F). (G–I) bFGF-treated cells exhibit both tyr-α-tubulin (G) and acet-α-tubulin (H) in the cell body and extending out into the distal processes. The codistribution of tyr-α-tubulin and acet-α-tubulin is apparent in the merged confocal images (I). Magnification, ×800.


Tyr-α-Tubulin
Acet-α-Tubulin
Tyr-α-Tubulin & Acet-α-Tubulin
CON
ISO
bFGF
A
B
C
D
E
F
G
H
I

by the same nuclear gene. This implies that the cell cycle gene product in some way affected the binding of colchicine to microtubules and cell cycle arrest was caused by some defect of microtubule formation at the restrictive temperature. This assumption could be tested using electron microscopy or immunofluorescence microscopy with tubulin antibodies. It is unknown, however, whether TS-60 or the other mutants are still extant.

4. Cdb Mutants

The Cdb mutants were isolated using the procedures outlined previously (see Section III,A; Harper *et al.*, 1995b). The mutants were so named in acknowledgment of the earlier described Cb mutants (Howell and Naliboff, 1973) and to avoid further confusion with the CDC mutants described in budding and fission yeast (Forsburg and Nurse, 1991). The 11 mutants described were shown to be defective in single nuclear genes. The mutants were backcrossed to wild type at least four times to ensure the cdb genes were in wild-type genetic backgrounds. DNA quantitation (see Section III,A,3,b) revealed that mutants were blocked in different phases of the cell cycle (i.e., G_1, G_2 and cytokinesis). Immunofluorescence microscopy using antibodies to centrin and tubulin showed that the mutants displayed complex terminal phenotypes (see Section I,B,1). For instance, despite being arrested in either G_1 or G_2, the mutants displayed contracted, uncontracted, or partially contracted NBBCs (Figs. 6A–C). Contraction of the NBBCs is believed to occur in preprophase (Salisbury *et al.*, 1998; J. Harper, personal observations; see Section II,C,2), pulling the nucleus closer to the flagellar–basal apparatus in preparation for the ensuing division. The fact that the NBBC can contract even though cells remain in G_1 is circumstantial evidence that DNA replication is not a requirement for NBBC contraction. Also, the microtubule cytoskeletons of arrested mutants show a number of different configurations (Figs. 6D–F). For instance, in the cdb2 mutant (Fig. 6E), which arrests in G_1, an array of microtubules is seen to emanate from the flagellar–basal region but only extends partially into the large cell. Presumably, the growth of microtubules in this arrested cell cannot keep pace with the continued growth of the cell. Other mutants which maintained interphase-like microtubule arrays on arrest at the restrictive temperature appeared to have more highly branched arrays than those seen in dividing cells (e.g., cdb8; Fig. 6D).

Of the seven G_1 arresting mutants, three (cdb4–6) had a propensity to form multinucleate cells at the permissive temperature. This was assumed to be due to the partial dysfunctioning of the mutated cell cycle gene at the permissive temperature. Another G_1 arresting mutant, cdb7, attempted cytokinesis if cells were binucleate when shifted to the restrictive temperature. One mutant (cdb11) arrested with multiple nuclei at the restrictive

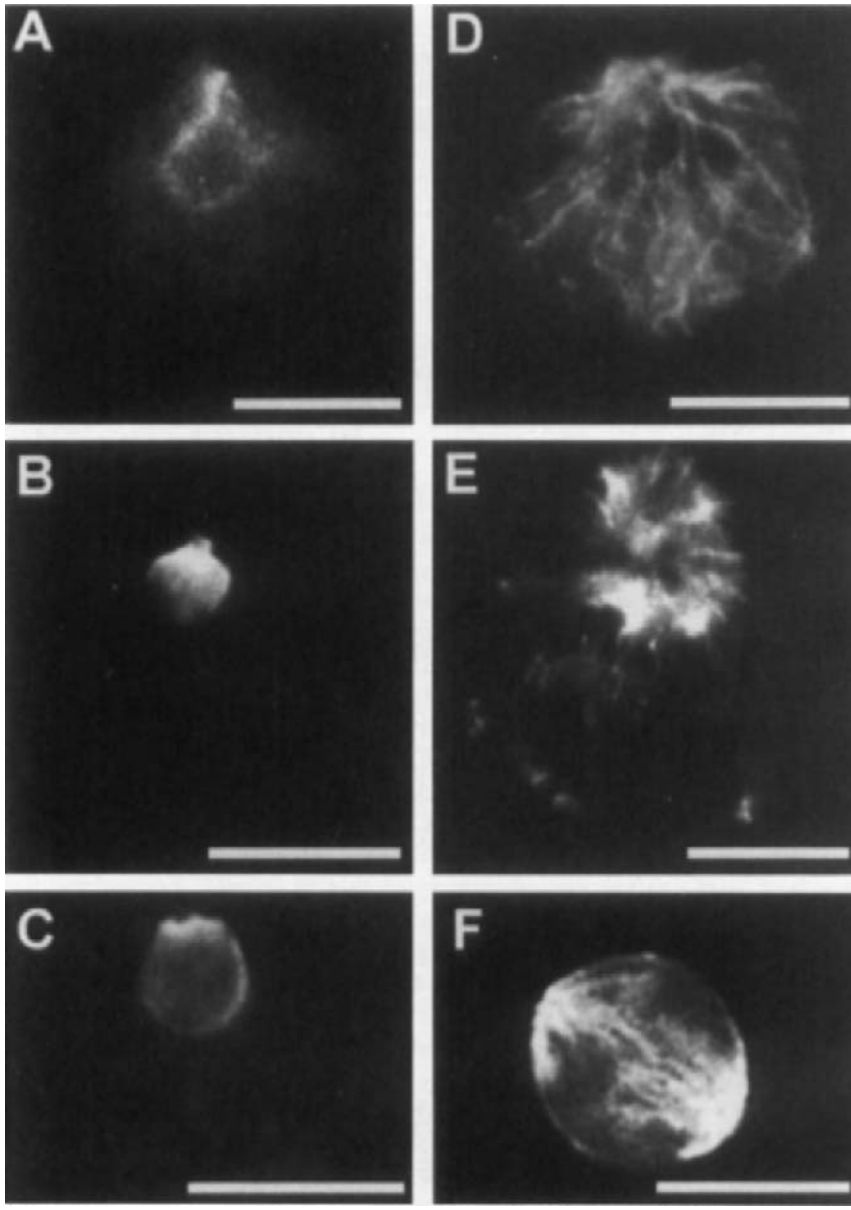


FIG. 6 Centrin (A–C) and microtubule (D–F) immunolocalization in different cdb mutants arrested at the restrictive temperature (33°C) for 24 h. A, cdb7 arrests in G_1 with an extended NBBC; B, cdb4 arrests in G_1 with a partially contracted NBBC; C, cdb5 arrests in G_1 with a contracted NBBC; D, cdb8 arrests in G_2 with an extensive interphase-like microtubule array; E, cdb2 arrests in G_1 with only a partially extended microtuble array; F, cdb11 cells blocked in cytokinesis arrest with multiple nuclei and aberrant phycoplasts. Scale Bars = 10 μm (reproduced with permission from Harper *et al.*, 1995b).

temperature and, although phycoplasts appeared to form, no cleavage furrows developed (Fig. 6F). Of the three G_2 arresting mutants, only cdb9 showed an increase in nuclear reactivity with the anti-phosphoprotein antibody MPM-2, indicating that it was arrested in late G_2 (Harper *et al.,* 1995b). MPM-2 has been shown to recognize nuclear phosphoproteins in mitotic *Chlamydomonas* cells (Harper *et al.,* 1990a).

The cdb8 mutant, which arrests in G_2 with an interphase-like array of microtubules and extended NBBCs, has been further characterized by immunofluorescence and electron microscopy. It was observed that incubation of cdb8 cells at the restrictive temperature for between 24 and 48 h resulted in the loss of the centrin-based nucleus connection (Fig. 7B). Electron microscopy revealed that the nucleus still had an undispersed nucleolus and the basal bodies appeared to be unduplicated (J. Harper, unpublished observations). The centrin-containing striated fibers between the basal bodies also appeared to have broken down. Incubation of cdb8 cells at the restrictive temperature under nongrowing conditions (in the dark in minimal medium) revealed that the NBBC remained intact. This suggests that the loss of the connector was not due to some general thermolability of the structure but rather occurred as cells continued to grow in the absence of cell division. Further characterization of these mutants using other biochemical assays and temperature-shift experiments with synchronous cultures is in progress. Gene mapping and cloning of the defective genes will also be valuable.

5. cAMP-Requiring Mutants

cAMP is a second messenger or signaling molecule. It was first discovered in animal cell systems, in which it is involved in hormone secretion (Sutherland, 1972), but has since been found in a wide variety of prokaryotes and eukaryotes. Signaling of cAMP is mediated by its activation of cAMP-dependent protein kinases and transcription (Katagiri *et al.,* 1989). cAMP is rapidly synthesized by adenylate cyclase and degraded by phosphodiesterase. Phosphodiesterase is in turn activated by calmodulin, which is activated when it binds calcium (Cohen and Klee, 1988).

Chlamydomonas has been shown to utilize cAMP as a signaling molecule during mating (Pasquale and Goodenough, 1987; Kooijman *et al.,* 1990) and an increase in cAMP accompanies flagellar withdrawal during the cell cycle (Jayaswal, 1991). The role of cAMP in cell division, however, is unclear. In a number of organisms, cAMP has been implicated in cell cycle progression. For example, in budding yeast, mutations which affect the activity of cAMP-dependent protein kinase or adenylate cyclase block cells in G_1 (Matsumoto *et al.,* 1982; 1983; Ishikawa *et al.,* 1985). However, Nurse (1985) questioned whether these mutations cause G_1 arrest in yeast because

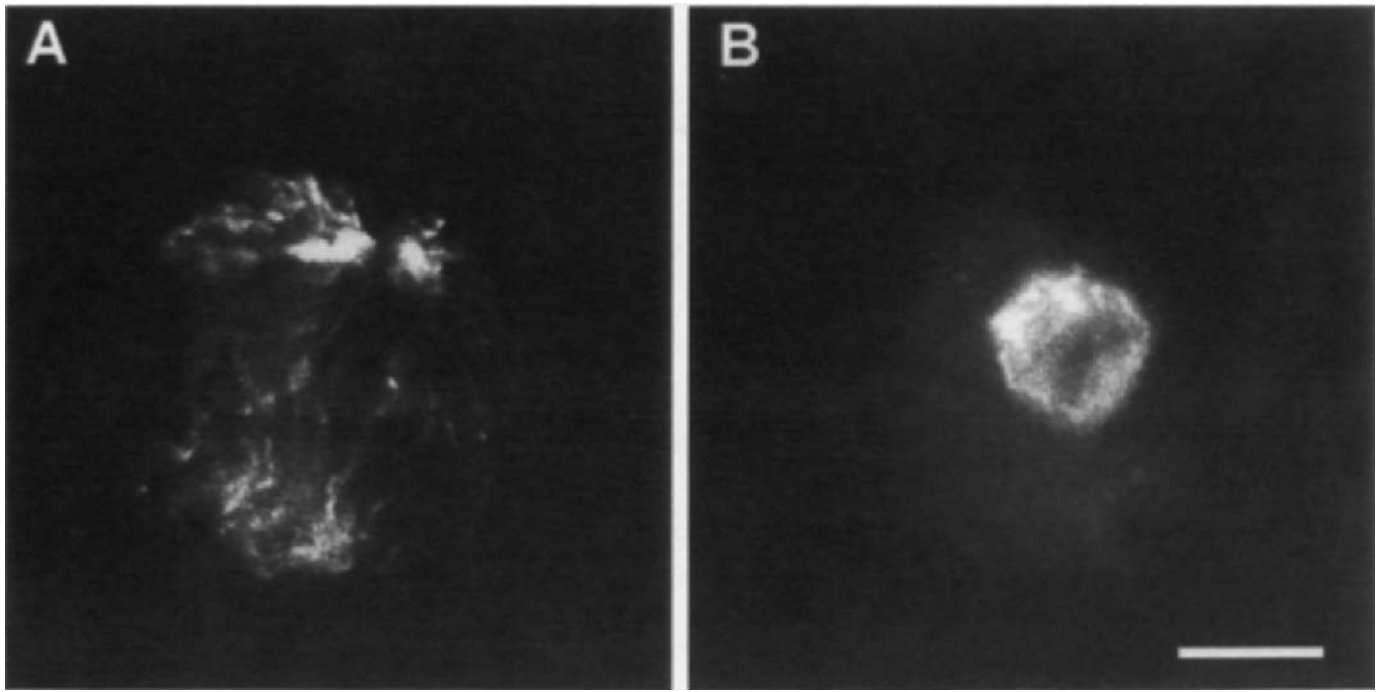


FIG. 7 cdb8 cells double labeled for microtubules (A) and centrin (B). Cells first arrest in G_1 with an extended NBBC (Harper *et al.*, 1995b). However, after prolonged incubation for 48 h at the restrictive temperature (33°C), cells lose the centrin-based NBBC (B). Anterior of cells is at the top of the photograph. Scale Bar = 10 μm.

cells are blocked in growth and fail to attain the necessary cell size for progression to START. *Chlamydomonas* cells blocked in cell cycle progress continue to grow. This growth in cell size in an organism which more than doubles its mass in a cell cycle (see Section II,B,1) may be more noticeable than that in yeast. Mutants defective in cAMP regulation which are blocked in division but not growth would be easier to screen because cells continuing to grow would become noticeably larger. Sakuanrungsirikul *et al.* (1996) isolated three temperature-conditional cAMP-requiring mutants in *Chlamydomonas* (M1–M3) which arrested at the restrictive temperature in G_1 but could be rescued for division by adding cAMP to the culture medium. The mutants were isolated using the EMS mutagenesis strategy described in Section III,A. In addition, a positive selection for cAMP mutants was used whereby the mutagenized cells were screened, on agar plates, for resistance to a known phosphodiesterase inhibitor (caffeine; Sutherland, 1972). This was followed by replica plating of the resistant cells at the permissive (21°C) and restrictive (33°C) temperatures to select temperature-conditional mutations blocked in cell division. Cells which arrested in cell division but continued to grow at 33°C were then shifted to the restrictive temperature in liquid medium in the presence of cAMP or an active analog, dibutyryl-cAMP; cells able to divide in the presence of these compounds were selected. Control mutant cultures were treated with the cAMP breakdown product, 5′-AMP, or butyrate and shifted to 33°C. These showed no rescue at 33°C. Also, wild-type cultures were not affected by added cAMP.

DNA measurements of the three mutants showed them to be arrested in G_1. Immunofluorescence labeling of microtubules and centrin indicated that cells were arrested with an interphase-like configuration of both cytoskeletal components (Fig. 3). Blocking of cell division in all three mutants was subsequently correlated with both low adenylate cyclase activity and low endogenous cAMP levels. Genetic analysis showed that the mutants were each defective in three nuclear genes which confounded their further biochemical and genetic dissection. Nevertheless, the positive screen for cAMP-defective mutants makes further analysis possible by the isolation of new mutant strains. It was concluded that because no single mutations were obtained which rendered cells cAMP dependent, division may need to be modified by mutation before becoming cAMP dependent. Another alternative is that conditional mutations may be difficult to obtain in proteins responsible for cAMP synthesis. This area deserves further study.

6. *Met1*

Met1 was isolated using the mutagenesis procedure described in Section III,A. Mutagenized cells were first screened for arrest in division at the

restrictive temperature (33°C) on agar plates. Cells were further screened in liquid medium and nuclear DNA was labeled with propidium iodide to find cells arrested in mitosis. The *met1* mutant was found to have arrested with chromosomes aligned at the metaphase plate. Preliminary characterization using anti-tubulin antibody showed that all cells had intact spindles (Fig. 8). Antibodies against the major rate-limiting cyclin-dependent kinase (CDK) or $p34^{cdc2}$-like kinase (John *et al.*, 1989) indicated that the levels of CDK were elevated in arrested *met1* cells (John and Wu, 1992). Further characterization revealed that the defect was in a single gene and mapping of *met1* extended the known limits of linkage group XIV. The high CDK levels were confirmed and it was further shown that *met1* cells arrest synchronously in metaphase with 2C levels of DNA and high levels of MPM-2 recognized phosphoprotein (Harper *et al.*, 1990a, 1993) and duplicated basal bodies (Wu *et al.*, 1997). Surprisingly, the mitotic spindles also became labeled with anti-acetylated α-tubulin antibody, which is usually associated with stable microtubule arrays such as the flagellar axonemes and roots (Le Dizet and Piperno, 1986). The number of spindles showing acetylation increased with time at the restrictive temperature, but this was thought not to be the primary cause of arrest. Evidence in support of this derives from two colchicine-resistant *Chlamydomonas* tubulin mutants (col^R 14 and col^R 15) which show acetylation of cortical and mitotic spindle microtubules but are able to divide normally (Schibler and Huang, 1991). The acetylation of the persistent *met1* spindles is explained by the model of Wilson and Forer (1989) in which tubulin subunits added at the kinetochore become acetylated with time. Centrin immunolabeling in arrested *met1* cells (J. Harper, unpublished observations) has revealed a spindle-like centrin labeling similar to that observed during metaphase in wild-type cells (Salisbury *et al.*, 1988) but possibly more extensive. It would be of interest to determine whether centrin is phosphorylated in arrested *met1* cells. In arrested *met1* cells partial cleavage furrows were also seen, indicating some progress toward cytokinesis despite arrest in metaphase. That cleavage furrows develop despite arrest in metaphase correlates with DNA inhibitor studies (Harper and John, 1986), in which cells attempted cytokinesis even though they were blocked in nuclear division, and supports the conclusion that progress to cytokinesis occurs in a sequence of events independent of the DNA division sequence (see Section II,C).

Evidence that the CDK from *met1* was not defective was provided by its purification and microinjection into *Tradescantia* stamen hair cells. The CDK was shown to both disassemble the preprophase band array of microtubules and accelerate progress to division (Hush *et al.*, 1996; Wu *et al.*, 1997). The time period during which the *met1* gene product performed its function was investigated by temperature-shift experiments in synchronous culture (Wu *et al.*, 1997). The execution point has been previously defined

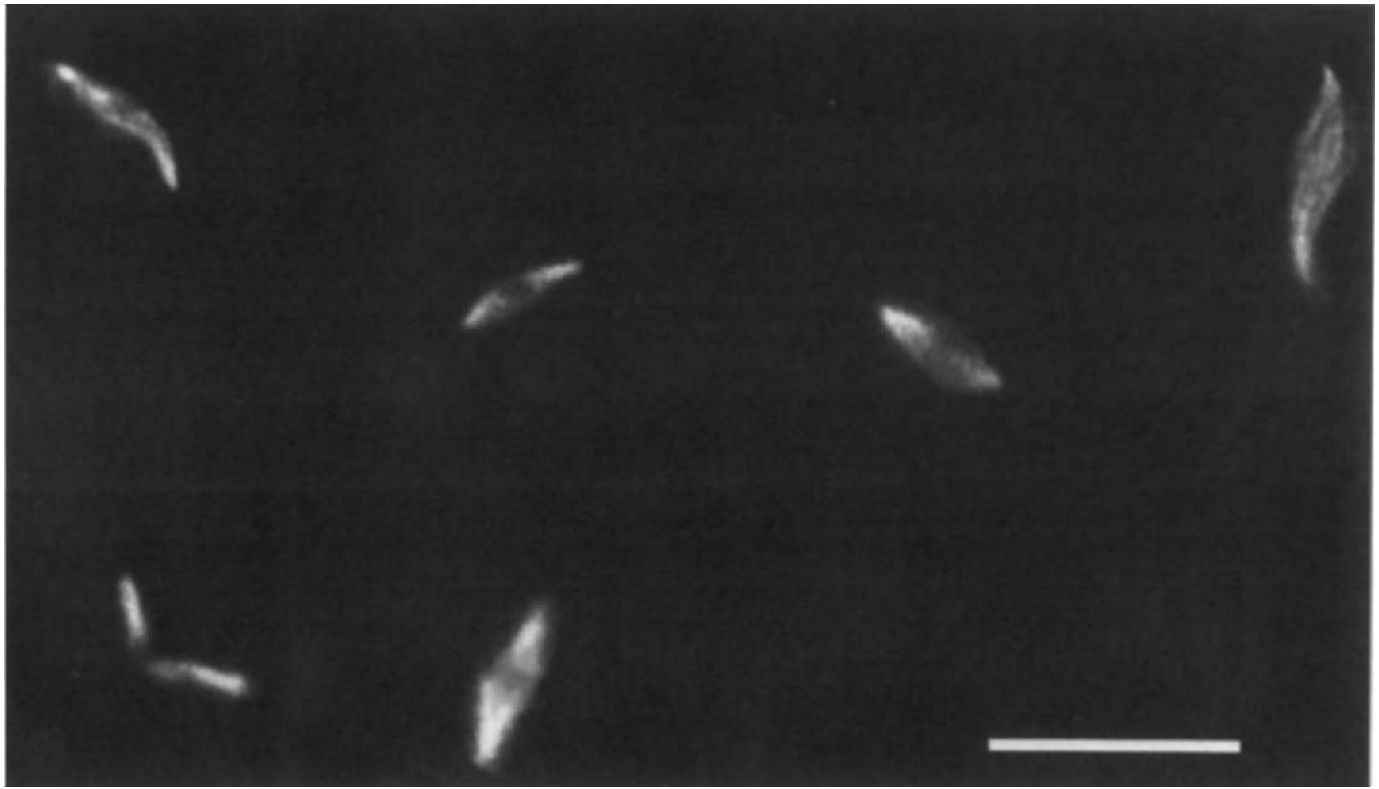


FIG. 8 *Met1* cells arrested at the restrictive temperature (33°C) during mitotic metaphase. Synchronous cells were shifted to 33°C at the beginning of the cell cycle and sampled after 17 h. Cells were processed for immunofluorescence labeling as described by Wu *et al.*, (1997) and labeled with anti-β-tubulin antibody. Note the dark region between the half spindles which is the position of the metaphase chromosomes. Scale bar = 20 μm.

as the time after which a shift up to restrictive temperature will no longer arrest cells in division. This is similar to the block point, which was defined by Howell and Naliboff (1973) for asynchronous cells (see Section III,B,2). The execution point is therefore a measure of when the defective gene product completes its function. A mean value of 17 h was obtained for the synchronous culture. Wu *et al.* (1997) also shifted synchronous cells back down from the restrictive to the permissive temperature at different times and coined the term catastrophe point, which was defined as the mean time when cells shifted back to permissive conditions lost the ability to complete the cell cycle. The catastrophe point was earlier than the execution point (15 h) and the time between these was argued to be when the *met1* gene product functioned. In a synchronous culture this timing was between 15 and 17 h, which indicates that *met1* functions after the first S (Donnan and John, 1983; Donnan *et al.*, 1985) and is probably directly involved in mitosis. It was concluded that, in *met1*, the failure to proceed from metaphase to anaphase was not due to a defective CDK but rather controls acting on the CDK may be altered in the mutant. Work is being directed towards cloning the *met1* gene and it is hoped that this will provide insight into the mode of action of the *met1* gene product.

C. Constitutive Mutants

Constitutive mutants (see Section I,B,2) are also useful tools in establishing the interdependency relationships of cell cycle events and the role of various components in the control and coordination of the *Chlamydomonas* cell cycle. These mutations have been described for a variety of cell functions and in some cases the genes concerned have been discovered. Most mutants described in Sections III,C,1–III,C,3 share the common feature of displaying cytokinesis defects.

1. Misdivision Mutants

Lewin (1952) mutagenized *C. moewusii* cells with ultraviolet radiation and isolated a range of mutants. Some were defective in photosynthesis or required vitamins for growth, whereas others had paralyzed or absent flagella.

Two mutants appeared to have cell division defects. Lewin called these "mutants exhibiting cell misdivisions." One mutant, M.470, resulted in "twinning" or "Siamese twins" which was a result of improper cleavage furrow formation (Fig. 9). The phenotype was present at a frequency of 5–10% in liquid cultures and many cells remained attached at their posterior ends. Some cells, having attempted to divide into four or more, exhibited

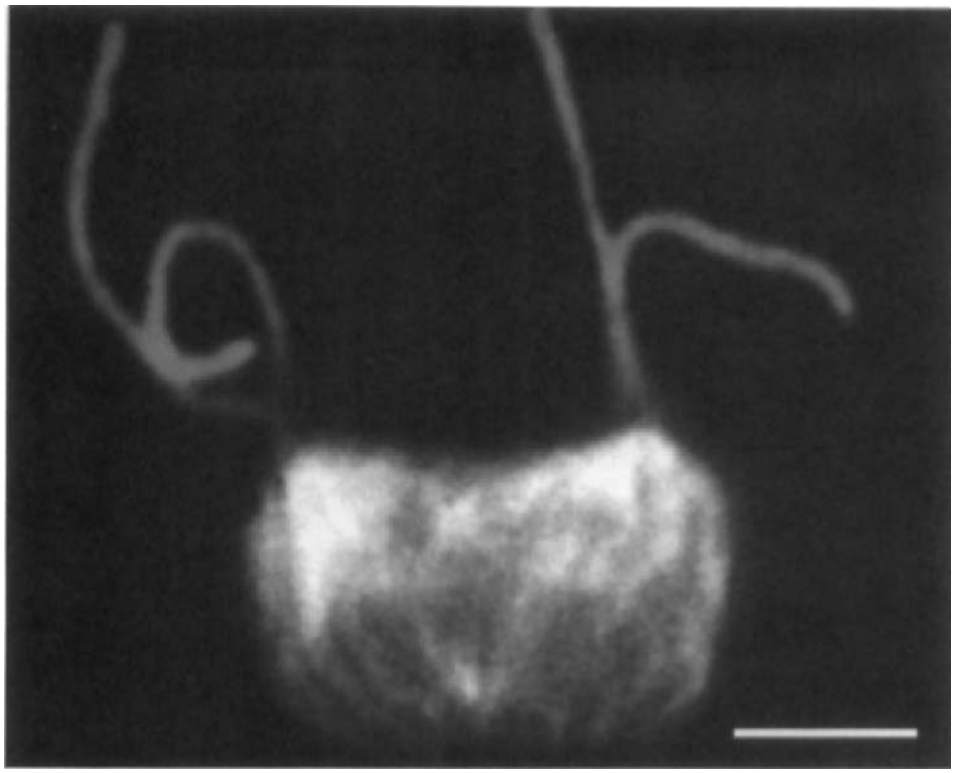


FIG. 9 An example of "twinning" as observed by Lewin (1952) and others. These cells from our culture collection were immunolabeled with anti-β-tubulin antibody to show flagella and arrays of microtubules. Scale bar = 5 μm.

more grotesque forms. The mutation for twinning (*t*) was found to be in a single gene which did not segregate with a sensitivity to sodium citrate found in the parent. This phenotype has been observed by many *Chlamydomonas* researchers, including Lewin, to occur in old wild-type cultures or in the presence of some subinhibitory concentrations of inhibitors.

Another mutant, M.202, was termed "monstrous" because many cells became very large and misshapen. These cells probably had a propensity to become blocked in division with continued growth. Some cells reached 50 μm in diameter (Fig. 5B). Many cells were lobed, indicative of defective cytokinesis, and had multiple nuclei and pyrenoids. Flagella were rare and when present were usually shorter than wild type. Although it was not mentioned, presumably the frequency of these monstrous cells in culture was relatively small compared to dividing ones because the mutant could be propagated.

2. Coordination of the Basal Body Cycle with the Cell Cycle

Jarvik and coworkers isolated and characterized several interesting mutants in *C. reinhardtii* which show variable flagellar number (*vfl*) and therefore have variable basal body numbers (Holmes and Dutcher, 1992). Each of these mutants offers insights into the coordination of the basal body cycle with the cell cycle. The mutants also show aberrant cytokinesis, which underscores the importance of the basal bodies and associated roots in the proper placement of the cleavage furrow. Each mutant will be described individually, but it should be kept in mind that each variable flagellar phenotype is brought about by mutations in different genes.

a. Vfl-1 The *vfl-1* mutant was isolated by Adams and coworkers (1982) and exhibits variable flagella numbers ranging usually from 0 to 4, but some cells have up to 10 (Adams *et al.*, 1985). The single gene mutation maps to linkage group VIII. The *vfl-1* mutant is unique in that it displays an uncoupling of the basal body–flagellar assembly cycle from the cell cycle. In wild-type cells, basal body and flagella production is restricted to discrete times during the cell cycle (Cavalier-Smith, 1974; Gould, 1975; Gaffal, 1988). In *vfl-1,* basal bodies and flagella are produced throughout the G_1 phase of the cell cycle. The flagella also appear at any location on the cell surface, not just at the anterior of the cell. The defect is therefore in both the temporal and the spatial regulation of the basal body cycle. In addition, cells display a wide range of sizes, which is likely due to unequal cytokinesis. For example, some extremely small nonviable cells of 1 μm in diameter were seen in culture.

Studies of division of single-agar immobilized cells revealed that after 24h microcolonies of *vfl-1* ranged in number from 2 to 23 cells per colony, whereas wild-type control colonies were much more uniform, containing 2–8 cells. The *vfl-1* colonies also exhibited odd numbers of cells, which indicated that some daughters were dying or failing to divide again. Six percent of mutants were actually dead and 17% of cells had not divided at all. Mutants also displayed aberrant flagellar beating even in cells with apparently anteriorly positioned flagella. Ultrastructural analysis revealed flagellar apparatus defects such as missing or striated fibers and reduced numbers of rootlet microtubules. It was proposed that the *vfl-1* defect could be revealing a regulatory gene involved in ensuring the correct timing and placement of developing basal bodies during the cell cycle.

b. Vfl-2 The *vfl-2* mutant was isolated by Kuchka and Jarvik (1982) and found to map to linkage group III. The number of flagella was shown to vary from 0 to 16. More strikingly, it was later discovered that *vfl-2* had a very labile centrin-based NBBC (Wright *et al.*, 1989). Immunofluorescence

microscopy using centrin antibodies did not detect NBBCs. Moreover, the centrin content in these cells was reduced by 75–80% compared to wild type (Wright *et al.,* 1989). It was concluded that the NBBC was involved in basal body segregation but did not have essential roles in mitosis or polarity determination (Wright *et al.,* 1989). In an elegant study, Taillon and coworkers (1992) showed that the *vfl-2* mutation was in the centrin gene by isolating the centrin gene from *vfl-2,* and they found that it varied from the wild-type gene by having lysine instead of glutamic acid at amino acid position 101. This mutation resulted in a loss of the fiber-forming ability of centrin. Revertants of *vfl-2* to wild-type-like genes showed that the NBBCs were intact. Cloning the centrin genes of these revertants showed either reversion at amino acid position 101 back to wild type or suppressor mutations at another portion of the gene (Taillon *et al.,* 1992). Further reversion analysis revealed that, like calmodulin, the central helix of centrin is important for its function (Taillon and Jarvik, 1995). It would be of interest to explore more closely cell cycle progression in *vfl-2* mutants.

*c. **Vfl-3*** This mutant was described as having incomplete or absent striated fibers (Wright *et al.,* 1983) which result in abnormal basal body number, orientation, and location. The mutation maps to linkage group VI, which indicates that it is distinct from *vfl-1* (VIII) and *vfl-2* (III). Similar to *vfl-1,* the *vfl-3* mutant exhibits abnormal cytokinesis which results in daughter cells of unequal sizes. Binucleate and bipyrenoidal cells were also observed. The primary defect of *vfl-3* was believed to be in the failure to position probasal bodies in the young cell (Gould, 1975; Wright *et al.,* 1983). Without proper placement of these it is proposed that striated fiber formation would be impaired, microtubular rootlets would be misaligned, and proper segregation of basal bodies would be impossible.

3. Other Cytokinesis Defects

Of the mutants described previously some already have been described as exhibiting defects in cytokinesis as well as other abnormalities. For instance, the constitutive mutants, M.470, M.202, *vfl-1,* and *vfl-3* have documented defects in cytokinesis. It is also noteworthy that of the cdb conditional mutations described in Section III,B,4, cdb4–6 have a tendency to form multinucleate cells at the permissive temperature, cdb7 cells attempt cytokinesis if binucleate when shifted to restrictive temperature, and cdb11 shows a conditional cytokinesis defect. These could all be classified as having some defect in cytokinesis. Because they are described elsewhere in this article, I will not deal with them further here.

a. Bald2 (bld2) The *bald2* mutant (now called *bld2*) was isolated by Goodenough and St. Clair (1975) and so named because it lacks flagella.

The mutation was believed to affect the formation of doublet and triplet microtubules (Goodenough and St. Clair, 1975). Electron microscopy revealed that most *bld2* cells lack basal bodies (>99%), with some cells exhibiting only rudimentary basal bodies (Goodenough and St. Clair, 1975; Ehler *et al.,* 1995). Because flagella are important in the signaling functions involved in *Chlamydomonas* mating (Harris, 1989), genetic analysis could not initially be performed. Later, added cAMP was found to stimulate mating reactions (Pasquale and Goodenough, 1987). This technique was refined (Dutcher, 1995b) and applied to a thorough tetrad analysis of *bld2* (more than 400 tetrads were dissected). It was revealed that the *bld2* mutant is defective in a single nuclear gene located on linkage group III (Holmes and Dutcher, 1992; Ehler *et al.,* 1995).

That the *bld2* mutation also affected cytokinesis in about 75% of cells was initially observed because the mutant exhibited a longer cell doubling time and cells showed irregularities in size. Multinucleate cells were also present (Holmes and Dutcher, 1992). In wild-type cells the position of the cleavage furrow is always correlated with the four-membered microtubule rootlet (Holmes and Dutcher, 1989; Gaffal and el-Gammal, 1990). A detailed study using immunofluorescence microscopy to localize microtubules, centrin, and actin revealed that the defect in cytokinesis was brought about by a misalignment of the flagellar rootlets (Ehler *et al.,* 1995). *Bld2* fails to position the cleavage furrow between the mitotic spindle poles. Thus, the position of the cleavage furrow and the mitotic spindle become random with respect to one another. The location of the *bld2* defect to linkage group III indicates that the defective gene product is not actin, the tubulins, or centrin (Ehler *et al.,* 1995). It is proposed that *bld2* reveals that basal bodies are needed for the organization of the cytoskeletal superstructure crucial for correct spindle and cleavage furrow placement.

b. Uni3 Is Defective in δ-Tubulin The *uni3* mutant was isolated by insertional mutagenesis (Tam and Lefebvre, 1993) and a microscopic screen for cells with single flagella (Dutcher and Trabuco, 1998). *Uni3* mutants have zero, one, or two flagella. Like *bld2* mutants, *uni3* cells also have a propensity to undergo aberrant cytokinesis (~15% of cells) which is further evidence for the role of the basal bodies in providing positional information. Cells are defective in a single nuclear gene located on linkage group III. Electron microscopic examination showed that *uni3* cells lack the c tubule in the microtubule triplet of the basal body. *Uni3* also affects basal body maturation. Pedigree analysis supports the proposition that a cell with no flagella grew one flagella in the next cell cycle and two flagella in the following cycle. This basal body maturation phenomenon has been observed in other algae (Melkonian *et al.,* 1987; Beech *et al.,* 1991). The cloning of

the *uni3* gene revealed a new member of the tubulin superfamily, which has been called δ-tubulin (Dutcher and Trabuco, 1998).

c. Cyt-1 The *cyt-1* mutation shows a partial blockage in cytokinesis. It was originally isolated after nitrosoguanidine mutagenesis by Warr (1968). Genetic analysis was hampered by the defect but *cyt-1* was shown to be defective in a single nuclear gene which maps to linkage group III. Cultures exhibit a range of phenotypes, including uni-, bi-, and multinucleate. The cells also appear lobed, indicating that cleavage furrows are attempted. Warr noted that there were two flagella per nucleus and proposed that they were somehow coordinated. Warr mentions that the presence of a fibrous ribosome-free area observed by Cavalier-Smith (1974) between the nucleus and basal bodies might be a physical connection. This observation was made before the rediscovery of the NBBC (Wright *et al.*, 1985; Salisbury, 1988). Because of the correlation between nuclear and flagellar numbers, I assume that the NBBC is not defective. It appears that in cultures that are allowed to go to stationary phase, the number of cytokinesis defective mutants decreases. Warr interpreted this to mean that *cyt-1* can complete cytokinesis if given enough time. Interestingly, the number of multinucleate cells also increased as the pH went from 6.0 to 8.0. Further studies revealed that the mean number of nuclei per cell was increased by addition of vitamin B_{12} or related compounds, such as benzimidazol or cobaltous chloride. *Cyt-1* was also more sensitive to the phosphodiesterase inhibitor caffeine than wild type. (Warr and Durber, 1971). Later studies showed that substances that raised the intracellular levels of free cysteine or cystine also increased the number of multinucleate cells in culture (Warr and Gibbons, 1973; Warr and Quinn, 1977). There were always a number of so-called moribund cells in culture which were very large and did not have flagella (Fig. 5B). The defect in *cyt-1* remains to be determined.

d. Occasional Cytokinesis Arrest Mutants Hirono and Yoda (1997) produced two mutations by insertional mutagenesis called *oca1* and *oca2* (*o*ccasional *c*ytokinesis *a*rrest). Large abnormally shaped cells with multiple flagella were seen in culture. Similar to the *cyt-1* mutant, nuclear numbers are correlated with pairs of flagella.

Time-lapse video microscopy revealed some surprising details about the mutants. It appears that some large multinucleate cells can undergo cytokinesis by producing multiple simultaneous cleavage furrows to divide up the cytoplasm. This is reminiscent of the mode of cell division in other green algae, such as *Chlorella* and *Scenedesmus* (Pickett-Heaps, 1975), or the cellularization process of insect eggs (Miller and Kiehart, 1995). Other mutants attempted to undergo multiple simultaneous cleavages and failed. The partially formed cleavage furrows could be seen on the surface of the

cells. The mutations reveal a different mode of cell division not observed in wild-type *Chlamydomonas.* The defects may be in regulatory genes, which usually schedule a cytokinesis between each nuclear division. That some mutants undergo simultaneous cytokinesis is remarkable and most certainly warrants further study. Moreover, mutagenesis and isolation of tagged genes (Tam and Lefebvre, 1993, 1995) should make it possible to easily identify the defective genes.

4. Size Control Defect

a. Mat3 During meiosis, in wild-type cells the mating type minus chloroplast DNA is actively destroyed, presumably by a zygote-specific nuclease. Meiotic progeny thus receive a chloroplast genome from the mating type plus parent (Harris, 1989). In the plus-linked nuclear mutation, *mat-3,* the selective destruction of chloroplast DNA in the minus strain is prevented. This results in a biparental inheritance pattern of chloroplast DNA (Gillham *et al.,* 1987). Armbrust and coworkers (1995) observed that *mat-3* cells are actually much smaller than wild-type cells. Compared to wild-type cells, which averages 10 μm in length, *mat-3* cells are about 3 μm in length. They also have greatly reduced amounts of both chloroplast and mitochondrial DNA. Armbrust and coworkers (1995) propose that this size control defect is the primary defect and that, because of the reduced chloroplast DNA, uniparental inheritance is inhibited.

5. Kinesin Defect

a. Fla10 *Fla10* was isolated in screens for temperature-sensitive flagellar assembly defective mutants (Huang *et al.,* 1977; Adams *et al.,* 1982). The *fla10* mutants showed a temperature-sensitive defect. Cells failed to assemble flagella at the restrictive temperature (32°C) and resorbed flagella already assembled at the permissive temperature (20°C). The *fla10* gene is located in linkage group XIX. Further genetic analysis isolated more mutations at the *fla10* locus and showed that some also exhibited cell division defects (Dutcher and Lux, 1989; Lux and Dutcher, 1991). Cells showed a significant percentage of chromosomal missegregations (Dutcher and Lux, 1989). More recently, the *fla10* gene product has been identified as a kinesin-homologous protein (KHP1; Walther *et al.,* 1994). The defect in the *fla10* mutant is rescued by transformation with the wild-type *KHP1* gene (Walther *et al.,* 1994). The gene product appears to be involved in flagellar assembly at the tip through a kinesin-driven motility called intraflagellar transport (IFT; Kozminski *et al.,* 1993; Vashishtha *et al.,* 1996; Cole *et al.,* 1998). Studies by Vashishtha and coworkers (1996) show that the defect in *fla10* is a C to A transversion, which alters amino acid 329 in

the KHP1 motor domain. Sequence comparisons show that KHP1 is part of a conserved family of kinesin-like proteins. Immunolocalizations of interphase cells with antibodies to KHP1 reveal that KHP1 is localized to basal bodies and the proximal region of flagella. During flagellar regeneration KHP1 labels along the flagella in a punctate fashion that extends to the flagellar tips, consistent with its role in IFT (Kozminski *et al.,* 1993; Vashishtha *et al.,* 1996; Cole *et al.,* 1998).

In dividing cells KHP1 remains associated with basal bodies throughout mitosis and localizes to the mitotic spindle. These observations, coupled with the observed defect in chromosome segregation of some *fla10* mutants (Lux and Dutcher, 1991), indicate that KHP1 has multiple roles in *Chlamydomonas.*

6. Organellokinesis: Chloroplast and Mitochondrial Division

It has been known for some time that organelles such as the chloroplast and mitochondria divide by binary fission (Kuroiwa *et al.,* 1998). These organelles also contain DNA which is specially packaged in discrete protein-containing structures called nucleoids. Division of these organelles involves replication and separation of the nucleoids followed by what has been called organellokinesis (Kuroiwa *et al.,* 1998). Much work to elucidate the mechanisms of chloroplast and mitochondrial division has been done on primitive red algae. It has been established that actin-like (FtsA) and tubulin-related (FtsZ) proteins are likely to be involved in the division machinery (Kuroiwa *et al.,* 1998). Considering the advanced genetics and molecular biology of *Chlamydomonas,* the isolation of mitochondrial and chloroplast division mutants may prove fruitful. Other organelles, such as the dictyosomes (Golgi apparatuses) in *Chlamydomonas,* are believed to divide by binary fission (Gruber and Rosario, 1979; Holmes and Dutcher, 1992; J. Harper, personal observations). With appropriate fluorescent probes for Golgi it may be possible to screen microscopically for mutants defective in Golgi division.

a. Chloroplast Division The large cup-shaped chloroplast of *Chlamydomonas* occupies 40–50% of the cell volume (Schotz *et al.,* 1972; Gaffal *et al.,* 1995) and its division timing can vary depending on the prevailing growth conditions (see Section II,C,1). To date, no mutations have been clearly identified which specifically disrupt the division of chloroplasts in *Chlamydomonas.* This has proved difficult because there are no probes available which make it easy to determine individual chloroplast numbers within cells. The only strategy used to date has been the laborious serial sectioning of cells embedded for electron microscopy (Schotz *et al.,* 1972; Gaffal, 1988). Another strategy might be to isolate chloroplasts from

division-arrested mutants and compare chloroplast, cell, and nuclear numbers. Inhibitor studies, using antibiotics which inhibit RNA biosynthesis in organelles, have raised the interesting possibility that the chloroplast contributes to the regulation of cell division in *Chlamydomonas* (Voigt and Munzner, 1989). Perhaps the isolation of mutants defective in chloroplast division will help answer this question.

b. Chloroplast Nucleoid Mutants The chloroplast genome of *Chlamydomonas* is present in 80–90 copies per cell localized into 6–10 discrete nucleoids (Ris and Plaut, 1962). The development and division of chloroplast nucleoids has been described in synchronous cultures of *Chlamydomonas* (Kuroiwa *et al.,* 1981; Ehara *et al.,* 1990). In general, in late G_1, compact nucleoids become thread-like and are dispersed to daughter cells during division. To date, no mutants have been described which have been specifically isolated for cell cycle defects in chloroplast nucleoid formation. However, the conditional cell division blocked mutant cdb9 (Harper *et al.,* 1995b) arrests with very few nucleoids. Nakumura and colleagues (1994) described two closely linked, single nuclear gene mutations, cond-1 and cond-2, that have defects in the normal condensation of nucleoids during growth cessation. In wild-type cells the nucleoids condense to form a single nucleoid when cells stop growing. However, in the mutants, there are between 10 and 20 nucleoids even after 7 weeks of culture. Measurement of oxygen evolution showed that the mutations did not affect photosynthesis. However, a high lipid content suggested that the nucleoids may increase lipid turnover and/or storage in cells. It would be of interest to determine the relationship between nucleoid condensation and the cell cycle in cond-1 and 2.

IV. Future Prospects

It is evident that there are a number of interesting cell cycle mutants, both conditional and constitutive, which will help unlock mysteries about how the *Chlamydomonas* cell cycle is controlled and coordinated. I believe that these mutants are a "gold mine" waiting to be tapped. In addition, strategies to use *Chlamydomonas* for the production and analysis of mutations are constantly being developed. There are many techniques available which could form a sizeable review in themselves (Dentler and Witman, 1995). I point out only a few techniques but believe that more will transpire as molecular and cell biology technologies advance.

A. Mapping

Mapping the mutations to the nuclear genome should be done by either conventional (Dutcher, 1995b) or molecular mapping (Silflow *et al.*, 1995).

B. Further Mutation of Alleles

It may be possible to isolate more mutations at a particular locus using the mutagenesis of diploid strains (Ebersold, 1967). Mutant strains could be crossed with wild-type cells and diploids screened for a reappearance of the mutant phenotype after a shift-up to restrictive conditions (Dutcher and Lux, 1989; Dutcher, 1995b).

C. Rescue of Mutant Phenotype by Complementation

Transformation systems in *Chlamydomonas* are becoming more refined (Nelson and Lefebvre, 1995), making it possible to rescue cell cycle defects by transformation and complementation with the wild-type gene.

D. Identification of Wild-Type Genes Complementary to Conditional Mutant Genes

A strategy to isolate particular conditional mutants could be to use transposon tagging (Schnell and Lefebvre, 1993) or integration of plasmid DNA by transformation (Tam and Lefebvre, 1993, 1995) to knock out the wild-type cell cycle gene in the diploid and then retrieve it for identification.

E. Isolation of Suppressor Mutations to Investigate Gene Interactions

Mutagenesis of mutants and recovery of pseudorevertants and the identity of these genes will help extend our knowledge of the interactions of gene products to carry out cell cycle functions. Yeast two-hybrid systems (Frederickson, 1998) may also aid in the recovery of interacting gene products.

F. Epitope Tagging and Green Flourescent Protein for Localization of Gene Product in Fixed and Living Cells

Epitope tagging, which involves tagging a gene sequence with antibodies against it to the gene product of interest, has been used successfully to

localize gene products and circumvents the need to raise antibodies (Diener, 1995). The jellyfish green fluorescent protein (GFP) is revolutionizing the localization of proteins in living cells. The technology has been successful in a number of organisms, including yeast (Shaw *et al.,* 1997). However, reports from a recent *Chlamydomonas* meeting (*http://www.botany.duke.edu/DCMB/chlamy.htm*) indicate that the GC coding bias of *Chlamydomonas* may be hampering expression of the AT-rich GFP in this organism. It is hoped that these hurdles will be overcome. GFP or another fluorescent molecule will be invaluable for monitoring the behavior of cellular or cytoskeletal components or cell cycle gene products in live cells.

G. Screens for Particular Types of Cell Cycle Mutants

For the isolation of different kinds of mutants, such as conditional chloroplast division defects, size control mutants, or anaphase arrest, specific screens could be set up to select for those types of mutant. In the case of the anaphase-arresting mutant, nuclear DNA staining could be used to identify cells which arrested with separated chromosomes. In the case of the size control mutants, cells could be size fractionated on sucrose gradients and only the most buoyant (smallest) cells selected.

V. Conclusions

Chlamydomonas is the most intensively studied alga. It has been called the green yeast (Goodenough, 1992) because of its amenability to cell and molecular genetic dissection. It has also been speculated that *Chlamydomonas* may find a niche in the biotechnology arena in the production of recombinant proteins (Stevens and Purton, 1997). With the rapid development of new molecular and cell biological techniques to explore the workings of *Chlamydomonas,* such as the recent initiative to sequence the *Chlamydomonas* genome, it is hoped that the genes "behind" the cell cycle mutants described in this review will be cloned and others isolated.

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References

Adams, G. M. W., Huang, B., and Luck, D. J. L. (1982). Temperature sensitive assembly defective flagella mutants of *Chlamydomonas reinhardtii. Genetics* **100,** 579–586.

Adams, G. M. W., Wright, R. L., and Jarvik, J. W. (1985). Defective temporal and spatial control of flagellar assembly in a mutant of *Chlamydomonas reinhardtii* with variable flagella number. *J. Cell Biol.* **100,** 955–964.

Armbrust, E. V., Ibrahim, A., and Goodenough, U. W. (1995). A mating type-linked mutation that disrupts the uniparental inheritance of chloroplast DNA also disrupts cell-size control in *Chlamydomonas. Mol. Biol. Cell* **6,** 1807–1818.

Beck, C. F., and Haring, M. A. (1996). Gametic differentiation of *Chlamydomonas. Int. Rev. Cytol.* **168,** 259–302.

Beech, P. L., Heimann, K., and Melkonian, M. (1991). Development of the flagellar apparatus during the cell cycle in unicellular algae. *Protoplasma* **164,** 23–37.

Buffaloe, N. D. (1958). A comparative cytological study of four species of *Chlamydomonas. Bull. Tor. Bot.* **85,** 157–178.

Cavalier-Smith, T. (1974). Basal body and flagellar development during the vegetative cell cycle and the sexual cycle of *Chlamydomonas reinhardtii. J. Cell Sci.* **16,** 529–556.

Cohen, P., and Klee, C. B. (1988). "Calmodulin." Elsevier, Amsterdam.

Cole, D. G., Diener, D. R., Himelblau, A. L., Beech, P. L., Fuster, J. C., and Rosenbaum, J. L. (1998). *Chlamydomonas* kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain proteins required for ciliary assembly in *Caenorhabditis elegans* sensory neurons. *J. Cell Biol.* **141,** 993–1008.

Coleman, A. W. (1982). The nuclear cycle in *Chlamydomonas* (Chlorophaceae). *J. Phycol.* **18,** 192–195.

Coss, R. A. (1974). Mitosis in *Chlamydomonas reinhardtii* basal bodies and the mitotic apparatus. *J. Cell Biol.* **63,** 325–329.

Craigie, R. A., and Cavalier-Smith, T. (1982). Cell volume and the control of the *Chlamydomonas* cell cycle. *J. Cell Sci.* **54,** 173–191.

Del Vecchio, A. J., Harper, J. D. I., Vaughn, K. C., Baron, A. T., Salisbury, J. L., and Overall, R. L. (1997). Centrin homologues in higher plant cells are prominently associated with the developing cell plate. *Protoplasma* **196,** 224–234.

Dentler, W., and Witman, G. (1995). "Methods in Cell Biology Vol. 47: Cilia and Flagella." Academic Press, San Diego.

Diener, D. R. (1995). Epitope tagging of flagellar proteins. *In* "Methods in Cell Biology" (W. Dentler and G. Witman, eds.), **47,** pp. 545–550. Academic Press, San Diego.

Donnan, L., and John, P. C. L. (1983). Cell cycle control by timer and sizer in *Chlamydomonas. Nature (London)* **304,** 630–633.

Donnan, L., Carvill, E. P., Gilliland, T. J., and John, P. C. L. (1985). The cell cycles of *Chlamydomonas* and *Chlorella. New Phytol.* **99,** 1–40.

Doonan, J. H. (1992). Cell division in *Aspergillus. J. Cell Sci.* **103,** 599–611.

Doonan, J. H., and Grief, C. (1987). Microtubule cycle in *Chlamydomonas reinhardtii;* An immunofluorescence study. *Cell Motil. Cytoskel.* **7,** 381–392.

Dutcher, S. K. (1995a). Flagellar assembly in two hundred and fifty easy-to-follow steps. *Trends Genet.* **11,** 398–404.

Dutcher, S. K. (1995b). Mating and tetrad analysis in *Chlamydomonas reinhardtii. In* "Methods in Cell Biology" (W. Dentler and G. Witman, eds.), Vol. 47, pp. 531–540. Academic Press, San Diego.

Dutcher, S. K., and Lux, F. G., III (1989). Genetic interactions of mutations affecting flagella and basal bodies in *Chlamydomonas. Cell Motil. Cytoskel.* **14,** 104–117.

Dutcher, S. K., and Trabuco, E. C. (1998). The *UNI3* gene is required for assembly of basal bodies of *Chlamydomonas* and includes δ-tubulin, a new member of the tubulin superfamily. *Mol. Biol. Cell* **9,** 1293–1308.

Ebersold, W. T. (1967). *Chlamydomonas reinhardtii:* Heterozygous diploid strains. *Science* **157,** 447–449.

Ehara, T., Ogasawara, Y., Osafune, T., and Hase, E. (1990). Behavior of chloroplast nucleoids during the cell cycle of *Chlamydomonas reinhardtii* (chlorophyta) in synchronized culture. *J. Phycol.* **26,** 317–323.

Ehler, L. L., Holmes, J. A., and Dutcher, S. K. (1995). Loss of spatial control of the mitotic spindle apparatus in a *Chlamydomonas reinhardtii* mutant strain lacking basal bodies. *Genetics* **141,** 945–960.

Ettl, H. (1976a). Die gattung *Chlamydomonas* Ehrenberg. *Beih. Nova Hedwigia* **49,** 1–1122.

Ettl, H. (1976b). Uber den teilungsverlauf des chloroplasten bei *Chlamydomonas. Protoplasma* **88,** 75–84.

Fernandez, E., Schnell, R., Ranum, L. P. W., Hussey, S. C., Silflow, C. D., and Lefebvre, P. A. (1989). Isolation and characterization of the nitrate reductase structural gene of *Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA* **86,** 6449–6453.

Forsburg, S. L., and Nurse, P. (1991). Cell cycle regulation in the yeasts *Saccharomyces cerevisiae* and *Shizosaccharomyces pombe. Annu. Rev. Cell Biol.* **7,** 227–256.

Frederickson, R. M. (1998). Macromolecular matchmaking—Advances in two-hybrid and related technologies. *Curr. Opin. Biotechnol.* **9,** 90–96.

Gaffal, K. P. (1987). Mitosis-specific oscillations of mitochondrial morphology in *Chlamydomonas reinhardtii. Endocytol. Cell Res.* **4,** 41–62.

Gaffal, K. P. (1988). The basal body–root complex of *Chlamydomonas reinhardtii* during mitosis. *Protoplasma* **143,** 118–129.

Gaffal, K. P., and el-Gammal, S. (1990). Elucidation of the enigma of the "metaphase band" of *Chlamydomonas reinhardtii. Protoplasma* **156,** 139–148.

Gaffal, K. P., el-Gammal, S., and Friedrichs, G. J. (1993). Computer-aided 3D reconstruction of the eyespot-flagellar/basal apparatus-contactile vacuoles-nuclear-associations during mitosis of *Chlamydomonas reinhardtii. Endocytol. Cell Res.* **9,** 177–208.

Gaffal, K. P., Arnold, C.-G., Friedrichs, G. J., and Gemple, W. (1995). Morphodynamical changes of the chloroplast of *Chlamydomonas reinhardtii* during the first division. *Arch. Protistenkd.* **145,** 10–23.

Gillham, N. W., Boynton, J. E., Johnson, A. M., and Burkhart, B. D. (1987). Mating type linked mutations which disrupt the uniparental transmission of chloroplast genes in *Chlamydomonas. Genetics* **115,** 677–684.

Goodenough, U. W. (1970). Chloroplast division and pyrenoid formation in *Chlamydomonas reinhardtii. J. Phycol.* **6,** 1–6.

Goodenough, U. W. (1992). Green yeast. *Cell* **70,** 533–538.

Goodenough, U. W., and St. Clair, H. S. (1975). Bald-2: A mutation affecting the formation of doublet and triplet sets of microtubules in *Chlamydomonas reinhardtii. J. Cell Biol.* **66,** 480–491.

Goodenough, U. W., and Weiss, R. L. (1978). Interrelationships between microtubules, a striated fiber, and the gametic mating structure of *Chlamydomonas reinhardtii. J. Cell Biol.* **76,** 430–438.

Goto, K., and Johnson, C. H. (1995). Is the cell division cycle gated by a circadian clock? The case of *Chlamydomonas reinhardtii. J. Cell Biol.* **129,** 1061–1069.

Gould, R. R. (1975). The basal bodies of *Chlamydomonas reinhardtii,* formation from probasal bodies, isolation and partial characterization. *J. Cell. Biol.* **65,** 65–74.

Gruber, H. E., and Rosario, B. (1979). Ultrastructure of the golgi apparatus and contractile vacuole in *Chlamydomonas reinhardtii. Cytologia* **44,** 505–526.

Harper, J. D. I., and John, P. C. L. (1986). Coordination of division events in the *Chlamydomonas* cell cycle. *Protoplasma* **131,** 118–130.

Harper, J. D. I., Rao, P. N., and John, P. C. L. (1990a). The mitosis specific monoclonal antibody MPM-2 recognizes phosphoproteins associated with the nuclear envelope in *Chlamydomonas reinhardtii* cells. *Eur. J. Cell Biol.* **51,** 271–278.

Harper, J. D. I., Rao, P. N., and John, P. C. L. (1990b). A monoclonal antibody to animal centrosomes recognizes components of the basal-body root complex in *Chlamydomonas. Protoplasma* **153,** 208–211.

Harper, J. D. I., McCurdy, D. W., Sanders, M. A., Salisbury, J. L., and John, P. C. L. (1992). Actin dynamics during the cell cycle in *Chlamydomonas. Cell Motil. Cytoskel.* **22,** 117–126.

Harper, J. D. I., Sanders, M. A., and Salisbury, J. L. (1993). Phosphorylation of nuclear and flagellar basal apparatus proteins during flagellar regeneration in *Chlamydomonas reinhardtii. J. Cell Biol.* **122,** 877–886.

Harper, J. D. I., Gubler, F., Salisbury, J. L., and Hardham, A. R. (1995a). Centrin association with the flagellar apparatus in spores of *Phytophthora cinnamomi. Protoplasma* **188,** 225–235.

Harper, J. D. I., Wu, L., Sakuanrungsirikul, S., and John, P. C. L. (1995b). Isolation and partial characterization of conditional cell division cycle mutants in *Chlamydomonas. Protoplasma* **186,** 149–162.

Harris, E. H. (1989). "The *Chlamydomonas* Source Book: A Comprehensive Guide to Biology and Laboratory Use." Academic Press, San Diego.

Hartwell, L. H. (1971). The genetic control of the cell division cycle in yeast II. Genes controlling DNA replication and its initiation. *J. Mol. Biol.* **59,** 183–194.

Hartwell, L. H. (1978). Cell division from a genetic perspective. *J. Cell Biol.* **77,** 627–637.

Hartwell, L. H. (1991). Twenty-five years of cell cycle genetics. *Genetics* **129,** 975–980.

Hirono, M., and Yoda, A. (1997). Isolation and phenotypic characterization of *Chlamydomonas* mutants defective in cytokinesis. *Cell Struct. Funct.* **22,** 1–5.

Holmes, J. A., and Dutcher, S. K. (1989). Cellular asymmetry in *Chlamydomonas reinhardtii. J. Cell Sci.* **94,** 273–285.

Holmes, J. A., and Dutcher, S. K. (1992). Genetic approaches to the study of cytoskeletal structure and function in *Chlamydomonas. In* "The Cytoskeleton of the Algae" (D. Menzel, ed.). CRC Press, Boca Raton, FL.

Howell, S. H. (1974). An analysis of cell cycle controls in temperature sensitive mutants of *Chlamydomonas reinhardtii. In* "Cell Cycle Controls" (G. M. Padilla, I. L. Cameron, and A. Zimmerman, eds.), pp. 235–249. Academic Press, New York.

Howell, S. H., and Naliboff, J. A. (1973). Conditional mutants in *Chlamydomonas reinhardtii* blocked in the vegetative cell cycle 1. An analysis of cell cycle block points. *J. Cell Biol.* **57,** 760–772.

Howell, S. H., Posakony, J. W., and Hill, K. R. (1977). The cell cycle program of polypeptide labelling in *Chlamydomonas reinhardtii. J. Cell Biol.* **72,** 223–241.

Huang, B. (1986). *Chlamydomonas reinhardtii* a model system for the genetic dissection of flagellar structure and motility. *Int. Rev. Cytol.* **99,** 181–215.

Huang, B., Rifkin, M. R., and Luck, D. J. (1977). Temperature-sensitive mutations affecting flagellar assembly and function in *Chlamydomonas reinhardtii. J. Cell Biol.* **72,** 67–85.

Huang, B., Mengersen, A., and Lee, V. D. (1988). Molecular cloning of cDNA for caltractin, a basal body-associated Ca^{2+}-binding protein: Homology in its protein sequence with calmodulin and the yeast *CDC31* gene product. *J. Cell Biol.* **107,** 133–140.

Hush, J. M., Wu, L., John, P. C. L., Hepler, L. H., and Hepler, P. K. (1996). Plant mitosis promoting factor disassembles the microtubule preprophase band and accelerates prophase progression in *Tradescantia. Cell Biol. Int.* **20,** 275–287.

Ishikawa, T., Uno, I., and Matsumoto, K. (1985). The role of cAMP in controlling yeast cell division. *BioEssays* **4,** 52–56.

Jayaswal, R. K., Handa, A. K., Bressan, R. A., Cherniack, J., and Filner, F. (1991). Physiological and heritable changes in cyclic AMP levels associated with changes in flagellar formation in *Chlamydomonas reinhardtii* (chlorophyta). *J. Phycol.* **27,** 587–591.

John, P. C. L. (1984). Control of the cell division cycle in *Chlamydomonas. Microbiol. Sci.* **1,** 96–101.

John, P. C. L. (1987). Control points in the *Chlamydomonas* cell cycle. *In* "Algal Development (Molecular and Cellular Aspects)" (W. Wiessner, D. G. Robinson, and R. C. Starr, eds.), pp. 9–16. Springer-Verlag, Berlin.

John, P. C. L., and Wu, L. (1992). Cell cycle control proteins in division and development. *In* "Cellular Basis of Growth and Development in Plants" (H. Shibaoka, ed.), Proceedings of the VII International Symposium, Osaka, Japan; pp. 65–84.

John, P. C. L., Sek, F. J., and Lee, M. G. (1989). A homolog of the cell cycle control protein $p34^{cdc2}$ participates in the division cycle of *Chlamydomonas,* and a similar protein is detectable in higher plants and remote taxa. *Plant Cell* **1,** 1185–1193.

Johnson, K. A., and Rosenbaum, J. L. (1993). Flagellar regeneration in *Chlamydomonas:* A model system for studying organelle assembly. *Trends Cell Biol.* **3,** 156–161.

Johnson, U. G., and Porter, K. R. (1968). Fine structure of cell division in *Chlamydomonas reinhardtii. J. Cell Biol.* **38,** 403–425.

Jones, R. F. (1970). Physiological and biochemical aspects of growth and gametogenesis in *Chlamydomonas reinhardtii. Ann. N. Y. Acad. Sci.* **175,** 648–659.

Katagiri, F., Lam, E., and Chua, N.-H. (1989). Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. *Nature* **340,** 727–730.

Kindle, K. L., Schnell, R. A., Fernandez, E., and Lefebvre, P. A. (1989). Stable nuclear transformation of *Chlamydomonas* using the *Chlamydomonas* gene for nitrate reductase. *J. Cell Biol.* **109,** 2589–2601.

Kooijman, R., De Wildt, P., ven den Briel, W., Tan, S.-H., Musgrave, A., and ven den Ende, H. (1990). Cyclic AMP is one of the intracellular signals during mating in *Chlamydomonas eugametos. Planta* **181,** 529–537.

Koutoulis, A., McFadden, G. I., and Wetherbee, R. (1988). Spine-scale reorientation in *Apedinella radians (Pedinellales, Chrysophyceae):* The microarchitecture and immunocytochemistry of the associated cytoskeleton. *Protoplasma* **147,** 25–41.

Kozminski, K. G., Johnson, K. A., Forscher, P., and Rosenbaum, J. L. (1993). A motility in the eukaryotic flagellum unrelated to flagellar beating. *Proc. Natl. Acad. Sci. USA* **90,** 5519–5523.

Kuchka, M. R., and Jarvik, J. W. (1982). Analysis of flagellar size control using a mutant of *Chlamydomonas reinhardtii* with a variable number of flagella. *J. Cell Biol.* **92,** 170–175.

Kuroiwa, T., Suzuki, T., Ogawa, K., and Kawano, S. (1981). The chloroplast nucleus: Distribution, number, size, and shape and a model for the multiplication of the chloroplast genome during chloroplast development. *Plant Cell Physiol.* **22,** 381–396.

Kuroiwa, T., Kuroiwa, H., Sakai, A., Takahashi, H., Toda, K., and Itoh, R. (1998). The division apparatus of plastids and mitochondria. *Int. Rev. Cytol.* **181,** 1–41.

Lechtreck, K.-F., and Melkonian, M. (1991). An update on fibrous flagellar roots in green algae. *Protoplasma* **164,** 38–44.

Lechtreck, K.-F., and Silflow, C. D. (1997). SF-assemblin in *Chlamydomonas:* Sequence conservation and localization during the cell cycle. *Cell Motil. Cytoskel.* **36,** 190–201.

Le Dizet, M., and Piperno, G. (1986). Cytoplasmic microtubules containing acetylated α-tubulin in *Chlamydomonas reinhardtii:* Spacial arrangement and properties. *J. Cell Biol.* **103,** 13–22.

Lefebvre, P. A., and Rosenbaum, J. L. (1986). Regulation of the synthesis and assembly of ciliary and flagellar proteins during regeneration. *Annu. Rev. Cell Biol.* **2,** 517–546.

Levy, Y. Y., Lai, E. Y., Remillard, S. P., Heintzelman, M. B., and Fulton, C. (1996). Centrin is a conserved protein that forms diverse associations with centrioles and MTOCs in *Naegleria* and other organisms. *Cell Motil. Cytoskel.* **33,** 298–323.

Lewin, R. A. (1952). Ultraviolet induced mutations in *Chlamydomonas moewusii Gerloff. J. Gen. Microbiol.* **6,** 233–248.

Lloyd, D., Poole, R. K., and Edwards, S. W. (1982). "The Cell Division Cycle. Temporal Organization and Control of Cellular Growth and Reproduction." Academic Press, London.

Loppes, R. (1968). Ethylmethane sulphonate (EMS): An effective mutagen in *Chlamydomonas reinhardtii. Mol. Gen. Genet.* **102,** 229–231.

Lux, F. G., III., and Dutcher, S. K. (1991). Genetic interactions of the *FLA10* locus: Supressors and sythetic phenotypes that affect the cell cycle and flagellar function in *Chlamydomonas reinhardtii. Genetics* **128,** 549–561.

Marcus, M., Fainsod, A., and Diamond, G. (1985). The genetic analysis of mammalian cell cycle mutants. *Annu. Rev. Genet.* **19,** 389–421.

Matsumoto, K., Uno, I., Oshima, Y., and Ishikawa, T. (1982). Isolation and characterization of yeast mutants deficient in adenylate cyclase and cyclic AMP dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **77,** 541–545.

Matsumoto, K., Uno, I., and Ishikawa, T. (1983). Control of cell division in *Saccharomyces cerevisiae* mutants defective in adenylate cyclase and cAMP dependent protein kinase. *Exp. Cell Res.* **146,** 151–161.

McAteer, M., Donnan, L., and John, P. C. L. (1985). The timing of division in *Chlamydomonas. New Phytol.* **99,** 41–56.

Melkonian, M. (1984). Flagellar root mediated interactions between the flagellar apparatus and cell organelles in green algae. *In* "Compartments in Algal Cells and Their Interactions" (W. Wiessner, D. G. Robinson, and R. C. Starr, eds.), pp. 96–108. Springer-Verlag, Berlin.

Melkonian, M., Reize, M. B., and Preising, H. R. (1987). Maturation of a flagellum/basal body requires more than one cell cycle in algal flagellates: Studies on *Nephroselmis olivacea* (Prasinphyceae). *In* "Algal Development" (W. Wiessner, D. G. Robinson, and R. C. Starr, eds.), pp. 102–113. Springer-Verlag, Berlin.

Melkonian, M., Beech, P. L., Katsaros, C., and Schulze, D. (1992). Centrin-mediated cell motility in algae. *In* "Algal Cell Motility" (M. Melkonian, ed.), pp. 179–221. Chapman & Hall, New York.

Miller, K. G., and Kiehart, D. P. (1995). Fly division. *J. Cell Biol.* **131,** 1–5.

Mitchison, J. M. (1971). "The Biology of the Cell Cycle." Cambridge Univ. Press, Cambridge, UK.

Nakamura, S., Sakihara, M., Chibana, H., Ikehara, T., and Kuroiwa, T. (1994). Mutations disturbing the condensation of plastid nucleoids in *Chlamydomonas reinhardtii. Protoplasma* **178,** 111–118.

Nelson, J. A., and Lefebvre, P. A. (1995). Transformation of *Chlamydomonas reinhardtii. In* "Methods in Cell Biology" (W. Dentler and G. Witman, eds.), Vol. 47, pp. 513–517. Academic Press, San Diego.

Norbury, C., and Nurse, P. (1992). Animal cell cycles and their control. *Annu. Rev. Biochem.* **61,** 441–470.

Nurse, P. (1985). Cell cycle control in yeast. *Trends Genet.* **1,** 51–55.

Paoletti, A., Moudjou, M., Paintrand, M., Salisbury, J. L., and Bornens, M. (1996). Most of centrin in animal cells is not centrosome-associated and centrosomal centrin is confined to the distal lumen of centrioles. *J. Cell Sci.* **109,** 3089–3102.

Pasquale, S. M., and Goodenough, U. W. (1987). Cyclic AMP functions as a primary sexual signal in gametes of *Chlamydomonas reinhardtii. J. Cell Biol.* **105,** 2279–2292.

Pickett-Heaps, J. D. (1975). "Green Algae—Structure, Reproduction and Evolution of Selected Genera." Sinauer, Sunderland, MA.

Pringle, J. R. (1975). Induction, selection and experimental uses of temperature sensitive and other conditional mutants of yeast. *Methods Cell Biol.* **12,** 233–272.

Pringle, J. R. (1978). The use of conditional lethal cell cycle mutants for temporal and functional sequence mapping of cell cycle events. *J. Cell Physiol.* **95,** 393–406.

Pringle, J. R. (1981). The genetic approach to the study of the cell cycle. *In* "Mitosis/Cytokinesis" (A. Zimmerman and A. Forer, eds.), pp. 3–28. Academic Press, New York.

Ringo, D. L. (1967). Flagellar motion and fine structure of the flagellar apparatus in *Chlamydomonas. J. Cell Biol.* **33,** 543–571.

Ris, H., and Plaut, W. (1962). Ultrastructure of DNA-containing areas in the chloroplast of *Chlamydomonas. J. Cell Biol.* **13,** 383–391.

Rochaix, J. D. (1995). *Chlamydomonas reinhardtii* as the photosynthetic yeast. *Annu. Rev. Genet.* **29,** 209–230.

Rollins, M. J., Harper, J. D. I., and John, P. C. L. (1983). Synthesis of individual proteins, including tubulins and chloroplast membrane proteins in synchronous cultures of the eukaryote *Chlamydomonas reinhardtii.* Elimination of periodic changes in protein synthesis and enzyme activity under constant environmental conditions. *J. Gen. Microbiol.* **129,** 1899–1919.

Sakuanrungsirikul, S. (1991). Cyclic AMP and the *Chlamydomonas* division cycle. PhD dissertation, Australian National University, Canberra.

Sakuanrungsirikul, S., Hocart, C. H., Harper, J. D. I., Parker, W., and John, P. C. L. (1996). Temperature conditional cAMP-requiring mutant strains of *Chlamydomonas reinhardtii* arrest in G1 and are rescued by added cAMP. *Protoplasma* **192,** 159–167.

Salisbury, J. L. (1988). The lost neuromotor apparatus of *Chlamydomonas:* Rediscovered. *J. Protozool.* **35**(4), 574–577.

Salisbury, J. L. (1995). Centrin, centrosomes and mitotic spindle poles. *Curr. Opin. Cell Biol.* **7,** 39–45.

Salisbury, J. L., Baron, A. T., Coling, D. E., Martindale, V. E., and Sanders, M. A. (1986). Calcium-modulated contractile proteins associated with the eukaryotic centrosome. *Cell Motil. Cytoskel.* **6,** 193–197.

Salisbury, J. L., Sanders, M. A., and Harpst, L. (1987). Flagellar root contraction and nuclear movement during flagellar regeneration in *Chlamydomonas reinhardtii. J. Cell Biol.* **105,** 1799–1805.

Salisbury, J. L., Baron, A. T., and Sanders, M. A. (1988). The centrin-based cytoskeleton of *Chlamydomonas reinhardtii:* Distribution in interphase and mitotic cells. *J. Cell Biol.* **107,** 635–641.

Sanders, M. A., and Salisbury, J. L. (1989). Centrin-mediated microtubule severing during flagellar excision in *Chlamydomonas reinhardtii. J. Cell Biol.* **108,** 1751–1760.

Sanders, M. A., and Salisbury, J. L. (1994). Centrin plays an essential role in microtulule severing during flagellar excision in *Chlamydomonas reinhardtii. J. Cell Biol.* **124,** 795–805.

Sato, C. H. (1976). A conditional cell division cycle mutant of *Chlamydomonas reinhardtii* having an increased level of colchicine resistance. *Exp. Cell Res.* **101,** 251–259.

Schibler, M. J., and Huang, B. (1991). The *col*^{R}14 and *col*^{R} 15 β-tubulin mutations in *Chlamydomonas reinhardtii* confer altered sensitivities to microtubule inhibitors and herbicides by enhancing microtubule stability. *J. Cell Biol.* **113,** 605–614.

Schiebel, E., and Bornens, M. (1995). In search of a function for centrins. *Trends Cell Biol.* **5,** 198–201.

Schmitt, R., Fabry, S., and Kirk, D. L. (1992). In search of molecular origins of cellular differentiation in *Volvox* and its relatives. *Int. Rev. Cytol.* **139,** 189–265.

Schnell, R. A., and Lefebvre, P. A. (1993). Isolation of the *Chlamydomonas* regulatory gene *NIT2* by transposon tagging. *Genetics* **134,** 737–747.

Schotz, F., Bathelt, H., Arnold, C.-G., and Schimmer, O. (1972). The architecture and organization of the *Chlamydomonas* cell. Results of serial section electron microscopy and a 3-dimensional reconstruction. *Protoplasma* **75,** 229–254.

Segaar, P. J., Gerritsen, A. F., and DeBakker, M. A. G. (1989). The cytokinetic apparatus during sporulation in the unicellular green flagellate *Gloeomonas kupfferi:* The phycoplast as a spatio-temporal differentiation of the cortical microtubule array that organizes cytokinesis. *Nova Hedwigia* **49,** 1–23.

Shaw, S. L., Yeh, E., Bloom, K., and Salmon, E. D. (1997). Imaging green flourescent protein fusion proteins in *Saccaromyces cerevisiae. Curr. Biol.* **7,** 701–704.

Silflow, C. D., Kathir, P., and Lefebvre, P. A. (1995). Molecular mapping of genes for flagellar proteins in *Chlamydomonas. Methods Cell Biol.* **47,** 525–530.

Spudich, J. L., and Sager, R. (1980). Regulation of the *Chlamydomonas* cell cycle by light and dark. *J. Cell Biol.* **85,** 136–145.

Stevens, D. R., and Purton, S. (1997). Genetic engineering of eukaryotic algae: Progress and prospects. *J. Phycol.* **33,** 713–722.

Sutherland, E. W. (1972). Studies on the mechanism of hormone action. *Science* **177,** 401–408.

Taillon, B. E., and Jarvik, J. W. (1995). Central helix mutations in the centrosome-associated EF-hand protein centrin. *Protoplasma* **189,** 203–215.

Taillon, B. E., Adler, S. A., Suhan, J. P., and Jarvik, J. W. (1992). Mutational analysis of centrin: An EF-hand protein associated with three distinct contractile fibers in the basal body apparatus of *Chlamydomonas. J. Cell Biol.* **119,** 1613–1624.

Tam, L.-W., and Lefebvre, P. A. (1993). Cloning of flagellar genes in *Chlamydomonas reinhardtii* by DNA insertional mutagenesis. *Genetics* **135,** 375–384.

Tam, L.-W., and Lefebvre, P. A. (1995). Insertional mutagenesis and isolation of tagged genes in *Chlamydomonas. Methods Cell Biol.* **47,** 519–523.

Traas, J., and Laufs, P. (1998). Cell cycle mutants in higher plants: A phenotypical overview. *In* "Plant Cell Division" (D. Francis, D. Dudits, and D. Inze', eds.), pp. 319–336. Portland Press, London.

Trainor, F. R., and Cain, J. R. (1986). Famous algal genera. I. *Chlamydomonas. Prog. Phycol. Res.* **4,** 82–127.

Triemer, R. E., and Brown, R. M. (1974). Cell division in *Chlamydomonas moewusii. J. Phycol.* **10,** 419–433.

van den Ende, H. (1994). Vegetative and gametic development in the green alga *Chlamydomonas. Adv. Bot. Res.* **20,** 125–161.

Vashishtha, M., Walther, Z., and Hall, J. L. (1996). The kinesin-homologous protein encoded by the *Chlamydomonas* FLA10 gene is associated with basal bodies and centrioles. *J. Cell Sci.* **109,** 541–549.

Voigt, J., and Munzner, P. (1987). The *Chlamydomonas* cell cycle is regulated by a light/dark-responsive switch. *Planta* **172,** 463–472.

Voigt, J., and Munzner, P. (1989). An extranuclear gene contributes to the regulation of cell division of the unicellular green alga *Chlamydomonas reinhardtii. Plant Sci.* **59,** 87–94.

Walther, Z., Vashishtha, M., and Hall, J. L. (1994). The *Chlamydomonas FLA10* gene encodes a novel kinein-homologous protein. *J. Cell Biol.* **126,** 175–188.

Warr, J. R. (1968). A mutant of *Chlamydomonas reinhardtii* with abnormal cell division. *J. Gen. Microbiol.* **52,** 243–251.

Warr, J. R., and Durber, S. (1971). Studies on the expression of a mutant with abnormal cell division in *Chlamydomonas reinhardtii. Exp. Cell Res.* **64,** 463–469.

Warr, J. R., and Gibbons, D. (1973). Effect of benzimidazole and cobalt on free cystine levels of *Chlamydomonas* wild type and cell division mutant strains. *Exp. Cell Res.* **78,** 454–456.

Warr, J. R., and Quinn, D. (1977). Low molecular weight sulphydryl compounds and the expression of a cell division mutant of *Chlamydomonas reinhardtii. Exp. Cell Res.* **104,** 442–445.

Weiss, R. L. (1984). Ultrastructure of the flagellar roots in *Chlamydomonas* gametes. *J. Cell Sci.* **67,** 133–143.

Wilson, P. J., and Forer, A. (1989). Acetylated alpha-tubulin in spermatogenic cells of the crane fly *Nephrotoma suturalis:* Kinetochore microtubules are selectively acetylated. *Cell Motil. Cytoskel.* **14,** 237–250.

Wolff, D. M. (1971). Isolation and partial characterization of temperature sensitive division mutants of *Chlamydomonas reinhardtii.* PhD thesis, Oregon State University, Corvallis.

Wright, R. L., Chojnacki, B., and Jarvik, J. W. (1983). Abnormal basal body number, location, and orientation in a striated fiber-defective mutant of *Chlamydomonas reinhardtii. J. Cell Biol.* **96,** 1697–1707.

Wright, R. L., Salisbury, J. L., and Jarvik, J. W. (1985). A nucleus-basal body connector in *Chlamydomonas reinhardtii* that may function in basal body localization or segregation. *J. Cell Biol.* **101,** 1903–1912.

Wright, R. L., Adler, S. A., and Jarvik, J. W. (1989). Nucleus-basal body connector in *Chlamydomonas:* Evidence for a role in basal body segregation and against essential roles in mitosis or in determining cell polarity. *Cell Motil. Cytoskel.* **14,** 516–526.

Wu, L., Hepler, P. K., and John, P. C. L. (1997). The *met1* mutation in *Chlamydomonas reinhardtii* causes arrest at mitotic metaphase with persisting p34 ^{cdc2}-like H1 kinase activity that can promote mitosis when injected into higher-plant cells. *Protoplasma* **199,** 135–150.

Cellular Aspects of Trophic Actions in the Nervous System

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During the past three decades the number of molecules exhibiting trophic actions in the brain has increased drastically. These molecules promote and/or control proliferation, differentiation, migration, and survival (sometimes even the death) of their target cells. In this review a comprehensive overview of small diffusible factors showing trophic actions in the central nervous system (CNS) is given. The factors discussed are neurotrophins, epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, insulin-like growth factors, ciliary neurotrophic factor and related molecules, glial-derived growth factor and related molecules, transforming growth factor-β and related molecules, neurotransmitters, and hormones. All factors are discussed with respect to their trophic actions, their expression patterns in the brain, and molecular aspects of their receptors and intracellular signaling pathways. It becomes evident that there does not exist "the" trophic factor in the CNS but rather a multitude of them interacting with each other in a complicated network of trophic actions forming and maintaining the adult nervous system.

KEY WORDS: Growth factors, Neurotrophins, Hormones, Neuritogenesis, Differentiation, Neurotransmitters.

I. Introduction

The adult brain is a complex intricate network of neurons which arises during development by an interaction of internal cell programs with environmental factors resulting in proliferation, commitment, migration, differentiation, and, in some instances, selective cell death. These interactions of environment with genetic determinants, however, do not cease in adulthood. Mature brain cells still critically depend on their environment and their genetic determinants. The actions of these environmental factors onto neurons could be termed trophic actions, and environmental factors show-

ing trophic actions could be termed trophic factors. The word trophic is derived from the greek word *trophikos,* meaning nourishment. Therefore, glucose, sodium, potassium, oxygen, and amino acids can also be regarded as trophic factors since they promote the survival of cells. However, in the sense that the term has been used during past decades, trophic factors must do more than simply ensure the basic cell metabolism. They must promote and/or control proliferation, differentiation, migration, and survival of their target cells. Since the term controlling also includes actions such as cessation of proliferation or migration, or initiation of programmed cell death, not only do trophic factors act in a positive manner but also the action of a trophic factor may result in a negative outcome for a given cell. In this context, a net "positive" influence is achieved via the attainment of appropriate cell numbers and connectional relationships.

In this review we will apply the latter definition of trophic action and describe a variety of molecules which control the proliferation, differentiation, migration, and survival of cells in the nervous system. Despite the exclusion of numerous nutrient molecules, there exists a plethora of factors in the nervous system which elicit specific trophic actions. Serious inquiry into neutrophic factors began with the discovery of nerve growth factor (NGF), more than 40 years ago and the extensive characterization of its actions. The identification and characterization of NGF stimulated further studies which identified other novel neurotrophic factors and novel neurotrophic actions of previously identified molecules. Novel proteins included other members of the now well-known family of neurotrophins, with distinct and overlapping actions in the brain, such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5. These factors have a relatively narrow target cell specificity. They act, for the most part, exclusively in the nervous system. Other factors primarily identified in the brain and exhibiting trophic actions are neurotransmitters, such as dopamine and noradrenalin, which exert their effects predominantly during development. In addition, peptide growth factors whose trophic actions were originally identified in "nonneural" tissues have been shown to act in the nervous system. These factors include epidermal growth factor (EGF) and members of its family, fibroblast growth factor (FGF) and members of its family, insulin and the insulin-like growth factors I and II (IGF-I and IGF-II), and platelet-derived growth factor (PDGF). These factors exert their effects primarily during early development and mostly act as mitogenic factors, as they do in other tissues. During the past several years cytokines derived from the hematopoetic and immune system have received increasing attention as trophic factors in the brain. In fact, there exist two cytokines which exert their actions predominantly in the nervous system: ciliary neurotrophic factor (CNTF) and glial-derived neurotrophic factor (GDNF). Other members of the cytokine family are also important for the develop-

ment and maintenance of proper neuronal activity. These factors include the transforming growth factor-β (TGF-β) superfamily, bone morphogenetic proteins (BMPs), hematolymphopoetic factors including the interleukins and the colony-stimulating factors, the tumor necrosis factor family, and the interferons. Finally, hormones, specifically estrogen and thyroid hormone, have also been shown to exert trophic actions in the nervous system. Proteins of the extracellular matrix and cell adhesion molecules also deserve mention. These are more critically involved in neuronal process formation and guidance.

It should be apparent from the previous discussion that any attempt to review the general area of "trophic actions" in the nervous system will necessarily require the consideration of a large variety of rather diverse molecular species. Furthermore, it is not possible to group these factors according to their actions (some factors initially identified by their survival-promoting activity may have other actions that are equally important), nervous system specificity (some factors show trophic actions in the brain but also in other tissues), and/or intracellular signaling components (many of the intracellular signaling components are not clearly understood). Finally, the trophic actions of certain factors have been shown only *in vitro,* and confirmatory *in vivo* studies are lacking. In order to achieve a balance between comprehensive treatment of the subject at hand and the great diversity of the molecules of interest, we will restrict this review to diffusable growth factors, thereby excluding the large group of proteins related to the extracellular matrix. We have organized the trophic agents into four major groups as summarized in Table I. The first group are the growth

TABLE I

Molecules Showing Trophic Actions in the Developing and Adult Brain

Growth factors		Cytokine-related family	Neurotransmitter	Hormone
Narrow specificity (neurotrophins)	Broad specificity			
NGF	EGFs	CNTF	Catecholamines	Estrogene
BDNF	FGFs	GDNF	GABA	Thyroid hormone
NT-3	PDGFs	TGF-β	Glutamate	
NT4/5	IGFs	BMPs	Neuropeptides	
NT-6		Interleukins	ACh	

[a] For details see text. NGF, nerve growth factor; BDNF, brain-derived growth factor, NT-3, NT4/5, NT-6, neurotrophins 3, -4/5, and -6; EGF, epidermal growth factor, FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; IGF, insulin-like growth factor; CNTF, ciliary neurotrophic factor; GDNF, glial-derived neurotrophic factor; TGF-β: transforming growth factor-β; BMP, bone morphogenetic proteins; GABA, γ-aminobutyric acid; ACh, acetylcholine.

factors acting through receptor tyrosine kinases. We further subdivided these into those factors that are relatively specific to the nervous system, the neurotrophins, and those with broader specificity. The second group constitutes the cytokine-related molecules. The remaining groups consist of neurotransmitters and hormones. Each group will be discussed in a way that the reader will be provided information about molecular aspects of the ligands and their receptors, the expression patterns of ligands and receptors in the central nervous system (CNS), the trophic actions, and aspects of their intracellular signaling pathways.

II. Growth Factors with Narrow Tissue Specificity: Neurotrophins

The neurotrophins are a well-studied family of structurally related polypeptides that promote differentiation and survival of specific neuronal populations. NGF was the first neurotrophin to be identified and characterized (Levi-Montalcini, 1987). Subsequently, BDNF, NT-3, and NT-4/5 were cloned and sequenced (Lewin and Barde, 1996). These proteins bear considerable sequence similarity to one another at both the nucleic acid and amino acid levels. Distinct high-affinity (TrkA, TrkB, and TrkC) and low-affinity (p75) neurotrophin receptors have also been identified (Ip and Yancopoulos, 1996; Barbacid, 1994). NGF was originally observed as a trophic activity released from a mouse sarcoma that increased the number and size of sensory and sympathetic neuronal perikarya as well as their fiber proliferation in the developing chick (Levi-Montalcini, 1987; Bueker, 1948; Levi-Montalcini and Hamburger, 1951). NGF was subsequently purified from the male mouse submaxillary gland, where it was secreted as a high-molecular-weight complex of sedimentation coefficient 7S (Varon *et al.,* 1967b) composed of (i) one β subunit (also known as β-NGF), which in turn is composed of two identical 118 amino acid chains (the β peptides) that are associated noncovalently (Varon *et al.,* 1967a); (ii) two γ subunits, which are serine proteases (Mason *et al.,* 1983); and (iii) two α subunits, which also bear homology to serine proteases but are catalytically inactive (Isaackson *et al.,* 1984). 7S NGF is biologically inactive, whereas dissociation of the α and γ subunits from the complex releases β-NGF which is active (Varon *et al.,* 1967b). The mature β-NGF is in some instances further processed through removal of the amino-terminal octapeptide to produce 2.5S NGF, which possesses identical biological activities to those of β-NGF (Moore *et al.,* 1974; Angeletti and Bradshaw, 1971; Mobley *et al.,* 1976). Cloning and sequencing of the cDNA (Scott *et al.,* 1983) and gene (Selby *et al.,* 1976) for the β peptide revealed that it is initially synthesized as a

large protein precursor coded by at least four mRNA transcripts generated by the use of alternate promoters and differential splicing. When the crystal structure of murine β-NGF was solved at 2.3 Å resolution, each NGF β peptide was found to contain three pairs of antiparallel β strands that are stabilized by hydrogen and disulfide bonds (McDonald *et al.,* 1991). These β strands form a flat interface at which two NGF β peptides associated through hydrophobic interactions to form the dimeric β-NGF. A total of four hairpin loops are also present, which are expressed on the surface of the β-NGF dimer. These exposed hairpin loops are the regions of greatest sequence divergence between different neurotrophin family members and participate in mediating specific interactions between particular neurotrophins and their cognate receptor(s) (McDonald *et al.,* 1991; Ibanez *et al.,* 1992).

BDNF was originally isolated from pig brain using a dorsal root ganglia neuronal survival assay (Barde *et al.,* 1982). NGF and BDNF were found to have partially overlapping biological effects since both are active on dorsal root ganglia neurons. Cloning and sequencing of the cDNA for BDNF revealed significant structural similarities with NGF (Leibrock *et al.,* 1989). The proteins share four regions of sequence identity or similarity, comprising >70% of the molecules, whereas the four hairpins show sequence divergence. Using the regions of sequence identity between NGF and BDNF to design oligonucleotide primers for PCR, NT-3 and NT-4/5 were subsequently identified. The amino acid sequences of NT-3 and NT-4/5 conform to the pattern of conserved and divergent sequences seen in NGF and BDNF. Finally, NT-6 has been identified in fish and appears to be the counterpart of NGF in this species (Gotz *et al.,* 1994).

The Trk proteins are receptor tyrosine kinases composed of extracellular ligand-binding domains and intrinsic cytoplasmic tyrosine kinase domains (Kaplan *et al.,* 1991a,b; Klein *et al.,* 1991a; Barbacid, 1994). A partial gene fragment for the first member of this family, *TrkA,* was originally isolated from a human colon carcinoma as a transforming chimeric gene produced by a fusion between a nonmuscle tropomyosin gene and a previously unidentified tyrosine kinase (Martin-Zanca *et al.,* 1986). The full-length receptor tyrosine kinase gene was subsequently isolated and called *Trk* (now *TrkA*) for tropomyosin receptor kinase. Analysis of the $p140^{trkA}$ primary amino acid sequence (Martin-Zanca *et al.,* 1989) revealed several features suggestive of a transmembrane receptor: (i) a 32-amino acid putative signal peptide; (ii) a 374-amino acid extracellular domain with 13 consensus N-glycosylation sites (Asn-X-Ser/Thr); (iii) a transmembrane domain; (iv) a cytoplasmic region with significant sequence similarity to previously identified tyrosine kinase domains, including an ATP-binding site and multiple tyrosine residues that may be targets for autophosphorylation; and (v) a

15-amino acid carboxy-terminal tail that also contains a tyrosine residue as has been seen in other growth factor receptors.

TrkB and TrkC were identified based on nucleotide sequence similarity with TrkA (Lamballe *et al.*, 1991; Klein *et al.*, 1989; Middlemas *et al.*, 1991). The structure of the full-length $p145^{trkB}$ protein is similar to that of $p140^{trkA}$, with an extracellular domain containing 12 N-glycosylation sites that is 37% identical to $p140^{trkA}$ at the amino acid level, a cytoplasmic tyrosine kinase domain that displays 75% amino acid identity to the analogous kinase domain in $p140^{trkA}$, and a relatively short 15-amino acid carboxy-terminal tail. Compared to TrkA, which is expressed exclusively as a 3.2-kb transcript (Martin-Zanca *et al.*, 1990), the TrkB is expressed as a series of at least eight mRNA transcripts of different sizes (Klein *et al.*, 1989; Middlemas *et al.*, 1991). These transcripts appear to represent three classes: (i) full-length receptor tyrosine kinases, (ii) receptors lacking either the kinase domain (whose protein product is designated $p95^{trkB}$) or the kinase and transmembrane domains, and (iii) cytoplasmic kinases lacking the extracellular receptor domain. The trkC protein product $p145^{trkC}$ displays approximately equivalent sequence similarity to both $p140^{trkA}$ and $p145^{trkB}$ (Lamballe *et al.*, 1991): The extracellular domain contains 14 N-glycosylation sites and is 54 and 53% similar to $p140^{trkA}$ and $p145^{trkB}$, respectively; the tyrosine kinase domain is 76 and 83% identical to $p140^{trkA}$ and $p145^{trkB}$, respectively; $p145^{trkC}$ also contains the short carboxy-terminal tail of 15 amino acids in which 8 amino acids are identical to those in the other two receptors, including a tyrosine five residues from the terminus. The *TrkC* gene is also expressed as multiple transcripts.

Some neurotrophins bind to multiple members of the Trk family and activate signal transduction, whereas some members of the Trk family are responsive to multiple neurotrophins. NGF binds exclusively to $p140^{trkA}$ (Klein *et al.*, 1991a; Kaplan *et al.*, 1991a), whereas BDNF and NT-4/5 bind exclusively to $p145^{trkB}$ (Klein *et al.*, 1991b, 1992; Soppet *et al.*, 1991; Squinto *et al.*, 1991; Berkemeier *et al.*, 1991; Ip *et al.*, 1992). NT-3 binds to $p145^{trkC}$ with high affinity (Lamballe *et al.*, 1991); in addition, NT-3 can also bind to $p140^{trkA}$ and $p145^{trkB}$ with somewhat lower affinities (Klein *et al.*, 1991b; Soppet *et al.*, 1991; Squinto *et al.*, 1991; Lamballe *et al.*, 1991). Nonetheless, NT-3 binding to these other two receptors can still elicit biological effects (Squinto *et al.*, 1991; Lamballe *et al.*, 1991; Cordon-Cardo *et al.*, 1991).

In addition to the high-affinity Trk receptors, all the neurotrophins can bind to a low-affinity receptor, $p75^{NTR}$, with dissociation constants of approximately 10^{-9} M (Ernfors *et al.*, 1990; Hallbook *et al.*, 1991; Rodriguez-Tebar *et al.*, 1990, 1992). The rat (Radeke *et al.*, 1987) and human (Johnson *et al.*, 1986) cDNA and genomic clones for this receptor were originally isolated using antibodies that could alter NGF–$p75^{NTR}$ interactions. The deduced amino acid sequence from the rat cDNA clone (Radeke *et al.*,

1987) predicts a protein of 426 amino acids with an extracellular domain containing four 25- to 28-amino acid long cystein-rich repeats and two putative N-glycosylation sites, a putative 22-amino acid transmembrane domain, and a cytoplasmic domain of 151 amino acids. The extracellular domain of the $p75^{NTR}$ has sequence similarity with the tumor necrosis factor (TNF) receptor and may function to increase TrkA signaling in the presence of low concentrations of NGF (Barker and Shooter, 1994). This possibility is consistent with the observation that $p75^{NTR}$-deficient mice display a decreased number of sensory neurons that are NGF dependent (Lee *et al.,* 1992). $p75^{NTR}$ may also reduce the responsiveness of TrkA to NT-3 (Clary and Reichardt, 1994). Activation of signaling pathways by $p75^{NTR}$ are discussed in more detail later.

Neurotrophins exert multiple effects during neuronal development and differentiation, including controlling proliferation of neuronal precursors (Barres *et al.,* 1994); regulating survival of neuroblasts and O_2A glial progenitors (Birren *et al.,* 1993; DiCicco-Bloom *et al.,* 1993; Barres *et al.,* 1994); regulating neuronal differentiation (Sieber-Blum, 1991; Nawa *et al.,* 1993; Ip *et al.,* 1993a); controlling axonogenesis, sprouting, and innervation (Hoyle *et al.,* 1993; Schnell *et al.,* 1994); and, in the mature nervous system, regulating long-term potentiation and other forms of synaptic communication (Korte *et al.,* 1995; Patterson *et al.,* 1996; Kang and Schuman, 1995). Studies aimed at deciphering the complexities of neurotrophin receptor signaling, and the subsequent biological effects, have relied heavily on the use of transgenic and "knock-out" mice, as well as *in vitro* model systems.

Approximately 50% of dorsal root ganglion (DRG) sensory neurons express TrkA (McMahon *et al.,* 1994), whereas there is an approximately 70% loss of DRG neurons in TrkA-deficient or NGF-deficient mice (Smeyne *et al.,* 1994; Crowley *et al.,* 1994). Interestingly, in NT-3-deficient mice there is a 60% loss of DRG neurons, including a marked deficit in group Ia afferent sensory neurons (Farinas *et al.,* 1994; Ernfors *et al.,* 1994; Airaksinen *et al.,* 1996). In comparison, there is only about a 20% loss of DRG neurons in TrkC-deficient mice (Klein *et al.,* 1994; Fagan *et al.,* 1996), suggesting important physiologic roles of NT-3 during development that may involve signaling through TrkA or TrkB. In support of this hypothesis, neurons from the trigeminal and nodose ganglions of TrkC-deficient mice are maintained by NT-3 *in vitro*, presumably via signaling through TrkA and TrkB receptors, respectively (Davies *et al.,* 1995). A similar mechanism appears to be present in sympathetic neurons. More than 95% of the superior cervical ganglion neurons are lost in both NGF- and TrkA-deficient mice (Smeyne *et al.,* 1994; Fagan *et al.,* 1996), whereas 50% are lost in NT-3-deficient, but not TrkC-deficient mice (Klein *et al.,* 1994; Fagan *et al.,* 1996; Farinas *et al.,* 1994; Ernfors *et al.,* 1994; Airaksinen *et al.,* 1996). In summary, development of many sensory and sympathetic neuronal popula-

tions is dependent on the classic target-derived neurotrophic factor NGF acting through TrkA; in addition, NT-3 functioning through its nonpreferred receptors TrkA or TrkB also appears to exert significant trophic effects on these populations. In contrast, there is no obvious deficit in motor neurons, which express TrkB and TrkC but not TrkA (Henderson *et al.,* 1993; Yan *et al.,* 1993), in mice deficient in BDNF, NT-3, or NT-4/5 (Ernfors *et al.,* 1994; Jones *et al.,* 1994; Farinas *et al.,* 1994; Conover *et al.,* 1995). These findings suggest that these neurotrophins are not critical for motor neuron development, which may instead be dependent on trophic agents such as CNTF, leukemia inhibitory factor (LIF), GDNF, or others (Li *et al.,* 1996; DeChiara *et al.,* 1995; Moore *et al.,* 1996; Sanchez *et al.,* 1996). The critical trophic interactions during the development of CNS neurons are also unclear. The neurotrophins and their receptors are expressed in diverse, and partially overlapping, neuronal populations in the cortex, including hippocampus, and the subcortex (Yan *et al.,* 1997; Katoh-Semba *et al.,* 1997; Hayashi, 1996; Katoh-Semba *et al.,* 1996; Anderson *et al.,* 1995). This complex pattern of expression suggests that rather than a strict "one neurotrophin–one target population" relationship, the neurotrophins may act in combinatorial or redundant fashions to support the development, differentiation, and survival of diverse neuronal populations. This appears to be the case in one well-studied neuronal circuit, the septohippocampal pathway. The basal forebrain cholinergic neurons, which are responsive to NGF and BDNF *in vitro,* express TrkA and TrkB receptors. In addition, NGF and BDNF are expressed in the hippocampal and cortical target fields of these cholinergic neurons. However, there are no obvious cholinergic neuronal deficits in mice with knockouts of NGF, BDNF, TrkA, and TrkB, supporting the redundancy of these trophic mechanisms. Recent studies suggest that there may be increased apoptosis in some CNS populations in TrkB knockouts and TrkB–TrkC double knockouts (Minichiello and Klein, 1996).

Neurotrophin signaling (Fig. 1) has been most well studied for NGF, in which binding of NGF to TrkA results in TrkA dimerization, tyrosine kinase activation, and autophosphorylation (Segal and Greenberg, 1996; Kaplan and Stephens, 1994). After tyrosine autophosphorylation, TrkA recruits three proteins to the cytoplasmic face of the PC12 cell plasma membrane (Basu *et al.,* 1994). The interactions necessary to form this tetrameric protein complex are largely dependent on the presence of phosphotyrosine residues and src homology (SH) domains in these proteins. This tetramer consists of (i) phosphorylated TrkA; (ii) phosphorylated Shc (*S*rc *h*omology and *c*ollagen, an "adapter" phosphoprotein with an SH2 domain); (iii) Grb2 (*g*rowth factor *r*eceptor-*b*ound protein 2, an SH2 and SH3 domain-containing adapter protein); and (iv) mSOS (*m*ammalian homolog of *Drosophila son of sevenless*, a guanine nucleotide exchange factor

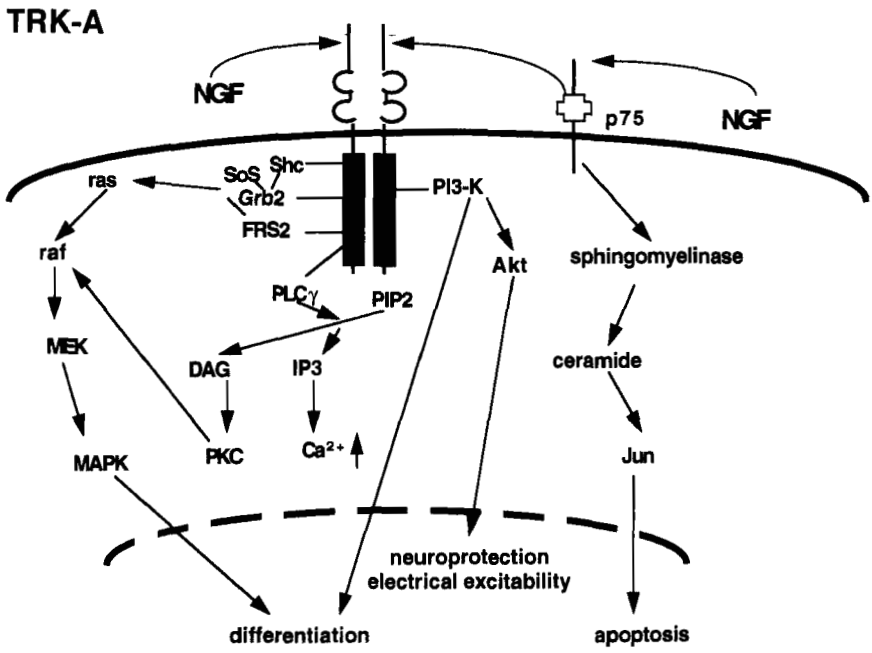

FIG. 1 Proposed intracellular signaling pathways for NGF-mediated neuronal differentiation, neuroprotection, excitability, and apopotosis (for details see text).

that stimulates Ras activity) (Basu *et al.*, 1994; Segal and Greenberg, 1996; Stephens *et al.*, 1994; Ohmichi *et al.*, 1994; Rozakis-Adcock *et al.*, 1992; Suen *et al.*, 1993). The SH2 domain of SHC allows it to bind to a specific phosphotyrosine residue on the cytoplasmic tail of TrkA. Upon association with phosphorylated TrkA, Shc also becomes tyrosine phosphorylated and can then be bound by the SH2 domain of Grb2. Grb2, in turn, associates through its SH3 domain with a proline-rich domain of mSOS, resulting in a membrane-anchored tetrameric complex (Egan *et al.*, 1993; Rozakis-Adcock *et al.*, 1992; Ohmichi *et al.*, 1994; Basu *et al.*, 1994; Segal and Greenberg, 1996). An analogous complex forms in cells treated with EGF composed of phosphorylated EGF receptor (EGFR)–Shc–Grb2–mSOS (Batzer *et al.*, 1994; Suen *et al.*, 1993). In some studies, Grb2 has also been shown to associate directly with the EGFR (Batzer *et al.*, 1994; Suen *et al.*, 1993), resulting in an EGFR–Grb2–mSOS heterotrimeric complex.

Once anchored to the cell membrane by either TrkA or EGFR, mSOS catalyzes the conversion of membrane-bound Ras, a small G protein, from the GDP-bound (inactive) to the GTP-bound (active) form. Ras activity is important for NGF signal transduction, as shown by studies in which

microinjection of anti-Ras antibodies into PC12 cells blocked neuritogenesis (Hagag *et al.,* 1986; Kremer *et al.,* 1991). In neuronal cells, such as PC12 cells, active GTP-Ras interacts with Raf-1 and B-Raf, members of the Raf serine/threonine kinase family (Jaiswal *et al.,* 1994). Small G proteins other than Ras, such as Rap and Rheb, can also regulate Raf kinases and may function to integrate growth factor and cAMP/PKA-mediated signaling (Vossler *et al.,* 1997; Yamagata *et al.,* 1994; Yee and Worley, 1997). Activated Raf then associates with Ras and activates mitogen-activated protein kinase kinase (MEK or MAPKK) (Ohmichi *et al.,* 1994). Once phosphorylated, MEK becomes active and phosphorylates mitogen-activated protein kinase (MAPK or ERK) (Chen *et al.,* 1993). MAPK is activated upon phosphorylation and in turn phosphorylates a number of cellular proteins, including ribosomal S6 kinase 2 (RSK2) (Chen *et al.,* 1992; Xing *et al.,* 1996). Both activated MAPK and RSK2 can translocate from the cytoplasm to the nucleus (Nguyen *et al.,* 1993), where studies suggest they can regulate various transcription factors (Chen *et al.,* 1992). The importance of this signaling pathway has been demonstrated by experiments in which NGF actions are blocked by dominant-negative mutants of Ras (Szeberenyi *et al.,* 1990; D'Arcangelo and Halegoua, 1993; K. Wood *et al.,* 1992; Szeberenyi *et al.,* 1992), Raf (Cosgaya and Aranda, 1996), MEK (Cowley *et al.,* 1994), or MAPK (Vossler *et al.,* 1997). In addition, constituitive overexpression of TrkA (Hempstead *et al.,* 1992), SHC (Rozakis-Adcock *et al.,* 1992), Ras (Bar Sagi and Feramisco, 1985; D'Arcangelo and Halegoua, 1993; Sassone-Corsi *et al.,* 1989; K. Wood *et al.,* 1992), Raf (D'Arcangelo and Halegoua, 1993; Wood *et al.,* 1993), MEK (Cowley *et al.,* 1994), or MAPK (Fukuda *et al.,* 1995) has been shown to produce effects similar to NGF treatment.

The previously described signaling pathway from Ras to MAPK and RSK2 cannot account for all actions of NGF. For example, while PC12 cells expressing constituitively active Ras, Raf, or MEK elaborate neurites, the kinetics are slower than those of wild-type cells treated with NGF (D'Arcangelo and Halegoua, 1993; Cowley *et al.,* 1994). These transfected PC12 cells also fail to express NGF-regulated genes such as Thy-1, a differentiation-related antigen, and sodium channels (D'Arcangelo and Halegoua, 1993; Leonard *et al.,* 1988). Thus, NGF must also activate other signaling mechanisms for complete differentiation responses. Recent studies have shown that in addition to the SHC adapter, NGF-activated TrkA also associates with phospholipase C-γ1 (PLC-γ1) (Obermeier *et al.,* 1993a; Stephens *et al.,* 1994; Vetter *et al.,* 1991) and phosphatidyl inositol 3-kinase (PI3-K) (Obermeier *et al.,* 1993b; Ohmichi *et al.,* 1992). PLC-γ1 catalyzes the formation of diacyl glycerol (DAG) and inositol triphosphate (IP3), thereby activating protein kinase C (PKC) and increasing intracellular calcium concentration (Berninger *et al.,* 1993; Berridge, 1993). PKC, in

turn, appears to phosphorylate and activate Raf (Kolch *et al.,* 1993). This pathway appears important since mutant TrkA receptors that cannot bind SHC can still activate Ras and produce neurite outgrowth. However, if TrkA is further mutated so that it also does not bind PLC-γ1, NGF-induced Ras activation and neurite outgrowth are blocked (Stephens *et al.,* 1994). In addition, selected aspects of the NGF response, such as transcription of the NGF-inducible gene peripherin, appear to be PLC-γ1 dependent and Ras independent (Loeb *et al.,* 1994). Several studies have also established an important role for PI3-K in NGF actions. Treatment of cells with the pharmacologic PI3-K inhibitor wortmannin, or transfection of cells with a dominant-negative p85 PI3-K regulatory subunit, blocks NGF-induced neurite outgrowth and also abolishes NGF-mediated PC12 survival (Jackson *et al.,* 1996; Kimura *et al.,* 1994). This latter finding is consistent with studies in which PI3-K has been shown to activate protein kinase B or Akt kinase), a protein that promotes cell survival by inhibiting apoptosis (Burgering and Coffer, 1995; Franke *et al.,* 1995).

Recently, p75-activated signal transduction pathways have been identified. Whereas TrkA primarily mediates the trophic actions of NGF (survival and neurite outgrowth), p75 appears to play a role in apoptosis by regulating sphingomyelinase activity, ceramide production, and nuclear factor-κB activation (Casaccia-Bonnefil *et al.,* 1996). In some cases the p75 pathway initiates apoptosis, but the specific response observed appears to vary with the type of neurotrophin and Trk present as well as the cell type (Bredesen and Rabizadeh, 1997; Dechant and Barde, 1997; Carter and Lewin, 1997).

NGF-induced differentiation of PC12 cells is blocked by inhibitors of RNA and protein synthesis (Greene *et al.,* 1982; Burstein and Greene, 1978). Thus, the regulation of gene expression appears to be critical for NGF actions. The genes that are upregulated in PC12 cells in response to NGF have been classified as immediate early genes (IEGs) and delayed response genes (DRGs). In addition, NGF also appears to control some cell cycle genes that may be important for neuronal differentiation (Yan and Ziff, 1995, 1997). The IEGs, including c-*fos,* c-*jun, egr*-1 (or *NGFI-A, TIS8, zif268,* and *krox24*) and *nur77* (or *NGFI-B* and *TIS1*), display rapid but transient activation of transcription within minutes of NGF exposure, in a manner independent of new protein synthesis (Greenberg *et al.,* 1985; Milbrandt, 1987; Bartel *et al.,* 1989). The NGF-induced expression of the IEG c-*fos* has been well characterized. After NGF treatment of PC12 cells, the c-*fos* promoter is bound by the transcription factors SRF (*s*erum *r*esponse *f*actor), Elk-1, and CREB (*c*AMP-*r*esponse *e*lement *b*inding protein) (Ginty *et al.,* 1994). CREB is a member of a large family of transcription factors characterized by the presence of both basic DNA-binding domains and leucine zipper dimerization motifs (Lee and Masson, 1993; Sassone-Corsi, 1995; Meyer and Habener, 1993). CREB is activated by phosphoryla-

tion (Ginty *et al.,* 1994) and appears to be important for c-*fos* induction since mutations in the CREB binding sites decrease NGF induction of c-*fos,* even when the other elements are intact (Bonni *et al.,* 1995). The participation of Elk-1 and CREB in the regulation of c-*fos* expression provides a link between activation of the Ras to MAPK–RSK2 cascade and changes in gene expression: As discussed previously, NGF-activated MAPK and RSK2 can translocate from the cytoplasm to the nucleus and have been shown to phosphorylate the Elk-1 and CREB transcription factors, respectively (Xing *et al.,* 1996; Chen *et al.,* 1992; Ginty *et al.,* 1994; Miranti *et al.,* 1995). While some of these IEGs, such as c-*fos,* may prove necessary for NGF effects, they are not sufficient since they are also induced by EGF (Greenberg *et al.,* 1985).

In contrast to IEGs, induction of DRGs, such as peripherin (Leonard *et al.,* 1988), *GAP43* (Federoff *et al.,* 1988), type IIa sodium channels (Mandel *et al.,* 1988), and *VGF* (De Rocco *et al.,* 1997; Hawley *et al.,* 1992; Luc and Wagner, 1997), exhibits more delayed kinetics and requires new protein synthesis. The protein products of DRGs are determinants of neuronal differentiation that mediate many of the phenotypic changes seen in NGF-treated PC12 cells. The promoters of several NGF-activated DRGs contain multiple CREB binding sites that appear necessary for NGF-induced expression (De Rocco *et al.,* 1997; Hawley *et al.,* 1992; Mandel *et al.,* 1988; Federoff *et al.,* 1988). It has been proposed that phosphorylated CREB may cooperate with IEG proteins to regulate expression of DRGs (Segal and Greenberg, 1996; Bonni *et al.,* 1995).

As stated previously, both NGF and EGF activate the Ras–RSK2 pathway and induce the expression of the same IEGs. However, while NGF causes a cessation of cell division and induction of differentiation, PC12 cells continue to proliferate in the presence of EGF. Thus, PC12 cells represent an ideal model for identifying which signaling components are critical for activating the program of neuronal differentiation and which represent a common response to all growth factors. Recent studies have demonstrated that one way PC12 cells may discriminate between differentiating and mitogenic factors is by the duration of the signal which they activate. While EGF activates Ras and MAPK for less than 1 h, NGF activates this signaling pathway for up to 48 h (Qiu and Green, 1992; Marshall, 1995). The difference in signal duration appears to be due to a recently identified protein, FRS2 (*F*GF *r*eceptor *s*ubstrate *2*), that is phosphorylated after NGF but not EGF treatment (Kouhara *et al.,* 1997). After ligand-induced autophosphorylation, both TrkA and EGFR are dephosphorylated, disrupting the multimeric complex that anchors mSOS (Kaplan *et al.,* 1991b). However, in NGF-treated cells, phosphorylated FRS2 can form a complex that may serve as an alternate membrane anchor to retain mSOS at the plasma membrane and maintain Ras activation.

FRS2 may be identical to SNT (*s*uc-associated *n*eurotrophic factor-induced *t*yrosine-phosphorylated target; Rabin *et al.*, 1993), a previously identified PC12 protein that is phosphorylated after NGF but not EGF treatment. FRS2(SNT) has been described as the "long-sought" (Kouhara *et al.*, 1997) protein that distinguishes the signaling pathways activated by NGF and EGF. FRS2(SNT) may also be critical for NGF-regulated changes in gene expression. For example, phosphorylation of CREB is slightly more prolonged in NGF than EGF-treated PC12 cells (2 h versus 30 min, respectively) (Bonni *et al.*, 1995). However, it has not been demonstrated that this difference in CREB phosphorylation is necessary for NGF-activated neuronal differentiation.

III. Growth Factors with Broad Tissue Specificity: EGFs, FGFs, PDGFs, and IGFs

A. Epidermal Growth Factor Family

EGF stimulates the proliferation of various types of cells of ectodermal and mesodermal origin (Carpenter and Cohen, 1979). It shares structural similarities with TGF-α-amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), β-cellulin (BTC), and the neu differentiation factor, also termed heregulin (NDF/HRG; Corfas *et al.*, 1995). NDF is identical to a factor with acetylcholine receptor-inducing activity (Falls *et al.*, 1993) and members of the neuregulin family. The following discussion will focus on EGF but point out important and interesting differences with the other trophic factors within this family.

EGF is a single polypeptide composed of 53 amino acid (aa) residues (Savage *et al.*, 1972), with three biologically important intramolecular disulfide bonds. It is derived from a 1217-aa glycosylated transmembrane protein precursor (Breyer and Cohen, 1990) by proteolytic processing. The large precursor also has the capability of binding to the EGF receptor (EGFR), which raises the possibility that these membrane-anchored precursors have an unknown physiological function (van der Geer *et al.*, 1994). The EGFR (Fig. 2) is a 170-kDa membrane-spanning protein, with two fibronectin III repeats at the extracellular sites and the catalytic site situated in the cytoplasm. Following receptor binding, the receptors undergo conformational changes and dimerize. The two cytoplasmic domains, which are intracellular tyrosine kinases, then cross- and/or autophosphorylate (van der Geer *et al.*, 1994). These intracellular tyrosine kinase domains show a high level of sequence homology with the oncogene product of the avain erythroblastosis virus, erbB (Hayman and Enrietto, 1991). Therefore, the receptor for EGF

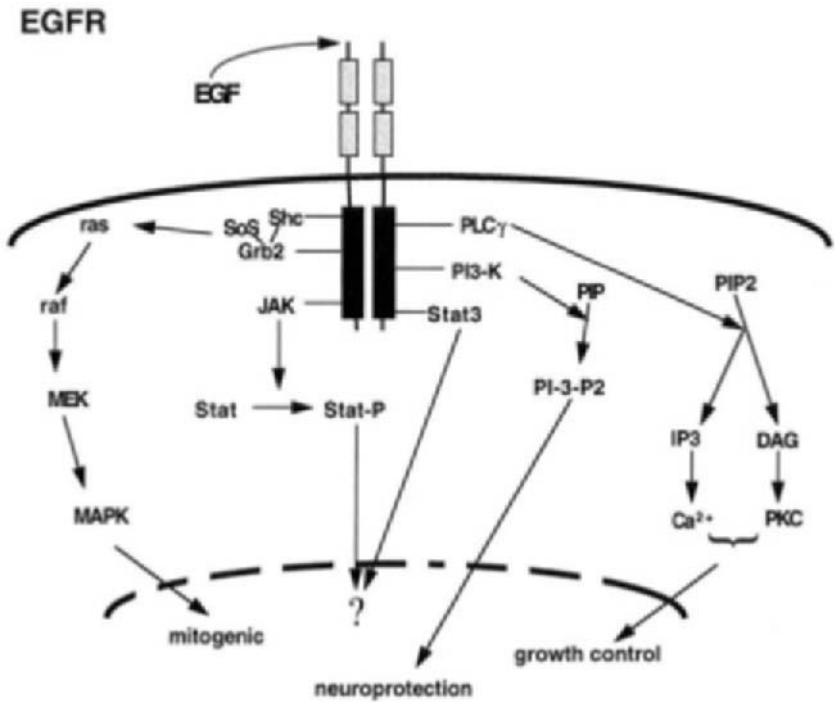

FIG. 2 Proposed intracellular signaling pathways for EGF-mediated proliferation and neuroprotection. The biological relevance of the activation of the Stat/Jak pathway is not yet determined (for details see text).

is also called ErbB-1. The EGFR family also consists of the structurally related receptors Neu/ErbB-2/HER2, ErbB-3/HER3, and ErbB-4/HER4. EGF, TGF-α, AR, HB-EGF, and BTC all bind to ErbB-1 (Shing *et al.,* 1993). NDF/HRG is the ligand for ErbB-3 and ErbB-4 (Plowman *et al.,* 1993). ErbB-2 is an orphan receptor but NDF/HRG can induce the tyrosine phosphorylation of ErbB-2 in some cells (Peles *et al.,* 1993). Following activation by their ligands the ErbB receptors heterodimerize following a strict hierarchy in which ErbB-2 is the preferred heterodimerization partner of all ErbB proteins (Graus-Porta *et al.,* 1997).

Both receptors and ligands are expressed in the CNS. EGF mRNA was detected by solution-hybridization ribonuclease protection assays throughout the brain—in the brain stem, cerebellum, cerebral cortex, hippocampus, basal hypothalamus, olfactory bulb, striatum, and thalamus. The highest amount of EGF mRNA is found in the olfactory bulb, striatum, and cerebellum. It is detected as early as E14 and continues to be expressed into adulthood. Interestingly, the mRNA of TGF-α, which also binds to EGFR

(ErbB-1), is detected in the same regions as EGF but at much higher levels during all developmental stages (Lazar and Blum, 1992). Furthermore it is also found in various regions of the white matter, such as the corpus callosum, anterior commissure, external and internal capsules, lateral olfactory tract, and optic tract, indicating its presence not only in neurons but also in glial cells. NDF/HRG mRNA is widely distributed and located in both neurons and glial cells in the rat brain (Pinkas-Kramarski *et al.,* 1994). One interesting feature of its distribution is that it is expressed in all cholinergic neurons throughout the brain, although it is not restricted to these populations (Corfas *et al.,* 1995).

Binding studies in brain extracts revealed that EGF binding sites were first detected at E15 and reach highest levels perinatally (Adamson and Meek, 1984). The total number of binding sites decreases with age. However, immunostaining for the EGFR revealed a decrease of immunoreactivity in the glial population, whereas immunoreactivity in neurons remains high into adulthood, suggesting that the observed decrease in binding sites is mainly due to a decrease in EGFR expression in glial cells. EGFR immunoreactivity was identified in cerebral cortical neurons, Purkinje cells, and the CA fields and dentate gyrus of the rat hippocampus (Gomez-Pinilla *et al.,* 1988; Tucker *et al.,* 1993). Seroogy *et al.* (1995) showed that EGFR is expressed in the forebrain ventricular/subventricular zone and cerebellar external granule layer, the principal germinal zones of the developing brain. Other members of the EGFR family—ErbB-2, ErbB-3, and ErbB-4—are also present in the CNS (Reynolds and Weiss, 1992). These localization studies strongly suggest that EGF plays a regulatory and modulatory role in the developing brain and that part of its trophic actions are still valid in the adult brain. Specifically, they suggest a trophic role of EGF family members on neural precursor cells.

Indeed, EGF has become well-known as a trophic factor in the developing and mature CNS because of its mitogenic effects on neural precursor cells (Reynolds and Weiss, 1992, 1996; Kuhn *et al.,* 1996, 1997; Craig *et al.,* 1996). A rapidly dividing population of stem cells in the subventricular zone (SVZ) of the lateral ventricle generates all neural cell types: neurons, astrocytes, and oligodendrocytes. Even in the adult, neural precursor cells continue to proliferate and differentiate (Kuhn *et al.,* 1996). Recently, several groups successfully isolated and propagated such adult neural progenitor cells from the SVZ and the hippocampus (Reynolds and Weiss, 1992; Gage *et al.,* 1995a,b). These cells require basic FGF (bFGF) and/or EGF for proliferation and long-term survival. Interestingly, Kuhn *et al.* (1997) showed that in contrast to the *in vitro* situation, EGF *in vivo* increases the number of glial cells (olfactory bulb and hippocampus) but has a limiting effect (antimitogenic) on the generation of neurons. These results suggest that the physiological effect, and probably the specific signal transduction elicited by

EGF, is cell type specific and/or modulated by the cell's specific environment.

In addition to mitogenic effects on precursor cells, EGF also acts on several postmitotic neuronal populations by enhancing their differentiation, maturation, and survival (Morrison, 1993). In these cases, however, it has to be determined whether EGF acts directly on target cells or indirectly via glial cells. A number of glial cell-mediated actions of EGF have been reported. These actions include an increase in the activity of choline acetyltransferase and the number of acetylcholinesterase-positive neurons in low-density cultures of fetal rat medial septal cells (Yokoyama *et al.,* 1994) as well as an increase in the survival rate and maturation of dopaminergic neurons. In these cells EGF also stimulates neurite outgrowth and dopamine uptake. It also inhibits MPP^+-induced death of dopaminergic neurons (Casper *et al.,* 1991, 1994; Park and Mytilineou, 1992). γ-Aminobutyric acid (GABAergic) mesencephalic neurons show increased GABA uptake following EGF treatment, and septal cells show increased glutamic acid decarboxylase (GAD) activity. These neurons possess specific EGF binding sites (Mazzoni and Kenigsberg, 1994) suggesting that these effects may be direct. Similarly, direct EGF actions have been proposed in mediating the protection of neonatal rat hippocampal neurons against anoxia and nitric oxide toxicity (Maiese *et al.,* 1993). The enhancement of survival and the stimulation of process outgrowth in cultured neonatal rat cerebellar (Morrison *et al.,* 1988) and cortical cultures (Kornblum *et al.,* 1990, 1995) is also thought to be a direct EGF effect. Other reported actions of EGF are an enhancement of long-term potentiation and NMDA receptor-mediated increase in intracellular calcium concentration (Abe *et al.,* 1991; Abe and Saito, 1992). Here, in regulating synaptic plasticity, EGF may be viewed more as neuromodulator than as a trophic factor.

EGF-mediated effects on glial cells appear to be both cell type and concentration dependent. In cultured astrocytes high concentrations of EGF are mitogenic, whereas low concentrations induce differentiation (Honegger and Guenbert-Lauber, 1983). EGF has also been reported to induce differentiation of oligodendrocytes as evidenced by the upregulation of the expression of the myelin basic protein (Almazan *et al.,* 1985).

As discussed previously the receptors for the EGF family become tyrosine phosphorylated following ligand-induced dimerization. These phosphorylated tyrosine residues serve as high-affinity binding sites for several secondary signaling molecules, such as Grb2, Shc, Nck, PLC-γ, Ras-GTPase-activating protein (Ras-GAP), and SHP-2, all of which contain the SH2 domain. These proteins transduce the activation of the EGFRs to a variety of intracellular signaling pathways (Fig. 2).

The best known signaling pathway activated by EGF is the MAPK pathway, as previously discussed. As stated previously, the activation of the

same signaling pathway by two different trophic factors (NGF and EGF) leading to different physiological responses is one of the puzzles of developmental neurobiology which remains to be solved. Cell type specificity of different signaling pathways could be one answer to this question. For example, in contrast to its action on PC12 cells, EGF acts on cultured cerebral cortical neurons not as a mitogen but as a differentiation factor. Furthermore, in these cells the activation of the MAPK pathway by EGF is sustained and not transient, as is the case for PC12 cells (Yamada *et al.,* 1995, 1996). However, like in PC12 cells, the mechanisms by which the activation of MAPK gets sustained remain to be elucidated.

In addition to the MAPK signaling pathway, other pathways are initiated by EGF. Again, these pathways are very similar to those activated by neurotrophins. Activated EGFR associates directly with PLC-γ, which becomes tyrosine phosphorylated. This phosphorylation increases the enzymatic activity of PLC-γ. Activated PLC-γ catalyzes the hydrolysis of the membrane phospholipid phosphatidylinositol-4,5-biphosphate, generating DAG and IP-3 (Fisher *et al.,* 1992). IP-3 releases calcium from intracellular stores, thereby affecting calcium-regulated processes in the cell. DAG activates members of the threonine/serine PKC family which are thought to be involved in growth control (Nishizuka, 1992). However, the role of this pathway in neurons is still under investigation.

PI3-K can also be activated by EGF (Raffioni and Bradshaw, 1992). This activation is mediated by phosphorylation of ErbB-3, which forms a heterodimer with EGFR. PI3-K then binds to the phosphorylated ErbB-3 and is thereby activated. There exist consensus sequences for the binding site of the SH2 domain of the regulatory subunit of PI3-K on ErbB-3 but not on EGFR (Soltoff and Cantley, 1996; Soltoff *et al.,* 1994). Activated PI3-K catalyzes the phosphorylation of the 3′ position of inositol in phosphatidylinositols, which then may act as second messengers and also activate members of the PKC family (Toker *et al.,* 1994). This signaling pathway induced by EGF could be involved in cell survival but not in differentiation (Yao and Cooper, 1995), as shown in PC12 cells stimulated with NGF.

Finally, EGF stimulates a pathway that involves activation of the nonreceptor tyrosine kinase Jak and the latent cytoplasmic transcription factors Stats. Activated Jak associates with the ligand receptor complex and phosphorylates the Stats, which translocate to the nucleus and induce gene transcription. EGF induces the tyrosine phosphorylation and activation of Stat1 and Stat3. *In vitro* Stat3 forms a stable complex with the receptor kinase (Zhong *et al.,* 1994; Sadowski *et al.,* 1993; Park *et al.,* 1996). The activation of the Stats may be controlled by EGF-activated Jak1 (Shuai *et al.,* 1993). However, the physiological significance of this pathway remains to be elucidated.

B. Fibroblast Growth Factor Family

Like the EGF family, members of the FGF family were first identified based on their broad mitogenic and cell survival activities (Eckenstein, 1994). The family is composed of at least 14 different members, which can be subdivided into two subgroups.

Members of the first subfamily of FGFs (FGF1–10), encoded by 10 genetically distinct genes, are between 155 and 267 aa residues in length, share a conserved central region of about 140 aa, forming a compact β-barrel with threefold symmetry. Interestingly, this structure closely resembles the structure of interleukin-1 (Zhu *et al.,* 1991). FGF-1 and FGF-2, also called acidic FGF (aFGF) and basic FGF (bFGF), exhibit the widest tissue distribution and were also the first to be identified and cloned (McKeehan *et al.,* 1998).

The second subfamily of FGFs [fibroblast growth factor homologous factors (FHF1–4)] has been described recently and members share about 30% amino acid identity with members of the first group, but 58–71% between themselves (Smallwood *et al.,* 1996). In contrast to the first subfamily, their biological activities in the nervous system are not as well defined. Like aFGF, and FGF-9, the FHFs lack the typical signal peptide for exocytosis at their amino terminus suggesting that they may be released from cells by an as yet not understood endoplasmic reticulum–Golgi independent secretory pathway (Smallwood *et al.,* 1996). In addition, like acidic and basic FGF, they contain clusters of basic residues, probably acting as nuclear localization signals. All FGFs exhibit a specific affinity for heparin and heparan sulfates, a characteristic which is important for their biological activity.

There exist two types of receptors for FGFs: the high-affinity receptor tyrosine kinases (FGFRs) and the low-affinity receptor cell surface proteoglycans (FGFHRs). The high-affinity, low-capacity FGFRs derive from a gene family composed of four members (FGFR1–FGFR4; McKeehan *et al.,* 1998; Partanen *et al.,* 1995). From these four genes a multitude of isoforms arises by alternative splicing and posttranslational modifications. The prototype of these receptors (Fig. 3) has three immunoglobulin-like domains in its extracellular ligand-binding part, followed by a transmembrane domain and the intracellular part containing two subdomains of the tyrosine kinase (ATP-binding site and catalytic domain) seperated by a short intervening sequence. Each receptor appears to be capable of binding a subset of growth factors and vice versa. Interestingly, the alternative splicing (more than 20 events described for FGFR1 and FGFR2) affects nearly every active domain (ligand binding and specificity, tyrosine kinase activity, etc.) of the receptors, resulting in differences in the receptors'

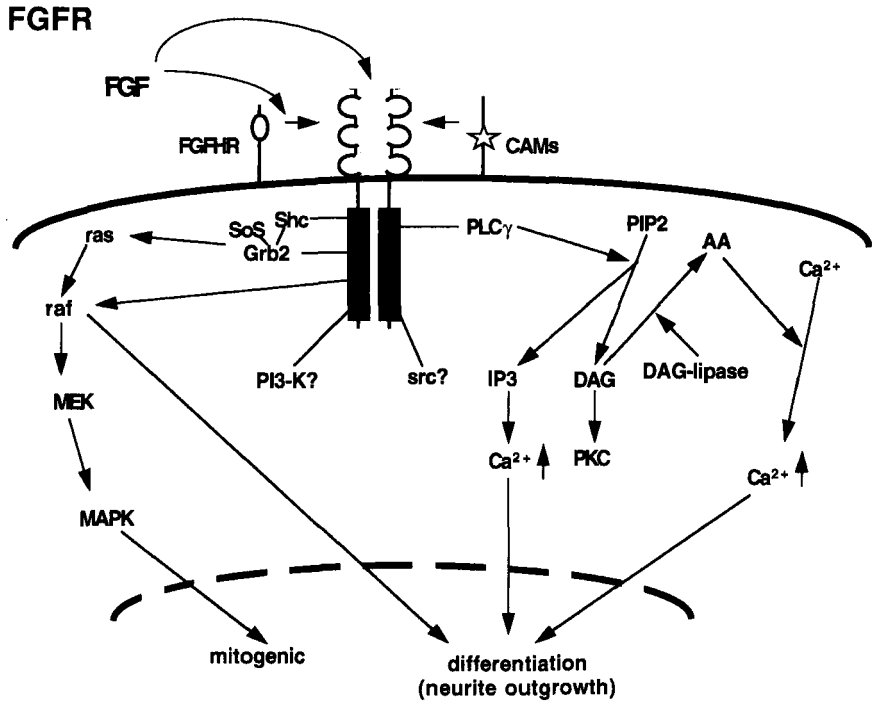

FIG. 3 Proposed intracellular signaling pathways for FGF-mediated proliferation and neuronal differentiation (for details see text). The biological relevance of the activation of P13-K and src kinases is not yet determined. Low-affinity receptors (FGFHR) cooperate with the high-affinity receptors (FGFRs), which can also be activated by cellular adhesion molecules (CAMs).

affinities for the distinct members of the ligand family and in many potentially functionally different receptors.

The low-affinity but high-capacity FGFHRs are proteoglycans containing heparan sulfates. The binding of these proteoglycans has been implicated in the stability of FGFs in the extracellular space since it has been shown that fragments of heparin exceeding five monosaccharides protect FGFs against proteolytic digestion (Luo *et al.*, 1996). Since a wide range of heparan sulfate-containing proteoglycans interact with FGFs and both types of FGF receptors have been shown to collaborate with each other, it has been suggested that the FGFHRs sequester FGFs in proximity to the FGFRs. The large number of these proteoglycans in tissues implies that FGFs are unlikely to exist in an unbound form in the extracellular space. This notion is reasonable since only low levels of free FGFs are found outside cells.

FGFs and FHFs are widely expressed in the developing and adult nervous system (Smallwood *et al.,* 1996; Grothe and Wewetzer, 1996; Ozawa *et al.,* 1996). The best studied examples, bFGF and aFGF, are expressed in the developing brain in a distinct spatiotemporal manner, with increases in FGF levels occurring during late embryonic stages (Caday *et al.,* 1990; Eckenstein *et al.,* 1991; Gomez-Pinilla *et al.,* 1992; Eckenstein, 1994; Weise and Grothe, 1993; Kuzis *et al.,* 1995). In the rat aFGF is present at E16 in sensory neurons in the midbrain followed by its expression in motor neurons. After Postnatal Day 7 it is also found in basal forebrain cholinergic neurons. On the other hand, bFGF is widely expressed at E16 in the developing cortex, striatum, brain stem, spinal cord, and spinal ganglia. This expression subsides and in the mature brain high levels of bFGF are found only in astrocytes and pyramidal cells of the CA2 area of the hippocampus and in subependymal cells. Interestingly, in cells exhibiting high levels of FGFs the growth factors are localized predominantly in their nuclei, whereas in cells with low FGFs levels, they are found in the cytoplasm. High levels of bFGF in the nucleus have been associated with "activity increased" cells, whereas low levels of bFGF in the nucleus indicate a "resting" state of the cell in question. The finding of nuclear FGFR1 with full kinase activity, and the fact that transfection of either bFGF or FGFR1 into cells not normally expressing these molecules results in an increase of cell proliferation, suggests that this intracrine mechanism of bFGF action is a novel way to control growth and proliferation of glial and neuronal cells (Stachowiak *et al.,* 1997).

With the exception of FGFR4, all the FGFRs are expressed in the nervous system. FGFR1–FGFR3 are expressed in nearly all areas of the CNS throughout development as well as in the adult brain (Kuzis *et al.,* 1995; Belluardo *et al.,* 1997). The expression of FGFR1 and FGFR2 is especially notable in the hippocampus and the dorsal root ganglia (Wanaka *et al.,* 1991). Interestingly, within the different regions these receptors seem to be expressed by different cell populations forming a basis for differential biological activity of their ligands (Belluardo *et al.,* 1997). These wide and nearly ubiquitous expression patterns of FGFR1–FGFR3 do not seem to be in agreement with the idea of a single function of their ligands in the brain but argue for a more complex action. In addition, the picture becomes more complex since FGFR1 can also be activated by extracellular matrix proteins, specifically cellular adhesion molecules such as neuronal cell adhesion molecule, N-cadherin, and L1 (Doherty and Walsh, 1996).

The trophic actions of bFGF on neurons are manyfold, but as for EGF, the best known seems to be its capacity to stimulate the proliferation of progenitor cells that have the potential to give rise to glia and neurons (Vescovi *et al.,* 1993; Palmer *et al.,* 1995; Shihabuddin *et al.,* 1997). Furthermore, bFGF promotes their survival and delays the differentiation into

neurons (Cavanagh *et al.*, 1997). Interestingly, bFGF increases the proliferation rate of neuronal progenitor cells without changing their cell cycle parameters (Cavanagh *et al.*, 1997). A stretch of 10 amino acids within the first receptor-binding domain of bFGF has been demonstrated to be critical for this effect (Ray *et al.*, 1997). Besides bFGF, only FGF4 of the FGF family can stimulate neuronal progenitor cells to proliferate (Ray *et al.*, 1997). These studies, however, were mostly performed *in vitro* on dissociated neuronal cultures and only recently have been verified *in vivo* (Tao *et al.*, 1997; Kuhn *et al.*, 1997). *In vivo,* it seemingly stimulates proliferation of neuronal precursor cells only (Kuhn *et al.*, 1997). The proliferative effects of bFGF can be antagonized by NT-3, which induces neuronal differentiation (Ghosh and Greenberg, 1995). In addition, the effect of increasing the number of newly formed neurons can be antagonized by activation of $GABA_A$ receptors. Interestingly, in proliferating neuroepithelial cells bFGF induces the expression of the α_1 subunit of $GABA_A$ receptors, indicating that within this system a GABA-mediated negative feedback loop for bFGF activity exists (Antonopoulos *et al.*, 1997).

The survival-promoting effect of bFGF on differentiated neurons is well documented (Dreyer *et al.*, 1989; Bouvier and Mytilineou, 1995; Lowenstein and Arsenault, 1996). bFGF has also been shown to be involved in various aspects of neuronal differentiation. It was shown to promote neurite outgrowth, possibly through increasing functional L-type Ca^{2+} channels (Gurney *et al.*, 1992; Shitaka *et al.*, 1996). However, it should be kept in mind that FGFs do cooperate with extracellular matrix proteins, which have been shown to be very potent inducers of neurite outgrowth and elongation (Doherty and Walsh, 1996). Other bFGF actions include the capacity to upregulate NGF expression in cultured hippocampal cells and the elevation of choline acetyltransferase activity in spetal cholinergic neurons (Ferhat *et al.*, 1997; Yokoyama *et al.*, 1994). bFGF also has shown neuroprotective effects during ischemia and excitotoxicity (Cuevas *et al.*, 1998).

In vitro, bFGF is a very potent mitogen for glial cells (Kniss and Burry, 1988; Bhat and Zhang, 1996). Supporting a role of bFGF in the proliferation of astrocytes is the fact that protein and mRNA expression correlates with the event of reactive gliosis (Gomez-Pinilla *et al.*, 1992; Chadi *et al.*, 1994) and the findings of an early study showing that bFGF induces glial fibrillary acidic protein (GFAP) expression in astrocytes (Morrison *et al.*, 1985). In contrast, bFGF treatment of cultured astrocytes results in a significant decrease in GFAP mRNA and protein, accompanied by a change from polygonal to stellate morphology (Reilly *et al.*, 1998). The discrepancies between these reports cannot readily be explained. In addition to these effects, a recent report demonstrated that bFGF treatment of astrocytes in culture resulted in a transient reduction of the gap junction protein connexin 43. This reduction leads to diminished intercellular communica-

tion (Reuss *et al.*, 1998). Whether this finding is relevant for the stimulation of astrocyte proliferation by bFGF or is an unrelated novel function of bFGF action remains to be elucidated.

Regarding other receptor tyrosine kinases, the activation of FGF receptors leads to an induction of a multitude of distinct signaling pathways leading to a cellular response. Not only are their different signaling pathways activated but also their pathways extensively interact with each other. However, signal transduction activated by FGFRs shows some distinct features (Fig. 3).

One of the most obvious features is the fact that high-affinity binding FGFRs cooperate with the low-affinity FGFHRs to become activated. Divalent cations (Ca^{2+} or Mg^{2+}) are required for the interaction between heparan sulfate proteoglycans and FGFRs (Kan *et al.*, 1996; McKeehan *et al.*, 1998). The second feature is that FGFRs can also be activated by CAMs. The interaction between CAMs and the FGFR is believed to take place through a CAM homology domain binding motif in the extracellular domain of the FGFR (Doherty and Walsh, 1996). This specific activation of FGFRs by CAMs is an important aspect of neuronal differentiation with respect to neurite outgrowth and elongation.

Signaling pathways initiated by the phosphorylation of the dimerized receptors are very similar to those described for EGF. The classical MAP kinase pathway is critically involved in the proliferative effect of FGFs on neurons and glial cells (Bhat and Zhang, 1996; Creuzet *et al.*, 1995). It also becomes activated during the neuronal differentiation process in PC12 cells and AS583-8 cells, a neuronal progenitor cell line of the basal forebrain (Kwon *et al.*, 1998). However, in PC12 cells and in another immortalized neuronal cell line derived from the hippocampus (H19-7), it was shown that MAP kinase activation alone is not sufficient for differentiation (neurite formation; Kuo *et al.*, 1996; Renaud *et al.*, 1996). In H19-7 cells, although MAP kinase is not sufficient for differentiation, raf is still required for differentiation. This findings raise the possibility of a raf-dependent but MAP kinase-independent pathway that remains to be defined.

The second major pathway induced by FGFRs is the PLC-γ-dependent signal transduction. This pathway also appears to be involved in neuronal differentiation (Bartlett *et al.*, 1995; Doherty *et al.*, 1995). Although PKC, a major protein kinase, is activated through DAG, the differentiation effect (neurite formation) seems to be mediated by a second DAG-involving pathway. DAG becomes converted to arachidonic acid by a DAG lipase. Arachidonic acid induces an increase in the intracellular calcium concentration, which appears to be the key event responsible for neuritogenesis induced by FGFR activation (Doherty *et al.*, 1995). Interestingly, in initial studies to show differences between the four major types of FGFRs, it has been shown that the different FGFRs seem to activate the two distinct

pathways (MAP kinase versus PLC-γ) to different extents (Shaoul *et al.,* 1995; Wang *et al.,* 1994). For example, FGFR1 activation induces a strong phosphorylation of PLC-γ, whereas FGFR4 fails to phosphorylate PLC-γ.

There are several other signaling pathways induced by FGF which have been less well studied. P13-K, for example, has been shown to be activated by bFGF in PC12 cells and has been associated with mitogenic effects (Raffioni and Bradshaw, 1992). However, in other studies, P13-K has been reported not to bind to FGFRs (Partanen *et al.,* 1995). The effect of FGFs on src kinases seems to be cell type dependent (Landgren *et al.,* 1995). Lung endothelial cells stimulated by bFGF show an increased autophosphorylation of src, whereas in aortic endothelial cells the opposite has been observed. In neural cells, the involvement of src kinases in bFGF responses remains to be determined.

C. Platelet-Derived Growth Factor

PDGF was one of the first growth factors to be identified. It was discovered to be the crucial protein in platelet extracts exerting mitogenic activity on smooth muscle cells and fibroblasts (Ross *et al.,* 1974; Brevitt and Clark, 1988). Since then, its role in many growth-dependent processes in a variety of tissues has become evident. In the nervous system it was first described as a mitogenic factor for glial cells (Westermark and Wasteson, 1976; Besnard *et al.,* 1987). PDGF is composed of two peptide chains, A and/or B. The major form in human platelets is the AB heterodimer, but the homodimers AA and BB have also been described (Antoniades, 1981; Ross *et al.,* 1986). The PDGF family also includes VEGF, PLGF, SCF-1, SCF, and Flt3 ligands, all of which form homodimers and bind to their corresponding members of the PDGF receptor subfamily (van der Geer *et al.,* 1994). However, we will concentrate on the prototypes of this family: PDGF-AA, PDGF-BB, and PDGF-AB and their receptors.

PDGFs bind to two distinct receptors, termed α- and β-PDGF receptors, which are very similar to the formerly discussed FGF receptors. They exists as single polypeptides of 160–180 kDa, with five immunoglobulin-like domains in the extracellular portion (Fig. 4), a single transmembrane domain, and an intracellular domain exhibiting the tyrosine kinase activity. As with the FGF receptors, tyrosine kinase activity requires two subdomains, the ATP-binding site and the catalytic site. Ligand binding leads to dimerization of the receptors, with each chain of the PDGF dimer binding one receptor molecule. α-PDGF receptor binds both A and B chains, whereas β-PDGF receptor binds only the B chain. Therefore, the BB-PDGF is a universal ligand for all possible PDGF receptor combinations (Fretto *et al.,* 1993; Hart *et al.,* 1988).

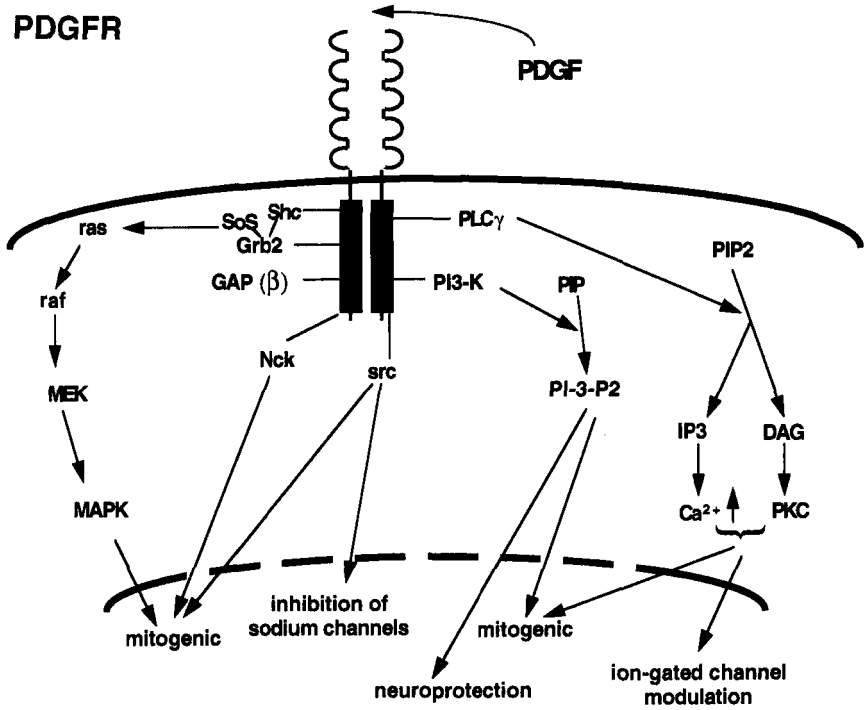

FIG. 4 Proposed intracellular signaling pathways for PDGF-mediated proliferation, neuroprotection, and modulation of ion channels (for details see text). Differences between the two PDGF receptors α and β could be mediated by the adaptor molecule GAP.

PDGF is widely expressed in the central nervous system (Valenzuela *et al.*, 1997). During development PDGF-A is first expressed in neurons of the spinal cord as early as E12 and is subsequently found throughout the brain. The expression of PDGF-A is also detectable in glia cells but at lower levels than in neurons (Yeh *et al.*, 1991). Like PDGF-A, PDGF-B is almost ubiquitously expressed in neurons of the CNS, with highest expression levels found in the hippocampus, cortex, cerebellum, brain stem, spinal cord, and olfactory bulb. The expression of a transgene (CAT under the control of the human PDGF-B promoter) was highest near the time of birth (Sasahara *et al.*, 1991).

α-PDGF receptor is detectable transiently in the developing brain from E9 onwards (in the neural tube). It is found by E13.5 in neurons of the cortex, brain stem, and spinal cord. Its expression decreases as the brain matures, but some mature neurons continue to produce it into adulthood. This "low-level" expression of α-PDGFR receptors in neurons can be found

almost everywhere in the adult CNS (Oumesmar *et al.,* 1997; Schatteman *et al.,* 1992; Vignais *et al.,* 1995). However, the predominant cells expressing α-PDGF receptor are oligodendrocyte progenitors in the developing CNS (Pringle and Richardson, 1993; Gotz *et al.,* 1994). In contrast to its neuronal expression, the oligodendroglial expression strongly decreases after Postnatal (P) Day 21 in the rat. Like α-PDGF receptors, β-PDGF receptors are expressed in neurons of almost all areas of the CNS (Smits *et al.,* 1991). The strongest expression is found in hippocampus, cerebellum, basal ganglia, spinal trigeminal nucleus, and neurons of the subventricular zone. Specifically, the latter location suggests a possible role in neuro- and/or gliogenesis.

In general, PDGFs and PDGFRs are widely expressed and produced locally in neuronal and glial cells of virtually every region of the CNS, making it difficult to assign a specific function to PDGF based on localization. Rather, these results imply a universal or even several roles of PDGFs in the developing and mature CNS. Furthermore, other members of the PDGF family, such as Flt3, are widely expressed in the brain (Rosnet *et al.,* 1991).

Several studies have shown that PDGFs have neurotrophic effects in cultured neurons. *In vitro* studies have demonstrated increases in the survival of GABAergic interneurons and mesencephalic dopaminergic neurons (Nikkhah *et al.,* 1993; Smits *et al.,* 1993). Furthermore, PDGFs induce neurite outgrowth in PC12 cells (Fanger *et al.,* 1995) and all three ligand isoforms support neuronal differentiation from stem cells from the fetal and adult nervous system (Johe *et al.,* 1996; Williams *et al.,* 1997). PDGF receptor activation in these cases leads to an immediate early gene response, which translates into neuronal differentiation after several days (Williams *et al.,* 1997). PDGF-BB, but not PDGF-AA, protects neurons from delayed neuronal death induced by ischemia (Cheng and Mattson, 1995; Kawabe *et al.,* 1997; Iihara *et al.,* 1997; Sakata *et al.,* 1998). In the mature nervous system PDGFs have been shown to modulate the function of ligand-gated ion channels, such as the $GABA_A$ and the NMDA receptors, by inhibiting the postsynaptic currents (Valenzuela *et al.,* 1995, 1996). Also, voltage-gated ion channels are thought to be modulatory targets of PDGFs (Timpe and Fantl, 1994). Furthermore, application of PDGF to PC12 cells inhibits sodium channel currents (Hillborn *et al.,* 1998). These effects on ion channels of nerve cells suggest that PDGF, in addition to its trophic actions, may play a prominent modulatory if not an acute physiological role in nerve cells.

The importance of PDGFs in the development of the nervous system has been demonstrated by studies of mutant and knockout mice. Homozygous Patch mice, which lack the α-PDGF receptor, normally die before the 11th day of gestation. However, those that survive until midgestation

exhibit major brain abnormalities, such as decreased metencephalon, a complete lack of the olfactory bulbs, collapsed ventricles, and abnormal choroid plexuses (Schatteman *et al.,* 1992). Heterozygous mice show normal protein levels of the α-PDGF receptor, resulting in no detectable abnormalities of the brain (Zhang and Hutchins, 1996). Deleting PDGF-B, PDGF-A, or the β-PDGF receptor by homologous recombination results in death of the mutant mice *in utero* but is not accompanied by gross anatomical CNS defects (Soriano, 1994; Leveen *et al.,* 1994; Bostrom *et al.,* 1996). These results suggest that the α-PDGF receptor plays an important role in CNS development.

In vitro and *in vivo* studies show that, besides its effects on neurons, PDGF is essential for the proliferation of oligodendrocyte progenitor cells (Ijichi *et al.,* 1996; Dutly and Schwab, 1991; Wolswijk *et al.,* 1991; Richardson *et al.,* 1988). PDGF was originally thought to be secreted solely by astrocytes and act on the oligodendrocyte precursor cells through their α-PDGF receptors, which are positively regulated by bFGF (Engel and Wolswijk, 1996; Raff, 1989; Richardson *et al.,* 1988; Raff *et al.,* 1988; McKinnon *et al.,* 1990). However, recently it has been shown that expression of PDGF by neurons may be important for the development of oligodendrocytes (Ellison *et al.,* 1996; Asakura *et al.,* 1997). In addition to its effects on oligodendrocytes, in the retina PDGF-A and the α-PDGF receptor seem to influence astrocyte proliferation (Fruttiger *et al.,* 1996).

As for the other receptor tyrosine kinases discussed previously, the best known and best studied signaling pathway induced by PDGFs is the MAPK pathway via the membrane-bound Ras (Claesson-Welsh, 1994; van der Geer *et al.,* 1994; Fig. 4). This pathway is likely to represent the major intracellular transduction mechanism for PDGF signaling leading to cell proliferation. However, other pathways are also activated by the PDGFs, including the PI3-K and PLC-γ pathways and the activation of src family kinases and phosphorylation of a small adaptor protein Nck. Both these pathways, as well as the src activation and Nck phosphorylation, are likely to be involved in the initiation of DNA synthesis upon PDGF stimulation (Claesson-Welsh, 1994). Recently, it has been shown that the src activation through the receptor tyrosine kinase is the pathway through which PDGF inhibits acute sodium channel currents in PC12 cells (Hillborn *et al.,* 1998). Activation of PLC-γ is involved in the modulation of the ligand-gated channels and probably in cell migration (Valenzuela *et al.,* 1995, 1996; Kundra *et al.,* 1994; Hu *et al.,* 1993). Activation of PI3-K may play a role in the protection from programmed cell death as has been proposed for other receptor tyrosine kinases (Yao and Cooper, 1995). Most of the studies revealing the intracellular signaling pathways elicited by PDGF were performed using the β-PDGF receptor, assuming that α-PDGF receptor functions similarly. However, recent evidence suggests functional differences

between these two receptors. For example, β-PDGF receptor can recruit ras-GAP, a regulator of the ras-induced MAPK pathway, whereas α-PDGF receptor cannot (Heidaran *et al.,* 1993). Recruitment of ras-GAP prevents the activation of PI3-K and PLC-γ (Klinghoffer *et al.,* 1996). How these differences are translated into different biological activities induced by the two receptors remains to be determined.

D. Insulin and Insulin-like Growth Factors I and II

Insulin, a hormone secreted by cells of the islets of Langerhans in the pancreas, is a polypeptide involved in the basic metabolism of the mammalian organism. Among its many-fold functions, the stimulation of glucose uptake by cells and the stimulation of fatty acid synthesis, particularly in liver, muscle, and fat cells, are the best known (Binoux, 1995). Structurally related to insulin, but with quite distinct functions, are IGF-I and -II. They regulate growth and development during embryogenesis. However, they can influence some metabolic activities (Stewart and Rotwein, 1996). A role for insulin and insulin-like growth factors as trophic factors in the CNS has been investigated during the past decade (D'Ercole *et al.,* 1996b; Folli *et al.,* 1996). Insulin is discussed in this section and not with the hormones because its receptor is a tyrosine kinase receptor and very similar to the receptor for IGF-I.

Insulin consists of two polypeptide chains: A chain (21 aa) and B chain (30 aa). During biosynthetic processing a single-chain proinsulin protein forms intrachain disulfide bridges and a connecting peptide (C-peptide) is removed, resulting in the mature insulin (Gold, 1989). In contrast to insulin, IGF-I and IGF-II retain the connecting C-peptide during biosynthetic processing, resulting in single-chain 7.5-kDa polypeptides. IGF-I and IGF-II exhibit approximately 70% sequence homology and, along with proinsulin, are products of successive duplications of a common ancestral gene. They are ubiquitously synthesized, with the liver producing the highest levels (Humbel, 1990).

The receptors to which these ligands bind are the insulin receptor (IR), the IGF-I receptor (IGF-IR), and the IGF-II receptor (IGF-IIR). Despite the fact that the ligands are closely related, the receptors are clearly distinct. IR and IGF-IR belong to the family of tyrosine kinase receptors (Ullrich and Schlessinger, 1990). In contrast to the other tyrosine kinase receptors, IR and IGF-IR are heterotetramers coupled by disulfide bonds (Fig. 5). Each receptor is composed of two α (135 kDa) and two β (95 kDa) subunits, which are derived by proteolytic cleavage from precursor molecules (van der Geer *et al.,* 1994). The α subunit contains the ligand-binding domain,

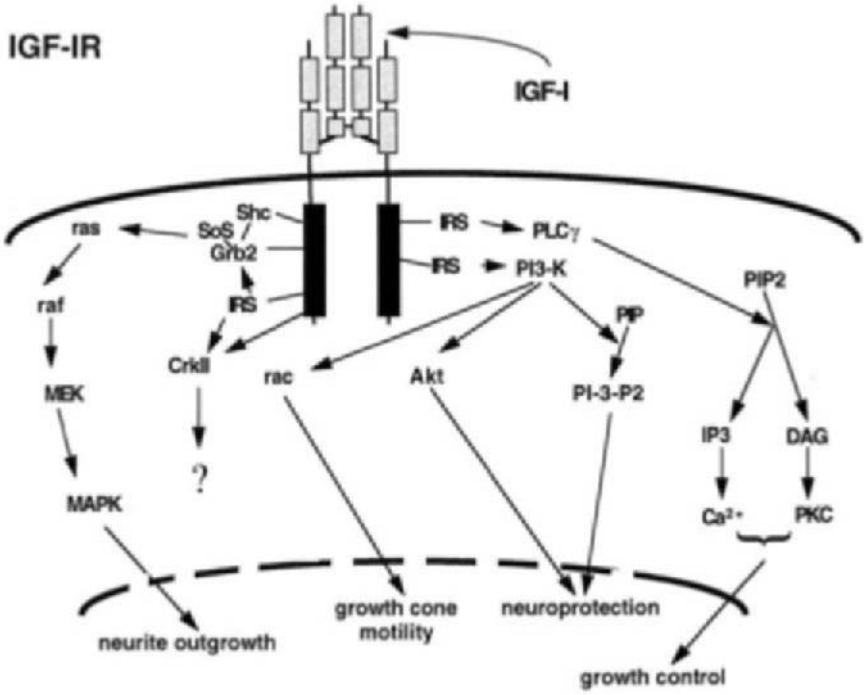

FIG. 5 Proposed intracellular signaling pathways for IGF-I-mediated proliferation, differentiation, neuroprotection, and effects on growth cone motility (for details see text). Differences between the insulin receptor and the IGF-I receptor could be mediated by the adaptor molecule Crk-II.

and the β subunit possesses tyrosine kinase activity. Ligand binding leads to an allosteric interaction of the two receptor halves rather than receptor dimerization. The catalytic subdomains autophosphorylate upon this interaction.

The IGF-IIR is structurally very distinct from IR and IGF-IR. In mammals it is identical to the cation-independent mannose-6-phosphate receptor (M6P-R). The IGF-IIR is a bifunctional monomeric glycoprotein with no tyrosine kinase activity, but it has been shown that this receptor mediates its signaling through heterotrimeric GTP proteins (Ikezu *et al.*, 1995). In addition to IGF-II, it also binds lysosomal enzymes bearing the M6P recognition marker (Kiess *et al.*, 1994).

The differences between the three ligands regarding their biological activity (metabolic vs growth and differentiation) are largely determined by their differing affinities for their cell surface receptors and the differential distribution of these receptors between tissues. For example, IGF-I binds

to the IR with a 10 times lower affinity (IGF-II, 50–100 times lower) than insulin. Insulin has 1000 times lower affinity to IGF-IR (IGF-II, 2 times lower) than IGF-I. In addition, the IR is the predominant receptor expressed on liver and fat cells, the principal target of insulin. Neither insulin nor IGF-I bind to IGF-IIR (Blakesley *et al.,* 1996).

Another level of control of IGF, but not insulin, function is provided by the expression of a family of insulin-like growth factor-binding proteins (IGFBPs), of which six molecular species ranging from 25 to 40 kDa have been identified (Binoux, 1995). Like IGFs, these proteins are synthesized ubiquitiously in a developmental pattern (Rechler and Brown, 1992). IGFBPs are located in the extracellular space and exhibit higher affinities for the IGFs than the IGF receptors. Probably >97% of the IGFs in the extracellular space are bound to IGFBPs (Shimasaki and Ling, 1992; Jones and Clemmons, 1995). The IGFs bound to IGFBPs are unavailable for interactions with their receptors. IGFs are released when IGFBPs bind to the plasma membrane and become dephosphorylated or degraded by serine proteases (Baxter and Martin, 1989). In addition, binding to IGFBPs has been proven to prolong the half-life of IGF-I. These characteristics render IGFBPs important regulators of the IGFs.

Surprisingly little is known about the distribution of insulin expression in the developing CNS. The existence of low levels of proinsulin mRNA has been reported in the neural tube and retina (Deltour *et al.,* 1993; Devaskar *et al.,* 1994). However, the nature of the resultant translation product(s) has not been characterized. In the adult brain insulin is localized in olfactory structures and the limbic system (Devaskar *et al.,* 1994). There is more information available on the distribution of IGF-I and -II in the developing and adult brain. In general, IGF-I expression is associated with neurons, whereas IGF-II mRNA is expressed predominantly in the meninges, choroid plexus, and mesenchymal cells surrounding the blood vessels (Stylianopoulou *et al.,* 1988a,b). This expression pattern for IGF-II likely accounts for the abundance of IGF-II in CSF. In the rat brain IGF-I mRNA is expressed first in the olfactory bulb and then becomes detectable in the thalamus, hippocampus, cerebellum, and retina between E16 and E20 (D'Ercole *et al.,* 1996a). Expression correlates with periods of cell proliferation in these regions. IGF-I mRNA is also found in the SVZ in early postnatal development (Bartlett *et al.,* 1992). Since this zone produces primarily glial cells at this point, IGF-I may be expressed by glial progenitor cells. In the adult brain IGF-I is expressed in many different regions, such as cortex, basal ganglia, basal forebrain, hipocampus, brainstem, cerebellum, spinal chord, and retina.

In the rat brain, binding sites for insulin, IGF-I, and IGF-II show a widespread but selective regional localization throughout the whole CNS at all stages of development (Kar *et al.,* 1993; D'Ercole *et al.,* 1996a; Folli

et al., 1996) and are often expressed concordantly with the spatiotemporal expression pattern of their ligands. The highest binding densities for all these receptors were found in the olfactory bulb, cortex, hippocampus, cerebellum, and choroid plexus. Within these structures the binding sites are distributed in a nonoverlapping fashion (Kar *et al.,* 1993, 1997). These findings are consistent with the hypothesis that the actions of these structurally related ligands are partially regulated by the differential distribution of their receptors. In addition to their distinct spatial distribution, they also show temporal differences in expression, with IGF-I and IGF-II binding sites generally peaking during late embryogenesis, whereas the levels of insulin-binding sites peak postnatally. IGF-IR-expressing cells also usually express IGF-I or are located in the proximity of an IGF-I-expressing cell. This suggests autocrine and paracrine actions of IGF-I. Like its ligand IGF-II, IGF-IIR is predominantly expressed in nonneuronal cells with the exception of hippocampal neurons (Dore *et al.,* 1996).

Expression of the IGFPs in the brain is more widespread than that of the ligands and their receptors. IGFBP-2, -4, and -5 are the major IGFBPs expressed in the brain. IGFBP-2 expression begins as early as E7 during rat embryogenesis in the ventral floor plate and the infundibulum (T. Wood *et al.,* 1992). Postnatally, its expression is restricted to astroglia (Lee *et al.,* 1993) and matches closely the expression of the IGF-II protein (Shimasaki and Ling, 1992). IGFBP-4 becomes evident by E14 in the choroid plexus and meninges. During development its expression extends to basal ganglia neurons, hippocampus, cerebral cortex, olfactory bulb, and amygdala (Brar and Chernausek, 1993). IGFBP-5 expression seems to be coordinated with IGF-I expression and is expressed by neurons (Bondy and Lee, 1993). The correlation of the expression between IGFs and IGFBPs suggests that the IGFBPs may influence IGF actions in the brain.

Most of the effects of insulin and the IGFs in the CNS are believed to be mediated by IGF-IR since tissue distribution studies have shown that the IR is less prominent in the brain. Therefore, the effect of insulin in the brain could be minor compared to the effects of IGF-I. IGF-I has been reported to promote the proliferation, survival, and maturation of sympathetic neuroblasts (Zackenfels *et al.,* 1995), the genesis of retinal neurons (Drago *et al.,* 1991), and the survival of CNS projection neurons and motor neurons after axotomy and nerve crush (Hughes *et al.,* 1993; Li *et al.,* 1994; Contreras and Steriade, 1995). In a recent report it was shown to be a differentiation factor for postmitotic CNS stem cell-derived neuronal precursors in which it may act together or sequentially with BDNF (Arsenijevic and Weiss, 1998). As suggested by its spatiotemporal distribution pattern during development, this factor may also play a role in neurogenesis. Indeed, when IGF-I was eliminated from the organism by knockout studies or reduced by antibody neutralization, the number of neurons generated

was reduced (Beck *et al.,* 1995; Frade *et al.,* 1996). Complementary to these results are the findings that the brains of hIGF-I transgenic mice, which overexpress hIGF-I, are enlarged. A detailed analysis of the neocortex in these mice has shown a 20% increase in neuronal number and enlarged neuronal circuitries (e.g., axonal terminal fields, dendritic arbors, and synapses; Matthews *et al.,* 1988; D'Ercole *et al.,* 1996b). This finding is consistent with *in vitro* findings in which it has been shown that neuronal cells treated with IGF-I are rescued from apoptosis and show vigorous neurite outgrowth and increased growth cone motility (Ishii *et al.,* 1991; Feldman *et al.,* 1997; Miller *et al.,* 1997; D'Mello *et al.,* 1997). Recently, it has been reported that a β-subunit variant of the IGF-IR is highly enriched and specifically localized in growth cone membranes (Mascotti *et al.,* 1997). Its expression is closely correlated with neurite outgrowth. Interestingly, the "normal" β subunit of the receptor is downregulated, when the variant becomes upregulated. This suggests that IGF-I actions in the nervous system can be regulated by differential expression of receptor variants. In addition to observed actions in the developing brain, IGF-I also exerts trophic effects in the adult brain. In studies of postmitotic CNS populations, IGF-I stimulated choline acetyltransferase activity in septal and pontine neuronal cultures. The uptake of dopamine is increased in cultures of ventral mesencephalic neurons (Knusel *et al.,* 1990). It has also been hypothesized that IGF-I acts as a neuromodulator of some higher brain functions, such as long-term potentiation and depression (Folli *et al.,* 1996).

As with IGF-I, IGF-II has been proposed in several studies to act as a growth and differentiation factor in the CNS (Lenoir and Honegger, 1983; Liu and Lauder, 1992; Zackenfels *et al.,* 1995). It has recently been shown to have some biological activities which are distinct from those of IGF-I in the brain: IGF-II potentiates potassium-evoked acetylcholine release, whereas IGF-I inhibits this response (Kar *et al.,* 1997).

In addition to their effects on neurons, IGFs have also been reported to influence glial cells, specifically oligodendrocytes. In the CNS developing oligodendrocytes need IGF-I to progress to mature, myelin-forming cells (McMorris and Dubois-Dalcq, 1988). IGF-I-overexpressing mice show increased myelination due to an increased number of myelinated axons and thicker myelin sheaths (which significantly contributes to the observed enlargement of the brains of the transgenic mice). Furthermore, the expression of oligodendrocyte-specific genes (e.g., myelin basic protein) is significantly increased (Carson *et al.,* 1993) suggesting direct actions of IGF-I on oligodendrocytes. In the peripheral nervous system IGF-I is required for Schwann cell mitogenesis and differentiation (Cheng and Feldman, 1997).

The intracellular signaling cascade elicited by insulin and IGF-I begins with the autophosphorylation of the receptors by ligand binding-induced tyrosine kinase activity (Fig. 5). However, in contrast to other tyrosine

kinase receptors, IR and IGF-IR do not interact directly with the Grb2–Shc–Sos complex but rather phosphorylate insulin receptor substrates (IRS-1 and/or IRS-2). Only these two phosphorylated substrates bind to the Grb2–Shc–Sos complex, which translocates to the plasma membrane, thereby initiating the classical MAP kinase cascade and ultimately leading to transcription of early response genes (Folli *et al.,* 1996; Blakesley *et al.,* 1996). The MAP kinase pathway has been implicated in the promotion of neurite outgrowth by IGF-I (Feldman *et al.,* 1997).

In addition to the activation of the classical MAP kinase pathway, activated IRSs also bind to PI3-K, initiating this particular signaling pathway. In neuronal cells, in which this specific pathway has been blocked, apoptosis is more likely to occur (Singleton *et al.,* 1996). Recently it has been shown that a serine threonine kinase, akt, becomes activated downstream of PI3-K and may be critical for the insulin and IGF-I-induced protection from apoptosis (Dudek *et al.,* 1997).

Furthermore, PI3-K may activate rac, a GTP-binding protein of the rho family, which then promotes cytoskeletal changes involving uncapping of the barbed ends of actin filaments (Feldman *et al.,* 1997). Interestingly, it has been shown that in a neuronal cell line IGF-I stimulates the tyrosine phosphorylation of focal adhesion kinase and paxillin, two components of focal adhesion complexes, showing that these complexes may play a crucial role in the insulin and IGF-I-dependent growth cone motility (Feldman *et al.,* 1997).

As stated previously, IR and IGF-IR are homolog receptors which can be activated by the same ligands and which initiate very similar signaling pathways. The question arises as to what regulates the divergent physiological functions of insulin and IGF (metabolic activity as opposed to mitogenic/differentiation activity). First, these differences may be explained by the distinct tissue distribution of the receptors and the regulation of the IGFs by the IGFBPs. However, it has recently been hypothesized that the time course of activation may play a determining role between these responses. As discussed previously, this argument has been used to explain the different responses of PC12 cells to EGF and NGF. A prolonged signal, brought about by the retarded rate of dissociation of IGF-I or insulin from their receptors, might preferentially initiate a mitogenic/differentiation pathway (DeMeyts *et al.,* 1994, 1995). In addition, a recent report identified a specific IGF-IR substrate, Crk-II. Crk-II is rapidly tyrosine phosphorylated by IGF-I treatment of cells but requires significantly more insulin to become phosphorylated. It associates with IRS-I and IRS-2 and also directly with the IGF-IR receptor. Its interaction with the C terminus of the IR is 50-fold lower, thus suggesting a possible mechanism for specific IGF-I responsiveness (Beitner-Johnson *et al.,* 1996). Recently, differences between IR and IGF-IR activation on the effector site of the signaling pathway have

been reported. IR, but not IGF-IR, signaling causes a rapid dephosphorylation of tyrosine residues on focal adhesion kinase (Knight *et al.,* 1995; Pillay *et al.,* 1995). The details of the apparently distinct effector signaling pathways and upstream signaling components, however, remain to be investigated.

In conclusion, the previously discussed growth factors, even though they show a broad tissue specificity, have been proven to be important trophic factors in the developing and adult brain. Despite this fact, they have not received nearly as much attention as the neurotrophins. One reason could be the fact that it is difficult to study the effects of depleting these factors *in vivo* since the consequences in the nervous system are very often "masked" or "minimized" by more general and more severe defects in other tissues. An interesting question to be answered is whether their action in the nervous system is based on tissue-specific cellular mechanisms.

IV. Ciliary Neurotrophic Factor and Related Molecules

A. Ciliary Neutrophic Factor

CNTF was first described as an *in vitro* survival factor for ciliary ganglion neurons. It subsequently was shown to induce differentiation of sympathetic precursors and to promote survival of cultured motor neurons, sensory neurons, and neurons isolated from hippocampus (Lin *et al.,* 1989; Martinou *et al.,* 1992; Thaler *et al.,* 1944; Ip *et al.,* 1991). In addition, CNTF regulates the *in vitro* differentiation and survival of nonneuronal cells such as astroglia and regulates gene expression in hepatocytes (Hughes *et al.,* 1988; Louis *et al.,* 1993; Schooltinck *et al.,* 1992). Based in part on its trophic effects on motor neurons, CNTF has been used in clinical trials for amyotrophic lateral sclerosis patients (Anonymous, 1995). Perhaps surprisingly, pharmacologic doses of CNTF often produce systemic side effects resembling a generalized inflammatory response, even though physiologic effects of the factor are thought to be limited to the nervous system due to restricted expression of CNTF receptors (Ip *et al.,* 1993b; Barinaa, 1994). As will be discussed, these clinical findings are likely to reflect the fact that CNTF is homologous to and shares signaling mechanisms with several widely acting cytokines, in particular leukemia inhibitory factor (LIF) and interleukin-6 (IL-6).

At the level of amino acid sequence, CNTF exhibits little homology with other proteins (only 15% with LIF, one of its closest relatives). However, at the level of tertiary structure, CNTF clearly belongs to the long-chain helical cytokine superfamily, which includes LIF and IL-6, as well as more distantly related peptides such as IL-11, oncostatin M, growth hormone,

and granulocyte colony-stimulating factor. The tertiary structure shared by this superfamily is a series of four helices arranged in an "up–up–down–down" orientation (Inoue *et al.,* 1996). CNTF, LIF, and IL-6 bind to cell membrane receptors composed of one ligand-specific α subunit and two signal-transducing β subunits. The β subunits, LIF receptor β (LIF-Rβ) and glycoprotein 130 (gp130), are shared in various combinations among the CNTF, LIF, and IL-6 receptors. CNTF signals are initiated by interaction with the CNTF receptor α (CNTF-Rα), a protein recently identified by specific binding activity toward epitope-tagged CNTF (Squinto *et al.,* 1990). CNTF-Rα is linked to the plasma membrane by a glycosyl phosphatidylinositol (GPI) anchor. Treatment of CNTF-responsive cells with phospholipase (which cleaves this glycolipid anchor) abolishes responsiveness to the ligand, while administration of excess soluble CNTF-Rβ restores responsiveness to phospholipase-treated cells (Davis *et al.,* 1991, 1993). After specific binding, the complex of CNTF and CNTF-Rα interacts with one molecule each of LIF-Rβ and gp130, thus forming the functional tetrameric signaling complex (CNTF, CNTF-Rα, LIF-Rβ, and gp130). In a similar fashion, IL-6 binds to the ligand-specific IL6-Rβ, a GPI-anchored protein homologous to CNTF-Rα. The complex of IL-6 and IL6-Rα then interacts with two gp130 molecules. LIF does not employ an α receptor; instead, it binds directly to one molecule of LIF-Rβ and one molecule of gp130 (Ip and Yancopoulos, 1996).

LIF-Rβ and gp130 are homologous transmembrane signaling proteins with no intrinsic enzymatic domains. These receptors instead recruit several intracellular signal transducers to the plasma membrane, including janus kinases (the JAK family of nonreceptor tyrosine kinases) and the Shc adapter protein involved in ras-MAPK activation (Fig. 6). LIF-Rβ and gp130 are bound constitutively to members of the JAK kinase family (Ip and Yancopoulos, 1992; Stahl *et al.,* 1994). When CNTF and CNTF-Rα link LIF-Rβ and gp130 at the plasma membrane, two JAK kinases are brought in proximity. As a result, the JAK molecules transactivate each other and phosphorylate specific tyrosine residues on the β subunits to which they are attached. The resultant phosphotyrosines on LIF-Rβ and gp130 serve as specific SH2 domain-docking sites for other cytoplasmic signaling molecules, in particular the signal transducer and activator of transcription (STAT) proteins. STAT proteins are key substrates for JAK kinases. Thus, as STATs are linked to LIF-Rβ and gp130, they become phosphorylated by nearby JAKs; the specific STAT factors activated by CNTF signals include STAT1, STAT3, and STAT5B (Stahl *et al.,* 1995; Boulton *et al.,* 1995). Phosphorylated STATs dissociate from LIF-Rβ and gp130, dimerize, translocate to the nucleus, and bind DNA in a sequence-specific fashion, thus activating gene transcription. The critical genes for the CNTF response remain unclear. However, specific DNA sequences,

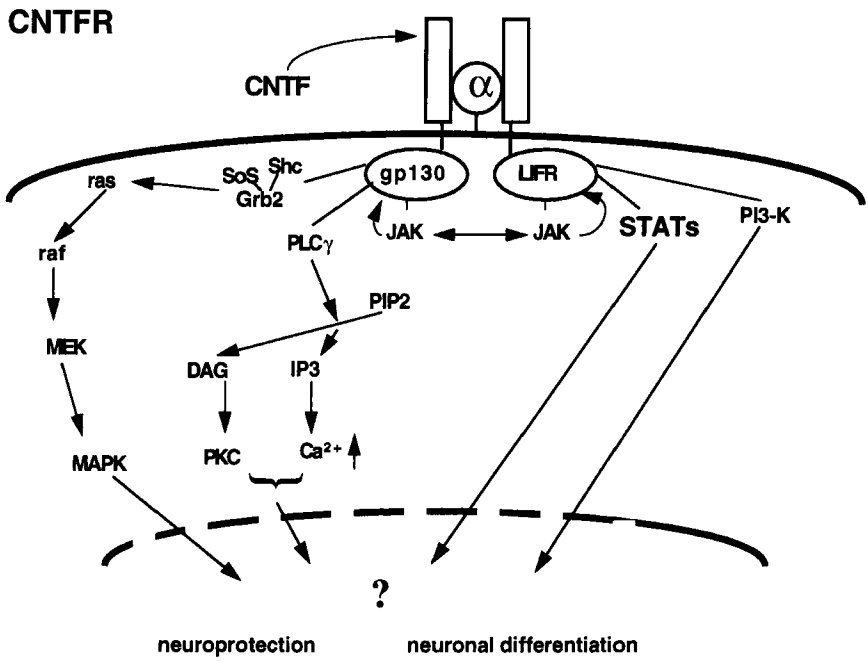

FIG. 6 Intracellular signaling pathways activated by CNTF (for details see text). The biological effects of CNTF (neuroprotection and neuronal differentiation) have yet to be assigned to one or more intracellular signaling pathways.

such as the IL-6 response element and the hematopoietin receptor response element, appear to be bound by STAT proteins after CNTF treatment (Shuai *et al.*, 1994).

Tyrosine-phosphorylated gp130 is also a binding substrate for the SH2 domain of the Shc adapter protein. As described previously, Shc binds Grb2 and mSos; mSos in turn activates the ras-GTP-binding protein and initiates the ras-MAPK signaling cascade. The combined activation of the JAK-STAT and the ras-MAPK pathways is likely to be important for cytokine signals. For example, several CNTF-responsive genes, such as vasoactive intestinal peptide, contain promoters with binding sites for MAPK-dependent transcription factors as well as for STAT transcription factors (Symes *et al.*, 1995). In addition, CNTF synergizes with FGF (which signals primarily through the ras-MAPK pathway) to activate MAPK and induce differentiation of the MAH neural progenitor cell line (Ip *et al.*, 1994).

Although exogenous CNTF is a widely acting neurotrophic factor *in vitro* (and possibly *in vivo*), experimental and clinical data suggest that

physiologic CNTF levels play a subtle role in nervous system development *in vivo*. For example, CNTF knockout mice grow normally and develop mild motor neuron deficits only late in adulthood (Masu *et al.*, 1993). Also, 2.5% of Japanese people contain a homozygous null mutation for CNTF; these people have normal life expectancies and no nervous system symptoms (Davis *et al.*, 1991). In contrast, CNTF-Rβ knockout mice die shortly after birth and exhibit profound loss of many motor neuron populations (DeChiara *et al.*, 1995), suggesting that while signals from the CNTF receptor are essential for nervous system development, the CNTF receptor can be bound by multiple physiologic ligands, including CNTF, which serve partly redundant functions.

Recent evidence has highlighted the possible role of CNTF in the nervous system response to injury. For example, following mechanical CNS trauma, the expression of CNTF and its receptor increases markedly around the wound site. Furthermore, while CNTF receptor expression is usually restricted to neurons, trauma appears to induce receptor expression in astrocytes and fibroblasts around the wound (Ip *et al.*, 1993c), suggesting that CNTF signaling could be involved in glial scar formation. In the PNS, CNTF is present at high levels in Schwann cell cytoplasm and is released into the extracellular space following nerve injury (Friedman *et al.*, 1992; Sendtner *et al.*, 1992b). CNTF lacks a signal sequence for secretion, and its release from Schwann cell cytoplasm may be dependent on cell disruption. CNTF appears to play a protective role for damaged peripheral nerves; its retrograde axonal transport increases markedly following neuronal injury and its presence inhibits degeneration of axotomized motor nerves (Sendtner *et al.*, 1992a; Curtis *et al.*, 1993). In addition, there is evidence that the function of CNTF as an injury factor is regulated by solubilization of CNTF-Rβ, secondary to phospholipase activation. The combined presence of liberated CNTF and soluble CNTF-Rα could result in circulating dimers that bind to cells expressing LIF-Rβ and gp130 and that otherwise would be responsive to LIF but not to CNTF (Ip and Yancopoulos, 1992).

B. Glial-Derived Neurotrophic Factor and Related Molecules

GDNF, a distant homolog of TGF-β, was initially characterized as a target-derived survival agent for midbrain dopaminergic neurons (Lin *et al.*, 1993). Subsequent expression studies in developing rats indicated that GDNF is expressed in nigrostriatal dopaminergic neurons, at times corresponding to the development of the nigrostriatal pathway (Poulsen *et al.*, 1994). In addition, exogenous GDNF exhibits neuroprotective effects in several animal models of Parkinson's disease (Lindsay, 1995). Based on these initial observations, it was thought that GDNF might function as a specific trophic

factor for dopaminergic neurons, with possible clinical utility in Parkinson's disease. However, a variety of knockout mouse systems have shown that GDNF is not necessary for development of the nigrostriatal pathway but is essential for development of sympathetic and enteric neurons and kidney (Sanchez *et al.,* 1996). To date, GDNF expression has been documented in numerous pre- and postnatal neuronal populations in the CNS and peripheral nervous system (PNS) (Widenfalk *et al.,* 1997). It also has been shown to exert trophic survival activity for peripheral motor and sympathetic neurons, in addition to the nigrostriatal population (Buj-Bello *et al.,* 1995; Oppenheim *et al.,* 1995; Yan *et al.,* 1995). GDNF has also been detected in nonneuronal tissues, in particular, the developing kidney, in which expression in nephrogenic mesenchyme is essential for initiating ureteric bud elongation (Sariola and Saino, 1997). It was suspected that GDNF would act at the cellular level in a manner similar to that of TGF-β, binding to a transmembrane receptor with serine/threonine kinase activity. However, it is now clear that GDNF binds to a GPI-linked membrane receptor (GDNF-Rα), and that signal transduction occurs by interaction of GDNF and GDNF-Rβ with the transmembrane receptor tyrosine kinase ret. In this regard, the signaling mechanism of GDNF is more similar to that of cytokines such as CNTF than to that of the GDNF structural homolog TGF-β.

Two independent groups identified GDNF-Rα using expression cloning strategies to isolate cDNAs that encode GDNF-binding molecules (Jing *et al.,* 1996; Treanor *et al.,* 1996). GDNF-Rα lacks transmembrane and cytoplasmic domains but does contain a consensus sequence for GPI modification. Treatment of GDNF-responsive neurons with phosphatidyl inositol phospholipase (PIPLC) to hydrolyze GPI moieties causes a significant reduction in the neurotrophic activity of GDNF. The addition of high concentrations of soluble GDNF-Rα restores GNDF responsiveness in the PIPLC-treated cultures. By analogy to CNTF and its receptor system, these findings indicate that GDNF-Rα is tethered to the outer surface of the cell membrane by a GPI anchor, and that GDNF signal transduction is likely to require the recruitment of an additional transmembrane protein(s). Full characterization of the GDNF receptor system has been facilitated by analysis of GDNF knockout mice. These mice have significantly reduced numbers of sympathetic and enteric neurons compared to normal mice, and show complete absence of kidney development (Sanchez *et al.,* 1996; Pichel *et al.,* 1996). As such, these mice closely resemble knockouts lacking the ret receptor tyrosine kinase (the loss of enteric neurons in ret knockouts is clinically interesting, given the frequent finding of ret loss of function mutations in familial Hirschsprung's disease; Pasini *et al.,* 1996). Jing *et al.* (1996), Treanor *et al.* (1996) and two additional groups (Trupp *et al.,* 1996; Durbec *et al.,* 1996) demonstrated that GDNF binds to a complex of cell proteins

that includes ret, and that binding results in ret tyrosine phosphorylation. In addition, ret promotes GDNF responsiveness when transfected into fibroblasts. Interestingly, ret expression is readily detected in the midbrain dopaminergic neurons of rats, correlating with the ability of GDNF to protect these neurons from 6-hydroxydopamine-induced death (Trupp *et al.*, 1996). Thus, even if GDNF is not absolutely required for nigrostriatal development, it may have clinical utility in adult nigrostriatal pathology. Recent studies indicate that activated ret may complex with Shc and Grb2, thus transducing the GDNF signal through the MAPK pathway (Ohiwa *et al.*, 1997). However, the details of GDNF signaling are poorly characterized.

The development of nigrostriatal neurons in GDNF knockout mice raises the possibility that homologs of GDNF with overlapping functions might exist. Indeed, two such homologs, termed neuriturin (NTN; Kotzbauer *et al.*, 1996) and persephin (Milbrandt *et al.*, 1998), have been isolated by functional assays and degenerate PCR. In addition, a GPI-linked receptor for NTN (NTN-R or GDNF-Rβ) has been identified by homology to GDNF-Rα (Buj-Bello *et al.*, 1997). A recent comparison of the expression patterns of GDNF, NTN, and their receptors shows partly overlapping expression in numerous regions of the developing and adult nervous system (Widenfalk *et al.*, 1997). GDNF and NTN are both present in developing striatum and brain stem, as well as in the developing enteric nervous system and kidney. GDNF, but not NTN, is detected in adult striatum and developing thalamus and hippocampus. Ret is expressed in all GDNF or NTN-expressing populations. GDNF-Rα, but not GDNF-Rβ, is expressed in striatum. However, both receptors are expressed widely throughout the remainder of the CNS, PNS, and developing gut and kidney. Persephin also appears to be widely expressed and, like GDNF and NTN, promotes survival of midbrain dopaminergic neurons and development of kidney. In contrast, persephin does not exert trophic activity on sympathetic and enteric neurons. In addition, fibroblasts cotransfected with ret and GDNF-Rα or GDNF-Rβ do not respond to persephin, suggesting that this molecule signals through a distinct receptor complex (Milbrandt *et al.*, 1998). As can be seen from these data, knowledge of the GDNF-related neurotrophic factors is expanding at a rapid rate, but a coherent model of how these factors interact, in unique or overlapping ways, is far from complete.

V. Transforming Growth Factor-β and Related Molecules

Several widely acting immune cytokines promote cell survival, differentiation, and proliferation in the nervous system. Some of these factors also

mediate inflammatory response via interaction with the nervous system, thus constituting a biochemical link between the immune and nervous systems. These molecules are produced both peripherally and within the nervous system. Peripherally synthesized cytokines are expressed mainly by immune cells; however, neither the cells nor the cytokines access the nervous system without damage to the blood–brain barrier. Within the nervous system, microglia and neurons express certain cytokines such as IL-1 and TGF-β-related proteins; the exact roles of these cytokines in neuronal physiology and pathology remain an active topic of research (Sei *et al.,* 1995; Rothwell *et al.,* 1996). Regardless of the site of production, several cytokines not considered classical neurotrophins have now been shown to exert trophic activity on neurons.

IL-1 is a survival factor for cultured spinal cord neurons and induces NGF expression by astrocytes (Brenneman *et al.,* 1992; Spranger *et al.,* 1990). On the other hand, IL-1 receptor antagonist inhibits neuronal death after ischemic damage, suggesting that while IL-1 exerts trophic activity in some systems, it also can mediate ischemic neuronal death (Relton and Rothwell, 1992). In addition, the function of IL-1 as endogenous pyrogen appears to at least partly involve direct interaction with the nervous system. IL-1 induces fever at much lower doses when injected intercerebrally or intracerebroventricularly than when injected peripherally (Rothwell *et al.,* 1996). In rats, anti-IL-1 antiserum inhibits fever when injected into the brain or ventricles. Interestingly, antisera against the β isoform of IL-1 retain this antipyretic activity, whereas antisera specific for the α isoform are without effect (Busbridge *et al.,* 1989).

In the CNS, TGF-β1 is produced by astrocytes, oligodendrocytes, and microglia. Its synthesis is enhanced by the α isoform of IL-1, mainly at the posttranscriptional level (Rothwell *et al.,* 1996). TGF-β1 inhibits NMDA-induced toxicity in cultured rat hippocampal neurons and TNF-β-induced toxicity in oligodendrocytes (Merrill and Zimmerman, 1991; Prehn *et al.,* 1994). Signaling by TGF-β1 [as well as the related bone morphogenetic proteins (BMPs)] occurs through a different mechanism than that used by the more distant homolog GDNF. The signals of TGF-β and BMPs are transduced by heteromeric transmembrane complexes containing distantly related receptor serine/threonine kinases (termed types I and II; Wrana *et al.,* 1994). TGF-β is synthesized as a precursor protein that is proteolytically processed to a disulfide-linked dimer that can be secreted. Secreted TGF-β appears to bind initially to two molecules of the type II receptor kinase. This complex then recruits two molecules of the type I receptor kinase. The type II receptors, which exhibit constitutive kinase activity, are thus enabled to transphosphorylate the type I receptors (Wrana *et al.,* 1994). The phosphorylated type I receptors then act to transduce the ligand signal further by phosphorylating MADR proteins (relatives of *Drosophila* moth-

ers against decapentaplegic protein) which translocate to the nucleus (Hoodless *et al.,* 1996). The functions of MADR proteins are not certain, but they appear to act as transcription factors. Immunophilins such as FKBP-12, the target of the immunosuppressant FK506, have also been shown to interact with the TGF-β receptor complex (Wang *et al.,* 1996). Again, the role of this interaction, particularly as it relates to the function of TGF-β in the nervous system, remains to be determined.

BMPs comprise a large and growing family of TGF-β homologs that are involved in developmental patterning in the embryo. BMPs and their receptors are expressed in several regions of the embryonic brain and neural crest-derived tissues. The functions of BMPs appear to depend in part on the stage of embryonic development and the location of expression. For example, while they inhibit formation of neuroectoderm during gastrulation, they promote cell differentiation in the neural tube. In the developing PNS, BMPs can serve as neuronal differentiation agents, whereas in the embryonic CNS, they induce astroglial differentiation (Mahler *et al.,* 1997). Similar to TGF-β, BMPs are synthesized as large precursors that are processed into disulfide-linked dimers (Bonini and Choi, 1995). The binding of secreted BMP dimers to surface receptors is slightly different then that described for TGF-β in that type II receptor kinases do not form the initial binding complex in the absence of the type I kinases (Yamashita *et al.,* 1996). After receptor binding, signal transduction appears to be similar to that of TGF-β, with phosphorylation of MADR-like proteins followed by translocation of these proteins to the nucleus. The BMP-activated MADRs are thought to influence the activity of cell cycle regulatory molecules, such as the p15 inhibitor of cyclin-dependent kinase. This in turn may regulate the activity of genes such as retinoblastoma and c-*myc,* which are important for determining the proliferation and differentiation status of the cell (Mahler *et al.,* 1997).

VI. Neurotransmitters

Numerous studies have suggested that neurotransmitters can exert neurotrophic actions in the developing nervous system (Whitaker-Azmitia, 1991; Lauder, 1993; Calabresi *et al.,* 1996). In particular, neurotransmitters have been shown to influence neuronal morphology, process outgrowth, and growth cone guidance (Lipton and Kater, 1989). Many of the so-called neurotrophic actions of neurotransmitters may be mediated via these latter mechanisms, as discussed later, and several very interesting observations have been made which strongly suggest that neurotransmitters play an important role in developmental events that take place both *in utero* and

in the early postnatal period (Levitt *et al.,* 1997; Berger-Sweeney and Hohmann, 1997). Virtually all the classical neurotransmitters, as well as neuropeptides, have been implicated in trophic actions (Lauder, 1993; Calabresi *et al.,* 1996). However, monoamines, particularly catecholamines are of special interest because of their distribution and developmental profile.

The catecholamines, dopamine (DA) and norepinephrine (NE), as well as the indole-amine, serotonin (5-HT), comprise several diffuse ascending systems from the brain stem to the forebrain which play major modulatory roles in forebrain activity, including arousal, memory, attention, and affective as well as other complex behaviors (Izquierdo and Medina, 1997; Goldman-Rakic and Selemon, 1997; Braun *et al.,* 1997; Arnsten, 1997; Robbins, 1997; Hasselmo, 1995). The arrival of these pathways in the forebrain takes place relatively early in development, well before synapse formation (Coyle and Molliver, 1977). For example, NE neurons of the locus coeruleus are generated as early as E10 (Lauder and Bloom, 1974), and ascending monoaminergic fibers coarse through the cortical and subcortical regions by E14 (Levitt and Moore, 1979). Similarly, β-adrenergic receptors are present in the forebrain as early as E13 and increase steadily until p23, with β_2 receptors representing the predominant receptor subtype in prenatal forebrain tissue (Erdtsieck-Ernste *et al.,* 1991, 1993). In detailed autoradiographic studies of α- and β-adrenergic receptor localization in the developing monkey visual cortex, unique profiles of these receptors were observed both in the developing cortical plate and within transient embryonic zones (Lidow and Rakic, 1994). High densities of α_1 receptors were observed in germinal zones in which there was intense proliferative activity, whereas β receptors were observed at time points subsequent to the generation of cortical neurons. The early appearance of these receptors and the differences in their pattern of distribution, as well as changes in the pattern of expression with time, suggested that they may play an important role in the regulation of cortical development. In a follow-up study, the distribution of various neurotransmitter receptors was studied in proliferative zones of the developing visual cortex from fetuses during the stage of cortical neurogenesis and also at a later stage (Lidow and Rakic, 1995). In cortical sections from fetuses in which cortical neurons were undergoing neurogenesis, high densities of 5-HT1, D1-DA, α_1- and α_2-adrenergic, and high-affinity kainate receptors were observed. These receptors were previously linked to cell proliferation responses in other systems (Seuwon *et al.,* 1988; Kahan *et al.,* 1992; Cruiso *et al.,* 1985; Nakaki *et al.,* 1989; Duncan *et al.,* 1990; Kennedy *et al.,* 1983; Ciani and Contestable, 1998; Slotkin and Bartolome, 1986). At a later period, following cortical neurogenesis, these receptors were no longer present in the deep cortical walls. Other subtypes of receptors, including 5-HT2, D2-DA, β-adrenergic, M_1-muscarinic cholinergic, GABA, *N*-methyl-D-aspartate (NMDA), and α-amino-3-hydroxy-5-

methyl-4-isoxoazole propionate, were not found in areas in which high levels of cell proliferation were occurring. Activation of these receptors, in some instances, has been shown to suppress proliferation (Nording *et al.,* 1992; Lloyd *et al.,* 1975; Floria *et al.,* 1992; Cruiso *et al.,* 1985; Nakaki *et al.,* 1989). These findings suggest that neuronal progenitor cells, during periods of active neurogenesis and proliferation, are capable of receiving signals from neurotransmitters that are present in the developing proliferative zones of the occipital cortex, and that these signals may influence neurogenesis. A note of caution must be made regarding possible overinterpretation of data derived solely from localization studies without direct confirmation of functional effects. Many neurotransmitter receptors are coupled to complex signaling pathways including G protein-coupled and cAMP-mediated signaling cascades (see Table 12-1 in Abe *et al.,* 1991). Therefore, the physiological response of receptor activation may vary considerably depending on the signaling apparatus expressed in a particular cell type (VanRenterghem *et al.,* 1994; Hafner *et al.,* 1994; Frodin *et al.,* 1994; Young *et al.,* 1994), as well as the physiological state of the cell type of interest (Abe and Saito, 1992).

Although it was previously proposed that ascending monoamine systems may potentially play some role in development (Lauder and Bloom, 1974), it is only in recent years that specific actions have been identified (Levitt *et al.,* 1997; Berger-Sweeney and Hohmann, 1997). One of the best studied examples of the possible influence of neurotransmitters and their receptors on development is the area of experience-dependent plasticity. In the visual system, it is known that synaptic connections in the visual cortex are modified by experience during a critical period of postnatal development (Hubel and Wiesel, 1970). In newborn kittens, for example, most cells in the primary visual cortex are responsive to stimuli presented to either eye. During this critical period, the synaptic connections are modified resulting in a characteristic distribution of monocularly versus binocularly responsive neurons. If one of the eyes is sutured shut during this critical period, the ocular dominance pattern "adapts" such that few neurons remain responsive to visual stimuli presented to the closed eye, and most of the neurons respond to stimuli presented to the normal eye. Bear and Singer (1986) demonstrated that acetylcholine and NE play a major modulatory role in this adaptation to monocular deprivation. Lesions of ascending NE and cholinergic pathways during this critical period block the capacity to initiate this adaptive response. Both neurotransmitter systems appear to be sufficient to mediate the response to monocular deprivation since neither cholinergic nor noradrenergic ablation alone alters the response. One possible final common pathway for this modulatory effect appears to be the NMDA subtype of glutamate receptors on visual cortical neurons. A subsequent study demonstrated that infusion of the NMDA receptor blocker D,L-2-

amino-5-phosphonovaleric acid also blocked the response to monocular deprivation (Kleinschmidt *et al.,* 1987; Bear *et al.,* 1990). Since NMDA receptors are permeable to Ca^{2+}, particularly under more sustained stimulation conditions, the activity-dependent remodeling in visual cortical synaptic connections is likely to involve a cooperative effect of normal thalamocortical projections and modulatory cholinergic/noradrenergic inputs (Kleinschmidt *et al.,* 1987; Bear, 1996; Kirkwood *et al.,* 1996).

Another example of neurotransmitter actions influencing development is in the barrel fields of the rodent somatosensory cortex, which are specialized areas that receive sensory input from the mistacial vibrissae or whiskers. The cortical barrels are circular clusters of neurons in layer IV of this region of cortex which represent a somatatopic map of the vibrissae, i.e., each barrel corresponds to an individual vibrissa. The barrels are known to develop postnatally under the influence of sensory stimulation of the vibrissae (Woolsey and Wann, 1976). In a transgenic mouse line deficient in monoamine oxidase A, an enzyme which catalyzes the oxidative deamination of 5-HT and NE, it was noted that the barrel fields failed to develop (Cases *et al.,* 1995). The possible role of "overproduction" of 5-HT in this instance was confirmed in a follow-up study in which restoration of barrel field development was accomplished through the administration of parachlorophenylalanine, which inhibits 5-HT synthesis (Cases *et al.,* 1996). These results suggest that "appropriate" levels of 5-HT innervation are essential for normal development of this cortical region.

One of the most interesting effects of neurotransmitters on brain development relates to the cortical DA innervation of the frontal and mesolimbic regions of cortex. It is well-known that psychomotor stimulants, such as cocaine, increase levels of synaptic monoamines (Adamson and Meek, 1984; Almazan *et al.,* 1985). In the case of cocaine, this agent binds to transporters for NE, DA, and 5-HT. It has been postulated that early exposure to these agents may result in long-term irreversible behavioral changes, but the anatomical correlates remain to be identified (Boguski and McCormick, 1993). Most of the previous work has focused on alterations in monoamine systems as a result of drug exposure, although considerable inconsistencies exist in the abnormalities reported (Meyer *et al.,* 1996; Casper *et al.,* 1994). Levitt and coworkers identified a potential effect of cocaine on the development of postsynaptic cortical neurons resulting from increased levels of DA following *in utero* cocaine exposure in rabbits (Levitt *et al.,* 1997; Breyer and Cohen, 1990). These changes include abnormal dendritic arborization of cortical pyramidal cells in frontal and cingulate cortical regions which receive heavy DA innervation and upregulation of the expression of the calcium-binding protein, parvalbumin, within interneurons in these cortical regions (Carpenter and Cohen, 1979; Casper *et al.,* 1991). Rabbits that have received early exposure to cocaine exhibit

significant behavioral abnormalities which impair attentional tasks and correlate well with the location of the neuroanatomical abnormalities (Lee *et al.,* 1993; Levitt *et al.,* 1997). The postulated mechanism for the abnormal dendritic arborizations has been shown to correlate with changes in the activation of dopamine D1 versus D2 receptors in response to elevated levels of DA. Normal stimulation of D1 receptors in the cortical neurons from these regions has been shown to retard neurite outgrowth, whereas activation of D2 receptors has an opposite effect (D'Ercole *et al.,* 1996a). In situations in which there is an excess of DA, the D1 receptor becomes uncoupled from its normal G protein signaling cascade such that the normal inhibitory effect of D1 receptor activation is markedly diminished (T. Wood *et al.,* 1992; Folli *et al.,* 1996). The result is an abnormal growth of cortical dendrites and presumed abnormalities in synaptic organization of the cortical regions receiving this DA innervation. While a considerable amount of work needs to be performed to further elucidate the mechanism(s) of the effect, the findings to date suggest that a single neurotransmitter may have multiple effects depending on the compliment of receptor subtypes that are present in a given brain region, and that neurotransmitters acting in concert with other signals play a very important role in sculpting brain architecture during development.

The effect of neurotransmitters on neuronal morphology, including process outgrowth and growth cone guidance, has been extensively studied (Lipton and Kater, 1989). Most of the studies describing neuritogenesis, as well as analyses of the complex cytoskeletal rearrangements that mediate process formation, have focused on process outgrowth in primary cultured neurons (Dotti *et al.,* 1988; Deitch and Banker, 1993). Developing neurons, when grown in primary cell culture, begin to establish a mature neuronal configuration within several days. As determined by light microscopic and ultrastructural studies, the establishment of a mature neuronal morphology occurs in four distinct stages: (i) the formation of membranous rufflings or lamellipodia around the cell periphery of newly plated neurons; (ii) the development of short protrusions or filopodia arising from the lamellipodia, some of which develop into neurites; (iii) the differentiation and outgrowth of a single axon from one of the short neurites; and (iv) the establishment of dendrites and the further maturation of all neuronal processes. Although it is well accepted that a variety of signals are likely to participate in neuronal process formation, including growth factors, neurotransmitters, cytokines, and cell adhesion molecules, the mechanisms by which these extracellular cues influence the establishment of a mature neuronal morphology are not fully understood. The fact that cultured neurons, even from early embryonic brains, will spontaneously initiate process outgrowth, suggests that important signaling events had occurred before the cells were harvested. It is therefore difficult, in these primary cultures, to study the

initiation and execution of these signaling events and the respective cytoskeletal changes that follow. Another problem that complicates analysis of primary cultures is the inherent heterogeneity of the cell populations harvested, even from careful dissection of specific brain regions.

Studies using organisms with simple nervous systems and the ability to examine "identified neurons" have facilitated investigations of neurite outgrowth. For example, Helisoma neurons demonstrate the multiple effects of neurotransmitters on neuronal process outgrowth. Neuron B19 of the buccal ganglion withdraws filopodia decreases lamellipodial surface area and ceases elongation in response to serotonin (Haydon *et al.,* 1984). Acetylcholine, while having no effect on neuron B19 alone, was shown to inhibit the effects of serotonin on neuron B19 (McCobb *et al.,* 1988). These two studies indicate that neurons are responsive to multiple neurotransmitters and indicate that the coordinated response to these environmental cues is necessary to direct process outgrowth. The ability of developing neurons to respond to multiple neurotransmitters is also evident in studies of *Xenopus* spinal neurons dissected from the neural tube tissue of 1-day-old embryos (Zheng *et al.,* 1994, 1996). Using time-lapse differential interference contrast microscopy, growth cone turning was shown to be directed in response to neurochemical gradients of acetylcholine and glutamate. The acetylcholine response was mediated by nicotinic acetylcholine receptors (Zheng *et al.,* 1996). Interestingly, growth cone turning in response to glutamate was correlated with an increased number of filopodia, and treatment with cytochalasin B, an agent which depolymerizes filamentous actin, resulted in an inhibition of growth cone turning indicating an important role of actin microfilaments. Other neurotransmitters have also been shown to influence neuronal process outgrowth *in vitro.* In dissociated cultures of chick retina, cerebral cortex, and optic tectum, significant increases in neuronal cell number and degree of process outgrowth were observed in response to GABA (Michler-Stuke and Wolff, 1987; Spoerri, 1988). NMDA has also been shown to induce process extension and growth cone formation in cerebellar granule cell cultures, whereas dopamine promotes neurite elongation and branching rat E18 cortical cultures (Todd, 1992). Dopamine also induces neurite retraction (Dos Santos Rodriguez and Dowling, 1993) and inhibits growth cone motility (Lankford and Letourneau, 1989) in cultured retina neurons. These effects may represent differential activation of D1 versus D2 receptors as discussed previously.

Two main signaling cascades have been found, among others, to participate in neuritogenesis: (i) receptor tyrosine kinase activation of the Ras-MAPK pathway leading to new gene expression and (ii) cAMP-dependent protein kinase (cAMP/PKA)-mediated signaling which may directly elicit neurite formation independent of new gene expression or feed into the MAPK pathway (Segal and Greenberg, 1996). While many peptide growth

factors can activate the MAPK pathway via activation of receptor tyrosine kinases, numerous neurotransmitters can activate or modulate cAMP/PKA signaling (Abe *et al.,* 1991).

Microtubules, as well as filamentous actin, comprise the major cytoskeletal elements in growing neuronal processes (Bray and Bunge, 1981; Bunge, 1973; Yamada *et al.,* 1971). These structures are composed of heterodimers of α-tubulin and β-tubulin subunits which are assembled into polar structures with kinetically distinct "plus" and "minus" ends (Gelfand and Bershadky, 1991). The assembly of microtubules in neurons and other cell types depends on several parameters: polymerization rate, depolymerization rate, rescue rate (disassembly to assembly transition), and the catastrophe rate (assembly to disassembly rate) (Mitchison and Kirschner, 1984). "Dynamic instability" refers to the frequency of interconversions of microtubules between phases of assembly and disassembly and could be influenced by many factors, including posttranslational modifications of tubulin and binding of specific microtubule-associated proteins. Several studies have demonstrated that posttranslationally modified tubulins are differentially expressed along the growing neurite shaft. Specifically, distal axons and growth cones are enriched with tyrosinated α-tubulin (tyr-α-tubulin), whereas the proximal axons and soma are enriched with the acetylated form of α-tubulin (acet-α-tubulin) (Dotti and Banker, 1991; Robson and Burgoyne, 1988; Ferriera and Caceres, 1989; Morfini *et al.,* 1994). This distribution has been observed in many neuronal cell populations, including hippocampal, dorsal root ganglion and superior cervical ganglion neurons, and cerebellar macroneurons. The expression of tubulin isoforms, as well as binding of microtubule-associated proteins, has been used as a reporter of microtubular dynamics during neuronal process outgrowth (Tanaka and Kirschner, 1991; Baas and Black, 1990; Black *et al.,* 1996; Rochlin *et al.,* 1996). Specifically, the tyrosination of α-tubulin has been correlated with increases in microtubule dynamics concomitant with enhanced growth cone migration and process outgrowth. The acetylation of α-tubulin has been correlated with the decrease in microtubule dynamics associated with slow consolidation of older, more stable processes. Despite these "correlations," there is no evidence to demonstrate that these modifications directly affect microtubule dynamics.

From the studies described previously it is clear that a multiplicity of process outgrowth effects may be ascribed to various neurotransmitters in the developing nervous system. It is also important to remember that a variety of other signals also participate in neurite outgrowth, including various growth factors and extracellular matrix molecules. It is therefore essential to understand mechanistically how neurotransmitters may work in concert with other signals to mediate this complex process. Our laboratory has generated a stable neuronal progenitor cell line, AS583-8, which elaborates neurites in response to cAMP-mediated β-adrenergic and recep-

tor tyrosine kinase-mediated FGF receptors (Kwon *et al.,* 1993, 1995, 1996). The AS583-8 neuronal progenitor cell line was generated from the basal forebrain of E15 rats using replication-defective retroviral constructs which express temperature-sensitive mutants of the SV40 large T antigen (T^{ts}) and the neomycin resistance (*neo*) gene (Eves *et al.,* 1992). These cell lines were selected in medium containing the neomycin analog G418 and screened for temperature-sensitive growth characteristics and the expression of a neuronal phenotype (Kwon *et al.,* 1996). AS583-8 cells satisfied all the criteria of the screen, i.e., the presence of neuronal and the absence of glial markers.

The transductant cells proliferate at 33°C, the permissive temperature for T^{ts} antigen function, but stop dividing at 39°C, the nonpermissive temperature at which the T^{ts} antigen is inactive (Kwon *et al.,* 1996). When the cells cease dividing at 39°C (at the nonpermissive temperature for T^{ts}), they do not exhibit spontaneous neurite outgrowth. With cell division arrested at 39°C, for agents that elevate cAMP, such as forskolin, there is a very rapid induction of process formation (e.g., minutes), whereas certain peptide growth factors, such as bFGF, elicit process formation over a longer time course (e.g., 24–48h). We examined the effects of a panel of neurotransmitters and growth factors on the AS583-8 cells. Neurotransmitters were selected on the basis of previous studies that demonstrated a putative role in the developing nervous system. Growth factors were selected on the basis of previous reports of possible trophic actions on basal forebrain neurons. We found that only catecholamine neurotransmitters could elicit rapid process formation (cAMP-mediated, 5 min–1 h), and that only bFGF and EGF elicit the slower form of process outgrowth (24–48 h). Both pharmacological and molecular studies demonstrated that the cell line expresses β_2-adrenergic and FGFR-1 receptors. Therefore, AS583-8 cells offer a unique opportunity to study the initial events in neuronal process formation that are mediated by two well-known receptor-coupled systems.

At the nonpermissive temperature (37°C) the cell line displays a flat, nonrefractile cell shape characterized by ruffling or lamellipodia surrounding the cell body. Isoproterenol (ISO), a nonselective β-adrenergic agonist, induces an initial cell body retraction within 5 min followed by the formation of multiple-branched processes and neurite extension with large growth cone-like structures by 2 h (Fig. 7). The ISO-treated cells revert to their original morphology within 24 h. Re-treatment of reverted cells with ISO induces another cycle of growth cone formation and process extension (Kwon *et al.,* 1996). In contrast, the cell line response to bFGF is not apparent within the first 2 h (Kwon *et al.,* 1993, 1995; Fig. 7). Rather, neurite outgrowth appears after 24 h and is characterized by the formation of fewer processes with little branching and smaller growth cone-like structures. This cell shape is maintained for several days in culture. To confirm our

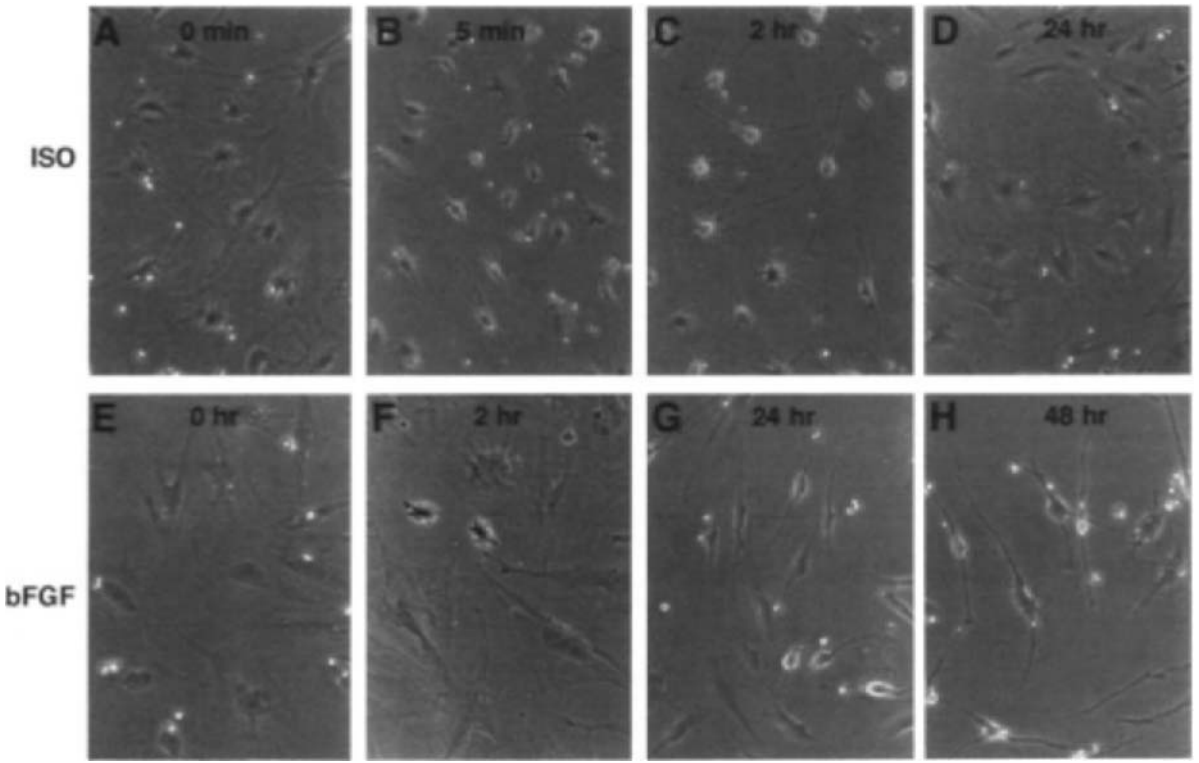

FIG. 7 Morphology of AS583-8 cells at different time points after ISO and bFGF addition. Photomicrographs of ISO-treated AS583-8 cells after 0 min (A), 5 min (B), 2 h (C), and 24 h (D) and bFGF-treated cells after 0 (E), 2 (F), 24 (G), and 48 h (H). Untreated cells exhibit a flat, nonrefractive morphology. ISO-treated cells exhibit cell body retraction within 5 min followed by the formation of multiple, highly branched processes within 2 h. The ISO-treated cells revert to their original morphology within 24 h. bFGF-treated cells do not exhibit process outgrowth until 12–24 h in culture (observations were made at 1, 2, 6, 12, and 24 h). After 24 h, the cells become somewhat spindle shaped with a few relatively unbranched, long processes. This morphology is maintained for several days. Magnification, ×500.

qualitative impressions we quantified the number of primary neurites after either ISO or bFGF treatment and found that the number was significantly ($p < 0.001$) higher in ISO-treated cells (6.38 ± 0.04 SEM) compared to bFGF-treated cells (4.11 ± 0.03 SEM) (Vogt Weisenhorn *et al.*, 1997).

To determine whether the cAMP-mediated process outgrowth requires new gene expression and protein synthesis, the effects of transcriptional and translational inhibitors were assessed (Kwon *et al.*, 1996; Fig. 8). The transcriptional inhibitor, actinomycin D (0.5 μg/ml), fails to inhibit the cAMP-mediated process outgrowth in AS583-8 cells whereas the [^{3}H]uridine incorporation was reduced by 99%. In addition, an inhibitor of both cytoplasmic and mitochondrial transcription, α-amanitin (5.0 μg/ml), also fails to inhibit the cAMP-mediated morphological differentiation. Similarly, the translational inhibitor, cycloheximide (5.0 μg/ml), fails to inhibit the cAMP-mediated process outgrowth while reducing the [^{35}S]methionine incorporation by 95%. Puromycin (100 μM), an inhibitor of both mitochondrial and cytoplasmic ribosomal function, also fails to inhibit the cAMP-mediated morphological differentiation. These results suggest that new gene expression is not required for the β-adrenergic cAMP-mediated process outgrowth and that the response may involve posttranslational events following activation of rapid intracellular signaling pathways. In contrast to the cAMP-mediated fast neurite initiation, transcriptional and translational inhibitors totally block the bFGF-mediated slow phase of neurite elongation and stabilization (Kwon *et al.*, 1995). Therefore, the mechanisms of the β_2-adrenergic receptor- and FGF receptor-mediated responses are likely to be quite distinct.

The differences observed in neurite initiation followed by β_2-adrenergic receptor and FGF receptor activation suggested that the receptor-mediated responses may involve the differential regulation of cytoskeletal protein expression, posttranslational modification, and/or rearrangements (Kwon *et al.*, 1996, 1998; Deltour *et al.*, 1993). Since actin and tubulin are known to be involved in neurite outgrowth, we performed confocal microscopic studies to localize filamentous actin (F-actin, labeled with rhodamine–phalloidin) and β-tubulin (labeled by indirect immunofluorescence using fluorescein; Kwon *et al.*, 1998). Following ISO treatment, F-actin becomes concentrated in the growth cone-like structures and the cell body, whereas β-tubulin becomes concentrated in the neurite shaft and in the cell body. Following bFGF treatment, a predominance of F-actin is observed in the growing neurite compared to ISO, whereas β-tubulin becomes localized to the cell body, with lesser intensity in neurite and growth cone-like structures. The distribution of α-tubulin was found to parallel the distribution of β-tubulin in response to ISO and bFGF.

To determine whether the posttranslationally modified tubulins exhibit different intracellular distributions in response to ISO and bFGF, dual

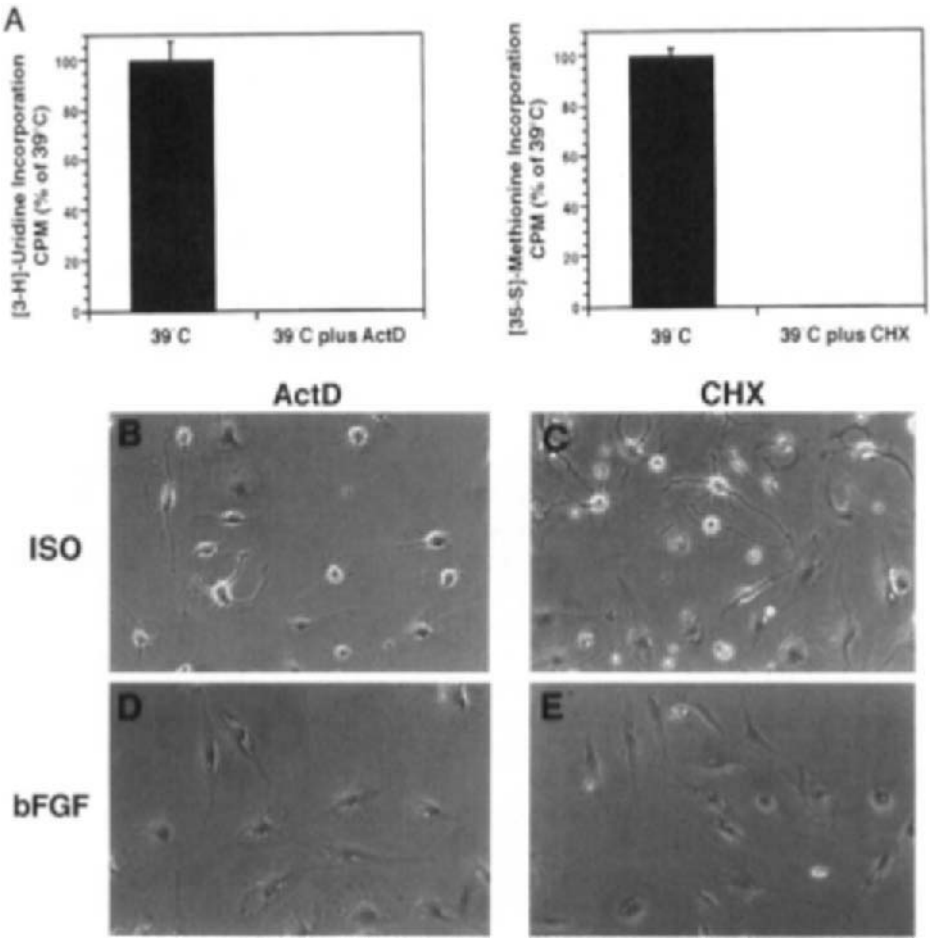

FIG. 8 Effects of transcription and translation inhibitors onto the ISO- and bFGF-induced morphologic response in AS583-8 cells. (A) A 24-h treatment with the transcriptional inhibitor, actinomycin D (ActD), decreases [^{3}-H] uridine incorporation to 2.8 ± 0.3% of control and a 24-h treatment with the translational inhibitor, cycloheximide (CHX), decreases [^{35}-S] methionine incorporation to 4.4 ± 0.4% of control. (B, C) Cells treated with ActD (B) or CHX (C) followed by ISO for 2 h still display a morphological response similar to that of cells treated with ISO alone. (D, E) Cells treated for 24 h with both bFGF and ActD (D) or CHX (E) continue to display the morphology of untreated cells. Magnification, ×500.

immunofluorescence confocal microscopy was performed to colocalize tyr-α-tubulin and acet-α-tubulin (Kwon *et al.,* 1998; Deltour *et al.,* 1993; Fig. 9, see color plate). In ISO-treated cells, tyr-α-tubulin is expressed in the cell body and extends into the distal neurites and growth cone-like structures,

whereas acet-α-tubulin is expressed predominantly in the proximal neurites. In contrast, bFGF-treated cells exhibit similar distributions of tyr-α-tubulin and acet-α-tubulin into the distal neurites. In order to assess these data quantitatively, we determined, using dual-immunofluorescence confocal microscopy, the area occupied by tyr-α-tubulin and acet-α-tubulin, respectively, in cells exposed to ISO or bFGF. We found, confirming our qualitative observations, that the ratio of the area occupied by acet-α-tubulin versus tyr-α-tubulin was significantly higher ($p < 0.05$) in bFGF-treated cells (0.87 $\pm$ 0.10 SEM) than in ISO-treated cells (0.59 $\pm$ 0.03 SEM) (Vogt Weisenhorn *et al.,* 1997; Kwon *et al.,* 1998).

These studies demonstrate that the distribution of tyr- versus acet-α-tubulin is differentially affected by ISO- versus bFGF-induced neurite outgrowth in the AS583-8 cell line. In the case of ISO, the distribution is similar to the pattern observed in neuronal processes that have enhanced microtubule dynamics and which are rapidly growing, whereas with bFGF, the distribution is similar to the pattern observed in more slowly growing processes with diminished microtubule dynamics (Rochlin *et al.,* 1996).

While the results presented previously suggest that adrenergic and FGF receptors can influence early neurite outgrowth, we believed that it would be important to demonstrate similar effects in primary neurons in order to verify the usefulness of the cell line as a model system. In initial experiments, we found that E16 basal forebrain neurons, the cell source for generation of the AS583-8 cell line, cultured for 24 h followed by a 1-h treatment with ISO exhibit an 83.8 $\pm$ 5.2% increase in growth cones that are intensely labeled for F-actin compared to untreated cells (Downen *et al.,* 1995; Kwon *et al.,* 1996). Although these primary cells elaborate processes spontaneously, in contrast to the AS583-8 cells, the time course of the enhanced growth cone response and character of the processes formed are identical to the responses observed in the cell line. We also examined the localization of posttranslationally modified α-tubulin in neurites arising spontaneously from E15 basal forebrain neurons (Vogt Weisenhorn *et al.,* 1997). In many neurons tyr-α-tubulin staining extends further into the distal neurite and growth cone that acet-α-tubulin. Quantitative analysis of three experiments revealed differences in the acet-/tyr-α-tubulin ratios in response to ISO versus bFGF treatment that are remarkably similar to those observed in AS583-8 cells (bFGF-treated cells, 0.84 $\pm$ 0.06 SEM; ISO-treated cells, 0.57 $\pm$ 0.01 SEM). However, these differences did not reach the level of statistical significance ($p = 0.09$). Such a result is not surprising since one might expect the cell populations present in primary cultures to be quite heterogeneous with respect to expression of FGF- and β-adrenergic receptors. In fact, examination of FGFR-1 and β_2-adrenergic receptor expression in primary neurons revealed that approximately 70% of the cells are positive for each marker. Based on this result we predict that at least

40% of the cells coexpress the two receptors. In contrast, approximately 95% of the AS583-8 cells stain positively for each receptor, suggesting a high incidence of coexpression. The similarity observed between the studies in the cell line and in the primary neuronal cultures supports the validity of the AS583-8 cell line as a model system.

Studies on the AS583-8 cell line have demonstrated two very different neurite outgrowth responses to β_2-adrenergic receptor versus FGF receptor activation. The responses differ with respect to time course, neurite morphology, requirements for new gene expression, the distributions of F-actin and tubulin, and the distributions of tyr- and acet-α-tubulin. Since both receptors are expressed in the developing nervous system, we hypothesize that both slow and rapid process outgrowth components contribute to the establishment of neuronal structure and connectional relationships.

Although most of the previous discussion focused on ways in which neurotransmitters may influence neuronal form, other types of actions pertinent to development have been described. One such action is related to the expression of neurotransmitter phenotype in the developing nervous system. An interesting example of this action is the development of postganglionic sympathetic innervation of eccrine sweat glands which, unlike other sympathetic pathways, are cholinergic instead of adrenergic (Habecker and Landis, 1994). Early in development the sweat glands are innervated by noradrenergic sympathetic fibers; however, following interactions with the sweat gland targets, the sympathetic afferents undergo a switch to a cholinergic phenotype. This switch has been demonstrated to be mediated via either α- or β-adrenergic receptors since the response can be blocked with appropriate pharmacological antagonists (Habecker and Landis, 1994). A diffusible factor has been postulated to mediate this action; however, known trophic factors which induce cholinergic differentiation such as CNTF or cardiotrophin-1 have been ruled out in this particular system (Francis *et al.,* 1997; Habecker *et al.,* 1995). However, in neuronal/sweat gland cocultures, the cholinergic differentiation activity was blocked by antibodies against the LIF β receptor (Habecker *et al.,* 1997). In addition, gland extracts stimulated phosphorylation of the LIF receptor and transcriptional activity in the STAT pathway, which is known to be activated by the LIF/CNTF family of neuropoietic cytokines. These results suggests that the sweat gland-derived activity may be a member of this family.

In addition to direct actions on developing neurons, neurotransmitter receptors are expressed on glial cells (Porter and McCarthy, 1997; Hosli and Hosli, 1982; Hosli *et al.,* 1983, 1995). The proposed consequences of receptor activation in this instance include alterations in glial energy metabolism (Magistretti and Pellerin, 1997) and release of neurotrophic growth factors from the glial cells (Schwartz and Mishler, 1990). In the latter

example, the neurotransmitters involved would be considered to be exerting "indirect neurotrophic effects."

To summarize, a variety of developmental programs have been shown to be influenced, or potentially influenced, by neurotransmitter systems and their receptors that are expressed early in development. In some instances the expression is developmentally regulated (i.e., not present in the mature nervous system) and is therefore more likely to play a role in the developmental program. The actions ascribed include control of neurogenesis in embryonic proliferative zones, the sculpting of neuronal form through influences on process outgrowth, growth cone guidance, activity-dependent synaptic plasticity (which may involve process outgrowth mechanisms), modulation of neurotransmitter phenotype, and various actions on glial cells. There is clearly much information that needs to be elucidated in this area, in particular how the signals elicited by neurotransmitters act in concert with other important developmental signals.

VII. Hormones

Hormones, produced by the endocrine system, represent another large group of small molecules exhibiting trophic actions in the brain and influencing behavior. There exist obvious overlaps between the nervous system and the endocrine system, even though both systems are morphologically distinct. The intimate connection between these two systems is evident by the fact that certain hormones can act as neurotransmitters (e.g., noradrenalin) and vice versa (e.g., neuropeptide Y). However, the field of neuroendocrinology is only emerging, and despite a myriad of reports describing actions of hormones onto the CNS, little is known about trophic actions of hormones in the nervous system with regard to the molecular and cellular mechanisms elicited by them. Among the many hormones studied, steroids, thyroid hormone, and insulin (previously discussed) represent important examples of hormones exerting trophic actions, and the following discussion will focus on these molecules. Notwithstanding, there also exists a growing number of studies showing trophic actions of other hormonal systems in the nervous system, including the renin–angiotensin system (Lenkei *et al.*, 1997), melantonin, oxytocin, and, of course, the "neuropeptide hormones"—neuropeptide Y, cholecystokinin, vasopressin, vasoactive intestinal peptide, enkephalin, and somatostatin.

A. Steroid Hormones

The function of steroid hormones, such as sex hormones (estrogens, progesterone, and androgens), glucocorticoids, and mineralocorticoids, is based

on the hormone reaching its target cell, crossing the plasma membrane, and binding to intracellular receptor proteins. The receptor is bound in its inactive state to an inhibitory protein complex. When the steroid hormone binds to its receptor, the inhibitory complex dissociates, thereby exposing the DNA binding site of the receptor. The receptor then binds to DNA and regulates transcriptional activity of genes. It is because of this mechanism that the onset of the action of these hormones has been traditionally thought to be slow and their actions to be prolonged.

In 1968 it was discovered that steroid hormones can reach the brain by crossing the blood–brain barrier (McEwen *et al.,* 1968). Follow-up studies verified the presence and distribution of intracellular steroid hormone receptors in the CNS (Ahima *et al.,* 1991; Chao *et al.,* 1989; Hagihara *et al.,* 1992; Pfaff and Keiner, 1973; Simerly *et al.,* 1990, Kawata, 1995). In addition to the systemic hormones reaching the brain, a group of steroids has been discovered which is synthesized directly in the CNS, either from precursors in the blood or *de novo* from cholesterol. These newly synthesized steroid hormones, such as pregnanolone or allopregnanolone, are called neurosteroids (Spindler, 1997). Interestingly, these neurosteroids bind preferentially to membrane-bound components. One of the best known membrane recognition sites which serves as a target for steroids is part of the $GABA_A$ receptor (Simmonds, 1991; Macdonald and Olsen, 1994). Furthermore, steroid hormones have modulatory effects on NMDA, kainate, and glycine receptors as well as on voltage-gated Ca^{2+} channels and the Mg^{2+}/Ca^{2+}-ATPase (Irwin *et al.,* 1994; Wong and Moss, 1994; Gazzaley *et al.,* 1996; Gu and Moss, 1996; ffrench-Mullen *et al.,* 1994). The modulation of kainate receptors and the voltage-gated Ca^{2+} channels appears to be a G protein-coupled process. This classical signal transduction mechanism can explain the recently described rapid changes in electrical properties of neurons evoked by steroid hormones (Joels, 1997) and implicates an important involvement of steroid hormones in the Ca^{2+} homeostasis of a subpopulation of neurons, which deserves further investigation. In addition, steroid hormones have been shown to modulate different neurotransmitter systems, such as the cholinergic, serotinergic, and catecholaminergic system (Joels and De Kloet, 1992; Hesen and Joels, 1993, 1996; Hu *et al.,* 1996). In these cases, and in the case of the later discussed estrogene-induced synapse formation, the hormone may cooperate with neurotrophins, specifically NGF. Estrogen receptors and NGF receptors are colocalized in neurons and estrogen influences the expression of NGF receptors in PC12 cells (Toran-Allerand *et al.,* 1992; Miranda *et al.,* 1994; Sohrabji *et al.,* 1994): Trk-A receptors become upregulated, and the low-affinity p75 receptor becomes downregulated. In addition, the binding of estrogen to these cells is enhanced by NGF. These findings suggest that estrogen and NGF cooper-

ate to amplify one another's trophic actions. In addition, this cooperation can also be brought about by the recently discovered estrogen interaction with the NGF-elicited MAP kinase signaling pathway (Migliaccio *et al.,* 1996; Singh *et al.,* 1997; Setalo *et al.,* 1997). One might assume that most of these processes take place in brain regions involved with regulation of the endocrine system, such as areas primarily involved in reproduction (in the case of ovarian steriods). However, neurons in areas as diverse as the basal forebrain, hippocampus, caudate putamen, midbrain raphe, and locus coerulus are also affected (McEwen *et al.,* 1997). In general, in the adult brain, steroids have important influences on synaptic plasticity, neuronal excitability, and neurotransmitter systems and therefore are implicated in learning processes as well as certain disease states such as epilotogenesis (Wickelgren, 1997).

Steroid hormones, specifically the sex steroids, also play an important role during the development of the nervous system (Lustig, 1996). Specifically, estrogen has been shown to augment neurite outgrowth and increase the formation of dendritic spines, membrane specializations which are involved in interneuritic structural contact. Interestingly, spine densities even vary with the estrous cycle in the adult rat, with the highest densities correlating with the highest plasma estradiol levels (Wooley and McEwen, 1993; Toran-Allerand *et al.,* 1983; Segarra and McEwen, 1991). These effects have been partially replicated *in vitro* using receptor-transfected PC12 cells as a model system (Lustig, 1996). Estrogen induces outgrowth of short neurites, enhances NGF-induced neurite elongation, and augments the production of neuritic spines, whereas testosterone has little effect on PC12 cells. The effects of estrogen were replicated in cultured hippocampal neurons, whereas neuritic development is altered by testosterone: It induces the production of more than one axon per cell (Lustig, 1996).

Sex differences related to sex steroids and their metabolism can be found early on in the brain. During a critical period of fetal development, testosterone produced by the testis is taken up by the brain and subjected to aromatization by a neuronal aromatase (P450 aromatase), which leads to the end product estradiol. In the hypothalamus the expression of P450 aromatase is induced by testosterone, and its activity is highest in the male during both the prenatal and the neonatal period. *In vitro,* sex differences concerning P450 activity can be found in that estradiol production is higher in "male" neurons than in "female" neurons. In addition, these gender-specific differences are area specific since they cannot be found in cortical cells (Tobet and Hanna, 1997). The "end product" of this testosterone influence on the brain during embryogenesis and perinatally is the sexually dimorphic brain. Gender differences are found in the volumes of nuclei due to differences mainly in absolute neuron numbers and density (Kawata,

1995). These neuroanatomic differences likely underlie functional sex differences in higher brain function in adulthood (Kimura and Hampson, 1993).

Steroid hormones also exhibit other classical neurotrophic actions, such as protection of neurons from apoptosis and enhancement of their survival. The best studied neural systems for these effects are the song control nuclei in canaries. Their survival during development is regulated by estrogens and androgens (Arnold, 1997). Studies demonstrating seasonal changes in the numbers of song control neurons in canaries also suggest that these hormones are crucially involved in this process. They appear to enhance the survival of the neurons (Alvarez-Buylla and Kirn, 1997). In mammals, steroids have been proven to protect neurons from injury-induced cell death (Singer *et al.,* 1996; Perez and Kelley, 1996). In axotomized motor neurons the proposed cellular mechanism involves the induction of the expression of calbindin, a calcium-binding protein which is involved in the regulation of cytoplasmic calcium (Perez and Kelley, 1997). However, to protect cells from toxic insults mediated by glutamate and β-amyloid, estrogen may also act through a novel, nuclear receptor-independent mechanism. In these cases estrogen acts as an anitoxidant eliminating free radicals (Green *et al.,* 1996, 1998; Behl *et al.,* 1995). Because of these neuroprotective effects and its ability to improve mental functions, estrogen has become very popular with regard to prevention or even treatment of Alzheimer's disease (Birge, 1997; Wickelgren, 1997).

In contrast to these numerous beneficial effects of steroid hormones, adrenal steroids have been reported to promote neural damage in the hippocampus (Houser *et al.,* 1983; Uno *et al.,* 1994). The mechanism by which these glucocorticoids exert this damaging action is unknown, but it seems to be connected to excitotoxicity and the calcium homeostasis in the neurons involved (Horner *et al.,* 1990). This effect of steroids on the brain might explain the described phenomenon in which individuals who have experienced prolonged periods of stress show cerebral cortical atrophy (Jensen *et al.,* 1982).

B. Thyroid Hormone

Thyroid hormone has become a target of research in neurobiology since it was discovered that thyroid hormone deficiency during prenatal and perinatal periods leads to severe cognitive impairment (Porterfield and Hendrich, 1993). Thyroid hormone is transported via a specific mechanism into the CNS involving transthyretin (formerly called prealbumin; Dratman *et al.,* 1991; Schreiber *et al.,* 1990). The primary biologically active thyroid

hormone 3,5,3'-triiodothyronine (T3) is derived by 5'-monodeiodination of 3,5,3,5'-tetraiodothyronine (thyroxine; T4) through enzymatic activity of 5'-deiodinases: 5'-deiodinase types I and II. In the CNS type II is predominant and primarily located in astrocytes. The presence of a "brain-specific" 5'-deiodinase provides the CNS with an independent mechanism to regulate thyroid hormone levels (Silva *et al.,* 1982; Visser *et al.,* 1982; Guadno-Ferraz *et al.,* 1997). The effect of T3 in thyroid-responsive tissue is mediated by binding to specific nuclear receptors, the α and β receptors, the products of two discrete genes (Lazar, 1993). Each gene product is expressed as two major isoforms and both are widely distributed in the body, including the CNS. High concentrations can be found in the hippocampus, cerbral cortex, the olfactory bulb, striatum, and some hypothalamic nuclei. The β receptor is found in high concentrations in the developing brain. The thyroid nuclear receptors belong, as do the steroid receptors, to the intracellular receptor superfamily, with which they share the highly conserved DNA-binding domain (Mangelsdorf *et al.,* 1995). Thyroid receptors act on thyroid hormone response elements in the regulatory sequences of different genes (e.g., tubulins, Na^+-K^+-ATPase, and growth hormone), thereby regulating their expression (Glass and Holloway, 1990; Desvergne, 1994). However, as is the case for steroid hormones, mechanisms for thyroid hormone action distinct from its regulation of gene expression cannot be excluded (Calza *et al.,* 1997).

In the mature brain the trophic actions of thyroid hormone have mainly been studied through examination of changes induced during the hypothyroid state. Prominent changes include up or downregulation of neuropeptides such as the vasoactive intestinal peptide and galanin (Calza *et al.,* 1997; Ceccatelli *et al.,* 1992; Giardino *et al.,* 1994). In the adult hippocampus gene expression regulation due to synaptic activity is altered in hypo- and hyperthyroid rats (Calza *et al.,* 1997). With increased synaptic activity, immediate early genes, such as c-*jun* and c-*fos,* become activated. These transcription factors in turn regulate the expression of NGF in the neurons in question. It is hypothesized that thyroid hormone induces changes in the activation and expression of the immediate early genes c-*fos* and c-*jun,* thereby possibly altering the expression of delayed response genes after increased synaptic activity. Interestingly, steroid hormones seem to regulate the expression of nuclear protooncogenes in the same fashion (Schuchard *et al.,* 1993; Calza *et al.,* 1996, 1997; Giardino *et al.,* 1995). Another interesting aspect of the action of thyroid hormone in the adult brain is the similar "phenotype" of hypothyroidism and the degeneration of the basal forebrain cholinergic system in Alzheimer's disease. Cholinergic degeneration correlates with changes in the expression of NGF and its low-affinity receptor p75, a situation which is also observed in hypothyroidism (Calza *et al.,*

1997). Furthermore, other neurotransmitter systems, such as the noradrenergic and serotonergic systems, are also affected by levels of thyroid hormone in the brain (Henley *et al.*, 1991, 1996; Andersson and Eneroth, 1987). Thyroid hormone is highly concentrated in noradrenergic nuclei and in their terminal fields. In the noradrenergic nuclei thyroid hormone is located in the cytosol and processes of neurons. In the terminal fields the hormone was prominent in cell nuclei. This distribution suggests that thyroid hormone may act as cotransmitters in the adrenergic nervous system (Dratman and Gordon, 1996). The association with the noradrenergic and serotonergic system may be why the thyroid hormone can influence mood. This has possibly led to its use as adjuncts for traditional antidepressant medications (Henley and Vladic, 1997).

The effect of hypothyroidism on the function and structural integrity of the developing brain is more severe and permanent than that in the adult brain. In humans, the classical signs and symptoms of congenital hypothyroidism develop progressively from the last trimester of gestation to 6–8 postnatal months. The signs and symptoms of hypothyroidism, with respect to the maturation of the CNS, are motor and cognitive disorders. In order to study the effects of hypothyroidism during development, animal models have been developed such as the hyt/hyt mouse model system, which has a mutation in the thyroid-stimulating hormone receptor gene, rendering it incapable of producing thyroid hormone. These mice show astonishingly similar phenotypes to untreated hypothyroidism in humans, such as delayed somatic and reflex development and permanent deficits in hearing and locomotor and adaptive motor behavior (Sher *et al.*, 1998).

The trophic actions of thyroid hormone on developing neurons and glial cells are many-fold. Most interest has focused on the promotion of cell survival and neurite outgrowth. Neuronal survival is promoted in embryonic and neonatal dorsal root ganglia, in cerebellar granule cells, and in the septum by thyroid hormone (Walter, 1996; Muller *et al.*, 1995; Filipcik *et al.*, 1994). This trophic action of thyroid hormone could be mediated by an influence of thyroid hormone on the expression of the bcl-2 protein, which is known to prevent cell death. In early granule neurons of the cerebellum, thyroid hormone induced a drastic increase of bcl-2 expression and in hypothyroid rats the bcl-2 content in the cerebellum was drastically reduced (Muller *et al.*, 1995). However, it is not clear whether thyroid hormone influences bcl-2 expression directly or through its known capacity to influence the expression of neurotrophins (through which it also induces the expression of AChE (Garza *et al.*, 1990). In contrast, in the same cells, but in late postnatal stages, thyroid hormone leads to cell death, despite the fact that NGF expression becomes induced. However, in parallel to an increase in NGF expression, NT-3 expression becomes depressed. Overexpression of NT-3 could rescue the normal phenotype (Neveu and Arenas,

1996), suggesting that NT-3 deficiency induced by hypothyroidism is the reason for the observed cell death.

The effect of thyroid hormone on neurite outgrowth and elongation is believed to be mediated by a thyroid hormone-induced regulation of the expression of cytoskeletal proteins, specifically actins and tubulins. Hypothyroidism results in a significant reduction in the steady-state levels of actin and tubulin mRNAs during the first postnatal week. There is also a delay in the decline of actin and tubulin mRNA levels that normally occurs later, resulting in older animals having high levels of these mRNAs. Actin mRNA levels are likely to be regulated at the transcriptional level, whereas tubulin mRNA has been shown to be regulated on a posttranscriptional level (Poddar *et al.,* 1996). A recent report demonstrated that thyroid hormone is required for dynein expression, a cytoskeletal protein involved in axonal transport (Barakat-Walter and Riederer, 1996).

Thyroid hormone also influences glial cells. For example, in astrocytes thyroid hormone induces GFAP expression, influences their morphology in a regional-specific manner, and induces their proliferation (Lima *et al.,* 1997; Trentin *et al.,* 1995). Whether these changes in astrocytes in turn mediate some of the effects of thyroid hormone onto neurons remains to be elucidated.

In summary, hormones, specifically steroids and the thyroid hormone, have a very profound impact on major developmental events in the brain and can influence to a large extent the function(s) of the adult brain. This justifies the classification of hormones as trophic factors, even though the intracellular mechanisms by which these hormones exert their functions are quite distinct from the classical trophic factors discussed previously.

VIII. Conclusion

Growth factors play a major role in the development of the nervous system and in the maintenance and proper function of neural circuitries in the adult brain. During the past few years, numerous novel and "well-known" factors have been shown to act as trophic factors in the brain. Even though remarkable progress has been made in elucidating their trophic actions and their molecular mechanisms, we still have much to learn. The intriguing question remaining to be solved is how these different factors work together in "nourishing" our developing and adult brain. The emerging evidence hints to a complicated network of interdependent actions on the cellular and systemic levels, which in itself is as fascinating and complex as the ultimate product of these actions—the nervous system.

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References

Abe, K., and Saito, H. (1992). Epidermal growth factor selectively enhances NMDA receptor-mediated increases of intracellular Ca^{2+} concentration in rat hippocampal neurons. *Brain Res.* **587,** 102–108.

Abe, K., Xie, F. J., and Saito, H. (1991). Epidermal growth factor enhances short-term potentiation and facilitates induction of long-term potentiation in rat hippocamplal slices. *Brain Res.* **547,** 171–174.

Adamson, E. D., and Meek, J. (1984). The ontogeny of epidermal growth factor receptors during mouse development. *Dev. Biol.* **103,** 62–70.

Ahima, R., Krozowski, Z., and Harlan, R. (1991). Type I corticosteroid receptor-like immunoreactivity in the rat CNS: Distribution and regulation by corticosteroids. *J. Comp. Neurol.* **313,** 522–538.

Airaksinen, M. S., Koltzenburg, M., Lewin, G. R., Masu, Y., Helbig, C., Wolf, E., Brem, G., Toyka, K. V., Thoenen, H., and Meyer, M. (1996). Specific subtypes of cutaneous mechanoreceptors require neurotrophin-3 following peripheral target innervation. *Neuron* **16,** 287–295.

Almazan, G., Honegger, P., Matthieu, J. M., and Guentert-Lauber, B. (1985). Epidermal growth factor and bovine growth hormone stimulate differentiation and myelination of brain cell aggregates in culture. *Brain Res.* **353,** 257–264.

Alvarez-Buylla, A., and Kirn, J. R. (1997). Birth, migration, incorporation, and death of vocal control neurons in adult songbirds. *J. Neurobiol.* **33**(5), 585–601.

Anderson, K. D., Alderson, R. F., Altar, C. A., DiStefano, P. S., Corcoran, T. L., Lindsay, R. M., and Wiegland, S. J. (1995). Differential distribution of exogenous BDNF, NGF, and NT-3 in the brain corresponds to the relative abundance and distribution of high-affinity and low-affinity neurotrophin receptors. *J. Comp. Neurol.* **357**(2), 296–317.

Andersson, K., and Eneroth, P. (1987). Thyroidectomy and central catecholamine neurons of the male rat. *Neuroendocrinology* **45,** 14–27.

Angeletti, R. H., and Bradshaw, R. A. (1971). Nerve growth factor from mouse submaxillary gland: Amino acid sequence. *Proc. Natl. Acad. Sci. USA* **68,** 2417–2420.

Anonymous (1995). A phase I study of recombinant human ciliary neurotrophic factor (rhCNTF) in patients with amyotrophic lateral sclerosis. The ALS CNTF Treatment Study (ACTS) Phase I–II Study Group. *Clin. Neuropharmacol.* **18,** 515–532.

Antoniades, H. N. (1981). Human platelet-derived growth factor: Purification of PDGF-1 and PDGF-II and separation of their reduced subunits. *Proc. Natl. Acad. Sci. USA* **78,** 7314–7317.

Antonopoulos, J., Pappas, I. J., and Parnavelas, J. G. (1997). Activation of the GABAA receptor inhibits the proliferative effects of bFGF in cortical progenitor cells. *Eur. J. Neurosci.* **9**(2), 291–298.

Arnold, A. P. (1997). Sexual differentiation of the zebra finch song system: Positive evidence, negative evidence, null hypotheses, and a paradigm shift. *J. Neurobiol.* **33,** 572–584.

Arnsten, A. F. (1997). Catecholamine regulation of the prefrontal cortex. *J. Psychopharmacol.* **11,** 151–162. [Review]

Arsenijevic, Y., and Weiss, S. (1998). Insulin-like growth factor-I is a differentiation factor for postmitotic CNS stem cell-derived neuronal precursors: Distinct actions from those of brain-derived neurotrophic factor. *J. Neurosci.* **18**(6), 2118–2128.

Asakura, K., Hunter, S. F., and Rodriguez, M. (1997). Effects of transforming growth factor-beta and platelet-derived growth factor on oligodendrocyte precursors: Insights gained from a neuronal cell line. *J. Neurochem.* **68**(6), 2281–2290.

Baas, P. W., and Black, M. M. (1990). Individual microtubules in the axon consist of domains that differ in both composition and stability. *J. Cell Biol.* **111,** 495–508.

Barakat-Walter, I., and Riederer, B. M. (1996). Triiodothyronine and nerve growth factor are required to induce cytoplasmic dynein expression in rat dorsal root ganglion cultures. *Brain Res.* **96**(1–2), 109–119.

Barbacid, M. (1994). The Trk family of neurotrophin receptors. *J. Neurobiol.* **25,** 1386–1403.

Barde, Y. A., Edgar, D., and Thoenen, H. (1982). Purification of a new neurotrophic factor from mammalian brain. *EMBO J.* **1,** 549–553.

Barinaa, M. (1994). Neurotrophic factors enter the clinic. *Science* **264,** 772–774.

Barker, P. A., and Shooter, E. M. (1994). Disruption of NGF-binding to the low affinity neurotrophin receptor p75LNTR reduces NGF binding to TrkA on PC12 cells. *Neuron* **12,** 203–215.

Barres, B. A., Raff, M. C., Gaese, F., Bartke, I., Dechant, G., and Barde, Y. A. (1994). A crucial role for neurotrophin-3 in oligodendrocyte development. *Nature* **367,** 371–375.

Bartel, D. P., Sheng, M., Lau, L. F., and Greenberg, M. E. (1989). Growth factors and membrane depolarization activate distinct programs of early response gene expression: Disassociation of *fos* and *jun* interaction. *Genes Dev.* **3,** 304–313.

Bartlett, P. F., Richards, L. R., Kilpatrick, T. J., Talman, P. T., Bailey, K. A., Brooker, G. J., Dutton, R., Koblar, S. A., Nurcombe, V., Ford, M. O., *et al.* (1995). Factors regulating the differentiation of neural precursors in the forebrain. *Ciba Foundation Symp.* **193,** 85–99.

Barlett, W. P., Li, X. S., and Williams, M. (1992). Expression of IGF-I mRNA in the murine subventricular zone during postnatal development. *Mol. Brain Res.* **12,** 285–291.

Bar Sagi, D., and Feramisco, J. R. (1985). Microinjection of the ras oncogene protein into PC12 cells induces morphological differentiation. *Cell* **42,** 841–848.

Basu, T., Warne, P. H., and Downward, J. (1994). Role of Shc in the activation of Ras in response to epidermal growth factor and nerve growth factor. *Oncogene* **9,** 3483–3491.

Batzer, A. G., Rotin, D., Urena, J. M., Skolnik, E. Y., and Schlessinger, J. (1994). Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. *Mol. Cell. Biol.* **14,** 5192–5201.

Baxter, R. C., and Martin, J. L. (1989). Biinding proteins for the insulin-like growth factors: Structure, regulation, and function. *Prog. Growth Factor Res.* **1,** 49–68.

Bear, M. F. (1996). NMDA-receptor-dependent synaptic plasticity in the visual cortex. *Prog. Brain Res.* **108,** 205–218. [Review]

Bear, M. F., and Singer, W. (1986). Modulation of visual cortical plasticity by acetylcholine and noradrenaline. *Nature* **320,** 172–176.

Bear, M. F., Kleinschmidt, A., Gu, Q. A., and Singer, W. (1990). Disruption of experience-dependent synaptic modifications in striate cortex by infusion of an NMDA receptor antagonist. *J. Neurosci.* **10,** 909–925.

Beck, K., Powell-Braxton, L., Widmer, H. R., Valverde, J., and Hefti, F. (1995). IGF-1 gene disruption results in reduced brain size, CNS hypomyelination, and loss of hippocampal granule and striatal parvalbumin-containing neurons. *Neuron* **14,** 717–730.

Behl, C., Widmann, M., Trapp, T., and Holsboer, F. (1995). 17-Beta estradiol protects neurons from oxidative stress-induced cell death in vitro. *Biochem. Biophys. Res. Commun.* **216**(3), 473–482.

Beitner-Johnson, D., Blakesley, V. A., Shen-Orr, Z., *et al.* (1996). The proto-oncogene product c-Crk associates with IRS-I and 4PS: Modulationi by IGF-I and enhanced IGF-1 signaling. *J. Biol. Chem.* **271,** 9287–9290.

Belluardo, N., Wu, G., Mudo, G., Hansson, A. C., Petterson, R., and Fuxe, E. (1997). Comparative localization of fibroblast growth factor receptor-1, -2, and -3 mRNAs in the rat brain: In situ hybridization analysis. *J. Comp. Neurol.* **379**(2), 226–246.

Berger-Sweeney, J., and Hohmann, C. F. (1997). Behavioral consequences of abnormal cortical development: Insights into developmental disabilities. *Behav. Brain Res.* **86,** 121–142.

Berkemeier, L. R., Winslow, J. W., Kaplan, D. R., Nikolics, K., Goeddel, D. V., and Rosenthal, A. (1991). Neurotrophin-5: A novel neurotrophic factor that activates trk and trkB. *Neuron* **7,** 857–866.

Berminger, B., Garcia, D. E., Inagaki, N., Hahnel, C., and Lindholm, D. (1993). BDNF and NT-3 induce intracellular Ca^{2+} elevation in hippocampal neurons. *Neuro Report* **4,** 1303–1306.

Berridge, M. (1993). Inositol triphosphate and calcium signaling. *Nature* **361,** 315–325.

Besnard, F., Perraud, F., Sensenbrenner, M., and Labourdette, G. (1987). Platelet-derived growth factor is a potent mitogen for glial cell but not neuronal rat brain cells in vitro. *Neurosci. Lett.* **73,** 287–292.

Bhat, N. R., and Zhang, P. (1996). Activation of mitogen-activated protein kinases in oligodendrocytes. *J. Neurochem.* **66**(5), 1986–1994.

Binoux, M. (1995). The IGF system in metabolism regulation. *Diabete Metab. (Paris)* **21,** 330–337.

Birge, S. J. (1997). The role of estrogen in the treatment of Alzheimer's disease. *Neurology* **48**(5, Suppl. 7),S36–S41.

Birren, S. J., Lo, L., and Anderson, D. J. (1993). Sympathetic neuroblasts undergo a developmental switch in trophic dependence. *Development* **119,** 597–610.

Black, M. M., Slaughter, T., Moschiah, S., Obrocka, M., and Fisher, I. (1996). Tau is enriched on dynamic microtubules in the distal region of growing axons. *J. Neurosci.* **16,** 3601–3619.

Blakesley, V. A., Scrimgeour, A., Esposito, D., and LeRoith, D. (1996). Signaling via the insulin-like growth factor-I receptor: Does it differ from insulin receptor signaling? *Cytol. Growth Factor Rev.* **7**(2), 153–159.

Boguski, M. S., and McCormick, F. (1993). Proteins regulating Ras and its relatives. *Nature* **366,** 643–654.

Bondy, C., and Lee, W. H. (1993). Correlation between insulin-like growth factor (IGF)-binding protein 5 and IFG-I gene expression during brain development. *J. Neurosci.* **13,** 5092–5104.

Bonini, N. M., and Choi, K.-W. (1995). Early decisions in Drosophila eye morphogenesis. *Curr. Opin. Genet. Dev.* **5,** 507–515.

Bonni, A., Ginty, D. D., Dudek, H., and Greenberg, M. E. (1995). Serine 133-phosphorylated CREB induces transcription via a cooperative mechanism that may confer specificity to neurotrophin signals. *Mol. Cell. Neurosci.* **6,** 168–183.

Bostrom, H., Willetts, K., Pekny, M., Leveen, L., Lindahl, P., Hedstrand, H., Pekna, M., Hellstrom, M., Gebre-Medhin, S., Schalling, M., Nilsson, M., Kurland, S., Tornell, J., Heath, J. K., and Besholtz, C. (1996). PDGF-A signalling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* **85,** 863–873.

Boulton, T. G., Zhong, Z., Wen, Z., Darnell, J. E., Jr., Stahl, N., and Yancopoulos, G. D. (1995). STAT3 activation by cytokines utilizing gp130 and related tranducers involves a secondary modification requiring an H7-sensitive kinase. *Proc. Natl. Acad. Sci. USA* **92,** 6915–6919.

Bouvier, M. M., and Mytilineou, C. (1995). Basic fibroblast growth factor increases division and delays differentiation of dopamine precursors in vitro. *J. Neurosci.* **15**(11), 7141–7149.

Brar, A. K., and Chernausek, S. D. (1993). Localization of insulin-like growth factor binding protein-4 expression in the developing and adult rat brain: Analysis by in situ hybridization. *J. Neurosci. Res.* **35,** 103–114.

Braun, A. R., Laruelle, M., and Mouradian, M. M. (1997). Interactions between D1 and D2 dopamine receptor family agonists and antagonists: The effects of chronic exposure on behavior and receptor binding in rats and their clinical implications. *J. Neural Transmission* **104,** 341–362. [Review]

Bray, D., and Bunge, M. B. (1981). Serial analysis of microtubules of cultures rat sensory neurons. *J. Neurocytol.* **10,** 589–605.

Bredesen, D. E., and Rabizadeh, S. (1997). $p75^{NTR}$ and apoptosis: Trk-dependent and Trk-independent effects. *TINS* **20,** 287–290.

Brenneman, D. E., Schultzberg, M., Bartfai, T., and Gozes, I. (1992). Cytokine regulation of neuronal survival. *J. Neurochem.* **58,** 454–460.

Brevitt, B., and Clark, J. J. (1988). Growth and transparency in the lens, an epithelial tissue, stimulated by pulses of PDGF. *Science* **242,** 777–779.

Breyer, J. A., and Cohen, S. (1990). The epidermal growth factor precursor isolated from murine kidney membranes. Chemical characterization and biological properties. *J. Biol. Chem.* **265,** 16564–16570.

Bueker, E. D. (1948). Implantation of tumors in the hind limb field of the embryonic chick and the developmental response of the lumbarsacral nervous system. *Anat. Rec.* **102,** 369–387.

Buj-Bello, A., Buchman, V. L., Horton, A., Rosenthal, A., and Davies, A. M. (1995). GDNF is an age-specific survival factor for sensory and autonomic neurons. *Neuron* **15,** 821–828.

Buj-Bello, A., Adu, J., Pinon, L. G., Horton, A., Thompson, J., Rosenthat, A., Chinchetru, M., Buchman, V. L., and Davies, A. M. (1997). Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. *Nature* **387**(6634), 721–724.

Bunge, M. (1973). Fine structure of nerve fibers and growth cones of isolated sympathetic neurons in cultures. *J. Cell Biol.* **56,** 713–735.

Burgering, B., and Coffer, P. (1995). Protein kinase B (Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376,** 599–602.

Burstein, D. E., and Greene, L. A. (1978). Evidence for RNA synthesis-dependent and -independent pathways in stimulation of neurite outgrowth by nerve growth factor. *Proc. Natl. Acad. Sci. USA* **75,** 6059–6063.

Busbridge, N. J., Dascombe, M. J., Tilders, F. J. H., van Oers, J. W. A. M., Linton, E. A., and Rodwell, N. J. (1989). Central activation of thermogenesis and fever by IL-1 beta and IL-1 alpha involves different mechanisms. *Biochem. Biophys. Res. Commun.* **162,** 591–596.

Caday, C. G., Klagsbrun, M., Fanning, P. J., Mirzabegian, A., and Finklestein, S. P. (1990). Fibroblast growth factor (FGF) levels in the developing rat. *Brain Res. Dev. Brain Res.* **51**(1–2), 241–246.

Calabresi, P., Pisani, A., Mercuri, N. B., and Bernardi, G. (1996). The corticostriatal projection: From synaptic plasticity to dysfunctions of the basal ganglia. *Trends Neurosci.* **19**(1), 19–25.

Calza, L., Giardino, L., Ceccatelli, S., and Hokfelt, T. (1996). Neurotrophins and their receptors in the adult hypo- and hyperthyroid rat after kainic acid injection: An in situ hybridization study. *Exp. Neurol.* **8,** 1873–1881.

Calza, L., Aloe, L., and Giardino, L. (1997). Thyroid hormone-induced plasticity in the adult rat brain. *Brain Res.* **44**(4), 549–557.

Carpenter, G., and Cohen, S. (1979). Epidermal growth factor. *Annu. Rev. Biochem.* **48,** 193–216.

Carson, M. J., Behringer, R. R., Brinster, R. L., and McMorris, F. A. (1993). Insulin-like factor I increases brain growth and central nervous system myelination in transgenic mice. *Neuron* **10,** 729–740.

Carter, B. D., and Lewin, G. R. (1997). Neruotrophins live or let die: Does $p75^{NTR}$ decide? *Neuron* **18,** 187–190.

Casaccia-Bonnefil, P., Carter, B. D., Dobrowsky, R. T., and Chao, M. V. (1996). Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. *Nature* **383,** 716–719.

Cases, O., Seif, I., Grimsby, J., Gaspar, P., Chen, K., Pournin, S., Muller, U., Aguet, M., Babinet, C., Shih, J. C., *et al.* (1995). Aggressive behavior and altered amounts of brain serotonin and norepinephrine in mice lacking MAOA [see comments]. *Science* **268,** 1763–1766.

Cases, O., Vitalis, T., Seif, I., De Maeyer, E., and Sotelo, C. (1996). Lack of barrels in the somatosensory cortex of monoamine oxidase A-deficient mice: Role of a serotonin excess during the critical period. *Neuron* **16,** 297–307.

Casper, D., Mytilineou, C., and Blum, M. (1991). EGF enhances the survival of dopamine neurons in rat embryonic mesencephalon primary cell culture. *J. Neurosci. Res.* **30,** 372–381.

Casper, D., Roboz, G. J., and Blum, M. (1994). Epidermal growth factor and basic fibroblast growth factor have independent actions on mesencephalic dopamine neurons in culture. *J. Neurochem.* **62,** 2166–2177.

Cavanagh, J. F., Mione, M. C., Pappas, I. S., and Parnavelas, J. G. (1997). Basic fibroblast growth factor prolongs the proliferation of rat cortical progenitor cells in vitro without altering their cell cycle parameters. *Cerebral Cortex* **7**(4), 293–302.

Ceccatelli, S., Giardino, L., and Calza, L. (1992). Response of hypothalamic peptide mRNAs to thyroidectomy. *Neuroendocrinology* **56,** 694–703.

Chadi, G., Cao, Y., Pettersson, R. F., and Fuxe, K. (1994). Temporal and spatial increase of astroglial basic fibroblast growth factor synthesis after 6-hydroxydopamine-induced degeneration of the nigrostriatal dopamine neurons. *Neuroscience* **61**(4), 891–910.

Chao, H. M., Choo, P. H., and McEwen, B. S. (1989). Glucocorticoid and mineralocorticoid receptor mRNA expression in rat brain. *Neuroendocrinology* **50,** 365–372.

Chen, R., Sarnecki, C., and Blenis, J. (1992). Nuclear localization and regulation of erk- and rsk-enclosed protein kinases. *Mol. Cell. Biol.* **12,** 915–927.

Chen, R. H., Tung, R., Abate, C., and Blenis, J. (1993). Cytoplasmic to nuclear signal transduction by mitogen-activated protein kinase and 90 kDa ribosomal 96 kinase. *Biochem. Soc. Trans.* **21,** 895–900.

Cheng, B., and Mattson, M. (1995). PDGFs protect hippocampal neurons against energy deprivation and oxidative injury: Evidence for induction of antioxidant pathways. *J. Neurosci.* **15,** 7095–7104.

Cheng, H.-L., and Feldman, E. L. (1997). Insulin-like growth factor-I (IGF-1) and IGF binding protein-5 in Schwann cell differentiation. *J. Cell Physiol.* **171,** 161–167.

Ciani, E., and Contestable, A. (1998). Ornithine decarboxylase is differentially induced by kainic acid during brain development in the rat. *Dev. Brain Res.* **71,** 258–260.

Claesson-Welsh, L. (1994). Platelet-derived growth factor receptor signals. *J. Biol. Chem.* **269,** 32023–32026.

Clary, D. O., and Reichardt, L. F. (1994). An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin 3. *Proc. Natl. Acad. Sci. USA* **91,** 11133–11137.

Conover, J. C., Erickson, J. T., Katz, D. M., Bianchi, L. M., Poueymirou, W. T., McClain, J., Pan, L., Helgren, M., Ip, N. Y., Boland, P., Friedman, B., Wiegand, S., Vejsada, R., Kato, A. C., DeChiara, T. M., and Yancopoulos, G. D. (1995). Neuronal deficits, not involving motor neurons, in mice lacking BDNF and/or NT4. *Nature* **375,** 235–238.

Contreras, D., and Steriade, M. (1995). Cellular basis of EEG slow rhythms: A study of dynamic corticothalamic relationships. *J. Neurosci.* **15,** 604–622.

Cordon-Cardo, C., Tapley, P., Jing, S., Nanduri, V., O'Rourke, E., Lamballe, F., Kovary, K., Klein, R., Jones, K. R., Reichardt, L. F., and Barbacid, M. (1991). The trk tyrosine protein kinase mediates the mitogenic properties of nerve growth factor and neurotrophin-3. *Cell* **66,** 173–183.

Corfas, G., Rosen, K. M., Aratake, H., Krauss, R., and Fischbach, G. D. (1995). Differential expression of ARIA isoforms in the rat brain. *Neuron* **14,** 103–115.

Cosgaya, J. M., and Aranda, A. (1996). Ras- and raf-mediated regulation of transforming growth factor β1 gene expression by ligand tyrosine kinase receptors in PC12 cells. *Oncogene* **12,** 2651–2660.

Cowley, S., Paterson, H., Kemp, P., and Marshall, C. (1994). Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* **77,** 841–852.

Coyle, J. T., and Molliver, M. E. (1977). Major innervation of newborn rat cortex by monoaminergic neurons. *Science* **196,** 444–447.

Craig, C. G., Tropepe, V., Morshead, C. M., Reynolds, B. A., Weiss, S., and van der Kooy, D. (1996). In vivo growth factor expansion of endogenous subependymal neural precursor cell populations in the adult mouse brain. *J. Neurosci.* **16**(8), 2649–2658.

Creuzet, C., Loeb, J., and Barbin, G. (1995). Fibroblast growth factors stimulate protein tyrosine phosphorylation and mitogen-activated protein kinase activity in primary cultures of hippocampal neurons. *J. Neurochem.* **64**(4), 1541–1547.

Crowley, C., Spencer, S. D., Nishimura, M. C., Chen, K. S., Pitts-Meek, S., Armanini, M. P., Ling, G. H., McMahon, S. B., Sheldon, D. L., Levinson, A. D., and Phillips, H. S. (1994). Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. *Cell* **76,** 1001–1011.

Cruiso, J. L., Keith, A. H., and Michalopoulos, G. K. (1985). Induction of DNA synthesis in cultured rat hepatocytes through stimulation of a1 adreno-receptors by norepinephrine. *Science* **227,** 749–751.

Cuevas, P., Carceller, F., Munoz-Willery, I., and Gimenez-Gallego, G. (1998). Intravenous fibroblast growth factor penetrates the blood–brain barrier and protects hippocampal neurons against ischemia–reperfusion injury. *Surg. Neurol.* **49**(1), 77–83.

Curtis, R., Adryan, K. M., Zhu, Y., Harkness, P. J., Lindsay, R. M., and DiStefano, P. S. (1993). Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury. *Nature* **365,** 253–255.

D'Arcangelo, G., and Halegoua, S. (1993). A branched signaling pathway for nerve growth factor is revealed by Src-, Ras-, and Raf-mediated gene inductions. *Mol. Cell. Biol.* **13,** 3146–3155.

Davies, A. M., Minichiello, L., and Klein, R. (1995). Developmental changes in NT3 signalling via TrkA and TrkB in embryonic neurons. *EMBO J.* **14,** 4482–4489.

Davis, S., Aldrich, T. H., Valenzuela, D. M., Wong, V., Furth, M. E., Squinto, S. P., and Yancopoulos, G. D. (1991). The receptor for ciliary neurotrophic factor. *Science* **253,** 59–63.

Davis, S., Aldrich, T. H., Ip, N. Y., Stahl, N., Scherer, S., Farruggella, T., DiStefano, P. S., Panayotatos, N., Gascan, *et al.* (1993). Released form of CNTF receptor alpha component as a soluble mediator of CNTF responses. *Science* **259,** 1736–1739.

Dechant, G., and Barde, Y.-A. (1997). Signalling through the neurotrophin receptor $p75^{NTR}$. *Curr. Opin. Neurobiol.* **7,** 413–418.

DeChiara, T. M., Vejsada, R., Poueymirou, W. T., Acheson, A., Suri, C., Conover, J. C., McClain, J., Pan, L., Stahl, N., Ip, N. Y., Kato, A., and Yancopoulos, G. D. (1995). Mice lacking the CNTF receptor, unlike mice lacking CNTF, exhibit profound motor neuron deficits at birth. *Cell* **83,** 313–322.

Deitch, J. S., and Banker, G. A. (1993). An electron microscopic analysis of hippocampal neurons developing in culture: Early stages in the emergence of polarity. *J. Neurosci.* **13**(10), 4301–4315.

Deltour, L., Leduque, P., Blume, N., Madsen, O., Dubois, P., Jami, J., and Bucchini, D. (1993). Differential expression of the two nonallelic proinsulin genes in the developing mouse embryo. *Proc. Natl. Acad. Sci. USA* **90,** 527–531.

DeMeyts, P., Bianco, A. R., and Roth, J. (1994). The structural basis of insulin and insulin-like growth factor-I (IGF-I) receptor binding and negative cooperativity, and its relevance to mitogenic versus metabolic signaling. *Diabetologia* **37,** 5135–5138.

DeMeyts, P., Christoffersen, C. T., Urso, B., *et al.* (1995). Role of the time factor in signaling specificity: Application to mitogenic and metabolic signaling by the insulin and insulin-like growth factor-I receptor tyrosine kinases. *Metabolism* **44,** 2–11.

D'Ercole, A. J., Ye, P., Calikoglu, A. S., and Gutierrez-Ospina, G. (1996a). The role of the insulin-like growth factors in the central nervous system. *Mol. Neurobiol.* **13**(3), 227–255.

D'Ercole, A. J., Ye, P., and Gutierrez-Ospina, G. (1996b). Use of transgenic mice for understanding the physiology of insulin-like growth factors. *Horm. Res.* **45**(Suppl. 1), 5–7.

De Rocco, G., Pennuto, M., Illi, B., Canu, N., Filocamo, G., Trani, E., Rinaldi, A. M., Possenti, R., Mandolesi, G., Sirinian, M. I., Jucker, R., Levi, A., and Nasi, S. (1997). Interplay of the E box, the cyclic AMP response element, and HTF4/HEB in transcriptional regulation of the neurospecific, neurotrophin-inducible *vgf* gene. *Mol. Cell. Biol.* **17,** 1244–1253.

Desvergne, B. (1994). How do thyroid hormone receptors bind to structurally diverse response elements? *Mol. Cell. Endocrinol.* **100**(1–2), 125–131.

Devaskar, S. U., Giddings, S. J., Rajakumar, P. A., Carnaghi, L. R., Menon, R. K., and Zahm, D. S. (1994). Insulin gene expression and insulin synthesis in mammalian neuronal cells. *J. Biol. Chem.* **269,** 8445–8454.

DiCicco-Bloom, E., Friedman, W. J., and Black, I. B. (1993). Nt-3 stimulates sympathetic neuroblast proliferation by promoting precursor survival. *Neuron* **11,** 1101–1111.

D'Mello, S. R., Borodezt, K., and Soltoff, S. P. (1997). Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: Possible involvement of PI3-kinase in IGF-1 signaling. *J. Neurosci.* **17,** 1548–1560.

Doherty, P., and Walsh, F. S. (1996). Review CAM–FGF receptor interactions: A model for axonal growth. *Mol. Cell. Neurosci.* **8,** 99–111.

Doherty, P., Fazeli, M. S., and Walsh, F. S. (1995). The neural cell adhesion molecule and synaptic plasticity. *J. Neurosci.* **26,** 437–446.

Dore, S., Krieger, C., Kar, S., and Quirion, R. (1996). Distribution and levels of insulin-like growth factor (IGF-I and IGF-II) and insulin receptor binding sites in the spinal cords of amyotrophic lateral sclerosis (ALS) patients. *Mol. Brain Res.* **41,** 128–133.

Dos Santos Rodriguez, P., and Dowling, J. E. (1993). Dopamine induces neurite retraction in retinal horizontal cells via diacylglycerol and protein kinase C. *Proc. Natl. Acad. Sci. USA* **87,** 9693–9697.

Dotti, C., and Banker, G. (1991). Intracellular organization of hippocampal neurons during the development of neuronal polarity. *J. Cell Sci.* **15**(Suppl), 75–84.

Dotti, C. G., Sullivan, C. A., and Banker, G. A. (1988). The establishment of polarity by hippocampal neurons in culture. *J. Neurosci.* **8,** 1454–1468.

Downen, M., Kwon, J., Roback, L., and Wainer, B. H. (1995). Noradrenergic receptor activation influences differentiation in the developing basal forebrain. *Soc. Neurosci. Abstr.* **21,** 32.

Drago, J., Murphy, M., Carroll, S. M., Harvey, R. P., and Bartlett, P. F. (1991). Fibroblast growth factor-mediated proliferation of central nervous system precursors depends on endogenous production of insulin-like growth factor-I. *Proc. Natl. Acad. Sci. USA* **88,** 2199–2203.

Dratman, M. B., and Gordon, J. T. (1996). Thyroid hormones as neurotransmitters. *Thyroid* **6**(6), 639–647.

Dratman, M. B., Crutchfield, F. L., and Schoenhoff, M. B. (1991). Transport of iodothyronines from bloodstream to brain: Contributions by blood:brain and choroid plexus–cerebrospinal fluid barriers. *Brain Res.* **554,** 229–236.

Dreyer, D., Lagrange, A., Grothe, C., and Unsicker, K. (1989). Basic fibroblast growth factor prevents ontogenetic neuron death in vivo. *Neurosci. Lett.* **99**(1–2), 35–38.

Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997). Regulation of neuronal survival by the serine–threonine protein kinase Akt. *Science* **275**(5300), 661–665.

Duncan, C. P., Seidler, F. J., Lappi, S. E., and Slotkin, T. A. (1990). Dual control of DNA synthesis by a- and b-adrenergic mechanisms in normotoxic and hypoxic neonatal rat brain. *Dev. Brain Res.* **55,** 29–33.

Durbec, P., Marcos-Gutierrez, C. V., Kilkenny, C., Grigoriou, M., Wartiowaara, K., Suvanto, P., Smith, D., Ponder, B., Costantini, F., Saarma, M., Sariola, H., and Pachnis, V. (1996). GDNF signaling through the ret receptor tyrosine kinase. *Nature* **381,** 789–793.

Dutly, F., and Schwab, M. E. (1991). Neurons and astrocytes influence the development of purified 0-2A progenitor cells. *Glia* **4**(6), 559–571.

Eckenstein, F., Woodward, W. R., and Nishi, R. (1991). Differential localization and possible functions of αFGF and βFGF in the central and peripheral nervous system. *Ann. N. Y. Acad. Sci.* **638,** 348–360.

Eckenstein, F. P. (1994). Fibroblast growth factors in the nervous system. *J. Neurobiol.* **25,** 1467–1480.

Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Weinberg, R. A. (1993). Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature* **363,** 45–51.

Ellison, J. A., Scully, S. A., and de Vellis, J. (1996). Evidence for neuronal regulation of oligodendrocyte development: Cellular localization of platelet-derived growth factor alpha receptor and A-chain mRNA during cerebral cortex development in the rat. *J. Neurosci. Res.* **45**(1), 28–39.

Engel, U., and Wolswijk, G. (1996). Oligondendrocyte-type-2 astrocyte (O-2A) progenitor cells derived from adult rat spinal cord: In vitro characteristics and response to PDGF, βFGF, and NT-3. *Glia* **16,** 16–26.

Erdtsieck-Ernste, E., Feenstra, M. G. P., and Boer, G. J. (1991). Pre- and postnatal developmental changes of adrenoceptor subtypes in rat brain. *J. Neurochem.* **57,** 897–903.

Erdtsieck-Ernste, E., Feenstra, M., and Boer, G. J. (1993). Perinatal influence of B-adrenergic drugs on the noradrenergic system of the rat brain. *Gen. Pharmacol.* **24**(5), 1069–1078.

Ernfors, P., Ibanez, C. F., Ebendal, T., Olson, L., and Persson, H. (1990). Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: Developmental and topographical expression in the brain. *Proc. Natl. Acad. Sci. USA* **87,** 5454–5458.

Ernfors, P., Lee, K. F., Kucera, J., and Jaenisch, R. (1994). Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. *Cell* **77,** 503–512.

Eves, E. M., Tucker, M. S., Roback, J. D., Downen, M., Rosner, M. R., and Wainer, B. H. (1992). Immortal rat hippocampal cell lines exhibit neuronal and glial lineages and neurotrophin gene expression. *Proc. Natl. Acad. Sci. USA* **89,** 4373–4377.

Fagan, A. M., Zhang, H., Landis, S., Smeyne, R. J., Silos-Santiago I., I., and Barbacid, M. (1996). TrkA, but not TrkC, receptors are essential for survival of sympathetic neurons in vivo. *J. Neurosci.* **16,** 6208–6218.

Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S., and Fischbach, G. D. (1993). ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the neu ligand family. *Cell* **72,** 801–815.

Fanger, G. R., Jones, J. R., and Maue, R. A. (1995). Differential regulation of neuronal sodium channel expression by endogenous and exogenous tyrosine receptors expressed in rat pheochromocytoma cells. *J. Neurosci.* **15,** 201–213.

Farinas, I., Jones, K. R., Backus, C., Wang, X. Y., and Reichardt, L. F. (1994). Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. *Nature* **369,** 658–661.

Federoff, H. J., Grabczyk, E., and Fishman, M. C. (1988). Dual regulation of GAP-43 gene expression by nerve growth factor and glucocorticoids. *J. Biol. Chem.* **263,** 19290–19295.

Feldman, E. L., Sullivan, K. A., Kim, B., and Russell, J. W. (1997). Insulin-like growth factors regulate neuronal differentiation and survival. *Neurobiol. Dis.* **4,** 201–214.

Ferhat, L., Represa, A., Zouaoui-Aggoun, D., Ferhat, W., and Ben-Ari, Y. (1997). FGF-2 induces nerve growth factor expression in cultured rat hippocampal neurons. *Eur. J. Neurosci.* **9**(6), 1282–1289.

Ferriera, A., and Caceres, A. (1989). The expression of acetylated microtubules during axonal and dendritic growth in cerebellar macroneurons which develop in vitro. *Dev. Brain Res.* **49,** 205–213.

ffrench-Mullen, J. M. H., Danks, P., and Spence, K. T. (1994). Neurosteroids modulate calcium currents in hippocampal CA1 neurons via a pertussis toxin-sensitive G-protein-coupled mechanism. *J. Neurosci.* **14,** 1963–1977.

Filipcik, P., Saito, H., and Katsuki, H. (1994). 3,5,3′-L-Triiodothyronine promotes survival and axon elongation of embryonic rat septal neurons. *Brain Res.* **647**(1), 148–152.

Fisher, S. K., Heacock, A. M., and Agranoff, B. W. (1992). Inositol lipids and signal transduction in the nervous system: An update. *J. Neurochem.* **58,** 18–38.

Floria, T., Pan, M. G., Newman, B., Harshberger, R. E., Civelli, O., and Stork, P. J. (1992). Dopaminergic inhibition of DNA synthesis in pituitary tumor cells is associated with phosphotyrosine phosphatase activity. *J. Biol. Chem.* **267,** 24168–24172.

Folli, F., Ghidella, S., Bonfanti, L., Kahn, C. R., and Merighi, A. (1996). The early intracellular signalling pathway for the insulin/insulin-like growth factor receptor family in the mammalian central nervous system. *Mol. Neurobiol.* **13**(2), 155–183.

Frade, J. M., Marti, E., Bovolenta, P., Rodriguez-Pena, M. A., Perez-Garcia, D., Rohrer, H., and Rodriguez-Tebar, A. (1996). Insulin-like growth factor-I stimulates neurogenesis in chick retina by regulating expression of the α6 integrin subunit. *Development* **122,** 2497–2506.

Francis, N. J., Asmus, S. E., and Landis, S. C. (1997). CNTF and LIF are not required for the target-directed acquisition of cholinergic and peptidergic properties by sympathetic neurons in vivo. *Dev. Biol.* **182,** 76–87.

Franke, T., Yang, S., Chan, T., Datta, K., Kazlauskas, A., *et al.,* (1995). The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81,** 727–726.

Fretto, L. J., Snape, A. J., Tomlinson, J. E., Seroogy, J. J., Wolf, D. L., LaRochelle, W. J., and Glese, N. A. (1993). Mechanism of platelet-derived growth factor (PDGF) AA, AB, and BB binding to α and β PDGF receptor. *J. Biol. Chem.* **268,** 3625–3631.

Friedman, B., Scherer, S., Rudge, J. S., Helgren, M., Morrissey, D., McClain, J., Wang, D. Y., Wiegand, S. J., Furth, M. E., Lindsay, R. M., *et al.,* (1992). Regulation of ciliary neurotrophic factor expression in myelin-related Schwann cells in vivo. *Neuron* **9,** 295–305.

Frodin, M., Peraldi, P., and Van Obberghen, E. (1994). Cyclic AMP activates the mitogen-activated protein kinase cascade in PC12 cells. *J. Biol. Chem.* **269,** 6207–6214.

Fruttiger, M., Calver, A. R., Kruger, W. H., Mudhar, H. S., Michalovich, D., Takakura, B., Nishikawa, S. I., and Richardson, W. D. (1996). PDGF mediates a neuron–astrocyte interaction in the development retina. *Neuron* **17,** 1117–1131.

Fukuda, M., Gotoh, Y., Tachibana, T., Dell, K., Hattori, S., Yoneda, Y., and Nishida, E. (1995). Induction of neurite outgrowth by MAP kinase in PC12 cells. *Oncogene* **11,** 239–244.

Gage, F. H., Coates, P. W., Palmer, T. D., Kuhn, H. G., Fisher, L. J., Suhonen, J. O., Peterson, D. A., Suhr, S. T., and Ray, J. (1995a). Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. *Proc. Nat. Acad. Sci. USA* **92**(25), 11879–11883.

Gage, F. H., Ray, J., and Fisher, L. J. (1995b). Isolation, characterization, and use of stem cells from the CNS. *Annu. Rev. Neurosci.* **18,** 159–192.

Garza, R., Puymirat, J., and Dussault, J. H. (1990). Influence of soluble environmental factors on the development of fetal brain acetylcholinesterase-positive neurons cultured in a chemically defined medium: Comparison with the effects of L-triiodothyronine (L-T3). *Brain Res. Dev. Brain Res.* **56**(2), 160–168.

Gazzaley, A. H., Weiland, N. G., McEwen, B. S., and Morrison, J. H. (1996). Differential regulation of NMDAR1 mNRA and protein by estradiol in the rat hippocampus. *J. Neurosci.* **16**(21), 6830–6838.

Gelfand, V. I., and Bershadky, A. D. (1991). Microtubule dynamics: Mechanism regulation and function. *Annu. Rev. Cell Biol.* **7,** 93–116.

Ghosh, A., and Greenberg, M. E. (1995). Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. *Neuron* **15**(1), 89–103.

Giardino, L., Ceccatelli, S., Zanni, M., Hokfelt, T., and Calza, L. (1994). Regulation of VIP mRNA expression by thyroid hormone in different brain areas of adult rat. *Mol. Brain Res.* **27,** 87–94.

Giardino, L., Ceccatelli, S., Hokfelt, T., and Calza, L. (1995). Expression of enkephalin and dynorphin precursor mRNAs in brain areas of hypo- and hyperthyroid rat: Effect of kainic acid injection. *Brain Res.* **687,** 83–93.

Ginty, D. D., Bonni, A., and Greenberg, M. E. (1994). Nerve growth factor activates a ras-dependent protein kinase that stimulates c-*fos* transcription via phosphorylation of CREB. *Cell* **77,** 713–725.

Glass, C. K., and Holloway, J. M. (1990). Regulation of gene expression by the thyroid hormone receptor. *Biochim. Biophys. Acta* **1032,** 157–176.

Gold, G. (1989). "Insulin Secretion." A. R. Liss, New York.

Goldman-Rakic, P. S., and Selemon, L. D. (1997). Functional and anatomical aspects of prefrontal pathology in schizophrenia [see comments]. *Schizophrenia Bull.* **23,** 437–458. [Review]

Gomez-Pinilla, F., Knauer, D. J., and Nieto-Sampedro, M. (1988). Epidermal growth factor receptor immunoreactivity in rat brain. Development and cellular localization. *Brain Res.* **438,** 385–390.

Gomez-Pinilla, F., Lee, J. W., and Cotman, C. W. (1992). Basic FGF in adult rat brain: Cellular distribution and response to entorhinal lesion and fimbria–fornix transection. *J. Neurosci.* **12**(1), 345–355.

Gotz, R., Koster, R., Winkler, C., Raulf, F., Lottspeich, F., Schartl, M., and Thoenen, H. (1994). Neurotrophin-6 is a new member of the nerve growth factor family. *Nature* **372,** 266–269.

Graus-Porta, D., Beerli, R. R., Daly, J. M., and Hynes, N. E. (1997). ErbB receptor is a mediator of lateral signaling. *EMBO J.* **16**(7), 1647–1655.

Green, P. S., Gridley, K. E., and Simpkins, J. W. (1996). Estradiol protects against beta-amyloid (25-35)-induced toxicity in SK-N-SH human neuroblastoma cells. *Neurosci. Lett.* **218**(3), 165–168.

Green, P. S., Gridley, K. E., and Simpkins, J. W. (1998). Nuclear estrogen receptor-independent neuroprotection by estratrienes: A novel interaction with glutathione. *Neuroscience,* **84**(1), 7–10.

Greenberg, M. E., Greene, L. A., and Ziff, E. B. (1985). Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. *J. Biol. Chem.* **260,** 14101–14110.

Greene, L. A., Burstein, D. E., and Black, M. M. (1982). The role of transcription-dependent priming in nerve growth factor promoted neurite outgrowth. *Dev. Biol.* **91,** 305–316.

Grothe, C., and Wewetzer, K. (1996). Fibroblast growth factor and its implications for developing and regenerating neurons. *Int. J. Dev. Biol.* **40**(1), 403–410.

Gu, Q., and Moss, R. L. (1996). 17β-estradiol potentiates kainate-induced currents via activation of the cAMP cascade. *J. Neurosci.* **16,** 3620–3629.

Guadno-Ferraz, A., Obregon, M. J., St. Germain, D. L., and Bernal, J. (1997). The type 1 iodothyronine deiodinase is expressed primarily in glial cells in the neonatal rat brain. *Proc. Natl. Acad. Sci. USA* **94**(19), 10391–10396.

Gurney, M. E., Yamamoto, H. Y., and Kwon, Y. (1992). Induction of motor neuron sprouting in vivo by ciliary neurotrophic factor and basic fibroblast growth factor. *J. Neurosci.* **12,** 3241–3247.

Habecker, B. A., and Landis, S. C. (1994). Noradrenergic regulation of cholinergic differentiation. *Science* **264,** 1602–1604.

Habecker, B. A., Pennica, D., and Landis, S. C. (1995). Cardiotrophin-1 is not the sweat gland-derived differentiation factor. *Neuro Report* **7,** 41–44.

Habecker, B. A., Symes, A. J., Stahl, N., Francis, N. J., Economides, A., Fink, J. S., and Landis, S. C. (1997). A sweat gland-derived differentiation activity acts through known cytokine signaling pathways. *J. Biol. Chem.* **272,** 30421–30428.

Hafner, S., Adler, H. S., Mischak, H., Janosch, P., Heidecker, G., Wofiman, A., Pippig, S., Lohse, M., Ueffing, M., and Kolch, W. (1994). Mechanism of inhibition of RAF-1 by protein kinase A. *Mol. Cell. Biol.* **14,** 6696–6703.

Hagag, N., Halegoua, S., and Viola, M. (1986). Inhibition of growth factor-induced differentiation in PC12 cells by microinjection of antibody to ras p21. *Nature* **319,** 680–682.

Hagihara, K., Hirata, S., Osada, T., Hirai, M., and Kato, J. (1992). Distribution of cells containing progesterone receptor mRNA in the female rat di- and telencephalon: An in situ hybridization study. *Mol. Brain Res.* **14,** 239–249.

Hallbook, F., Ibanez, C. F., and Persson, H. (1991). Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. *Neuron* **6,** 845–858.

Hart, C. E., Forstrom, J. W., Kelly, J. D., Seifert, R. A., Smith, R. A., Ross, R., Murray, M. J., and Bowen-Pope, D. F. (1988). Two classes of PDGF receptor recognize different isoforms of PDGF. *Science* **240,** 1529–1531.

Hasselmo, M. E. (1995). Neuromodulation and cortical function: Modeling the physiological basis of behavior. *Behav. Brain Res.* **67,** 1–27. [Review]

Hawley, R. J., Scheibe, R. J., and Wagner, J. A. (1992). NGF induces the expression of the VGF gene through a cAMP response element. *J. Neurosci.* **12,** 2573–2581.

Hayashi, M. (1996). Neurotrophins and the primate central nervous system: A minireview. *Neurochem. Res.* **21**(7), 739–747.

Haydon, P. G., McCobb, D. P., and Kater, S. B. (1984). Sectonin selectively inhibits growth cone motility and synaptogenesis of specific identified neurons. *Science* **226,** 561–564.

Hayman, M. J., and Enrietto, P. J. (1991). Cell transformation by the epidermal growth factor receptor and v-erbB. *Cancer Cells* **3,** 302–307.

Heidaran, M. A., Beeler, J. F., Yu, J.-C., Ishibashi, T., LaRochelle, W. J., Pierce, J. H., and Aaronson, S. A. (1993). Differences in substrate specificaties of α and β platelet-derived growth factor (PDGF) receptors. *J. Biol. Chem.* **268,** 9287–9295.

Hempstead, B. L., Rabin, S. J., Kaplan, L., Reid, S., Parada, L. F., and Kaplan, D. R. (1992). Overexpression of the trk tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. *Neuron* **9,** 883–896.

Henderson, C. E., Camu, W., Mettling, C., Gouin, A., Poulsen, K., Karihaloo, M., Rullamas, J., Evans, T., McMahon, S. B., Armanini, M. P., Berkemeier, L., Phillips, H. S., and Rosenthal, A. (1993). Neurotrophins promote motor neuron survival and are present in embryonic limb bud. *Nature* **363,** 266–270.

Henley, W. N., and Vladic, F. (1997). Hypothyroid-induced changes in autonomic control have a central serotonergic component. *Am. J. Physiol.* **41,** 11894–11903.

Henley, W. N., Chen, X., Klettner, C., Bellush, L. L., and Notestine, M. A. (1991). Hypothyroidism increases serotonin turnover and sympathetic activity in the adult rat. *Can. J. Physiol. Pharmacol.* **69,** 205–210.

Henley, W. N., Harness, P. I., Koehnle, T. J., and Bellush, L. L. (1996). Central serotonin metabolism correlates with autonomic control and thyroid status. *Soc. Neurosci. Abst.* **22,** 606.

Hesen, W., and Joels, M. (1993). Modulation of carbachol responsiveness in rat CA1 pyramidal neurons by corticosteroid hormones. *Brain Res.* **627,** 159–167.

Hesen, W., and Joels, M. (1996). Cholinergic responsiveness of rat CA1 hippocampal neurons in vitro: Modulation by corticosterone and stress. *Endocrinology* **69,** 915–924.

Hillborn, M. D., Vaillancourt, R. R., and Rane, S. G. (1998). Growth factor receptor tyrosine kinases acutely regulate neuronal sodium channels through the src signaling pathway. *J. Neurosci.* **18**(2), 590–600.

Honegger, P., and Guentert-Lauber, B. (1983). Epidermal growth factor (EGF) stimulation of cultured brain cells. I. Enhancement of the developmental increase in glial enzymatic activity. *Brain Res.* **313,** 245–251.

Hoodless, P. A., Haerry, T., Abdollah, S., Stapleton, M., O'Connor, M. B., Attisano, L., and Wrana, J. L. (1996). MADRI, a MAD-related protein that functions in BMP2 signaling pathways. *Cell* **85,** 489–500.

Horner, H. C., Dackan, D. R., and Sapolsky, R. M. (1990). Glucocorticoids inhibit glucose transport in cultured hippocampal neurons and glia. *Neuroendocrinology* **52,** 57–64.

Hosli, E., and Hosli, L. (1982). Evidence for the existence of α- and β-adrenoceptors on neurones and glial cells of cultured rat central nervous system—An autoradiographic study. *Neuroscience* **7,** 2873–2881.

Hosli, E., Stauffer, S., and Hosli, L. (1995). Autoradiographic and electrophysiological evidence for the existence of neurotensin receptors on cultured astrocytes. *Neuroscience* **66,** 627–633.

Hosli, L., Hosli, E., Zehntner, C., Lehmann, R., and Lutz, T. W. (1983). α- and β-adrenoceptors on cultured glial cells. *In* "CNS Receptors—From Molecular Pharmacology to Behavior" (P. Mandel and F. V. DeFeudis, Eds.), pp. 417–420. Raven Press, New York.

Houser, C. R., Crawford, G. D., Barber, R. P., Salvaterra, P. M., and Vaughn, J. E. (1983). Organization and morphological characteristics of cholinergic neurons: An immunocytochemical study with a monoclonal antibody to choline acetyltransferase. *Brain Res.* **266,** 97–119.

Hoyle, G. W., Mercer, E. H., Palmiter, R. D., and Brinster, R. L. (1993). Expression of NGF in sympathetic neurons leads to excessive axon outgrowth from ganglia but decreased terminal innervation within tissues. *Neuron* **10,** 1019–1034.

Hu, P., Mondino, A., Skolnik, E. Y., and Schlessinger, J. (1993). Cloning of a novel, ubiquitously expressed human phosphatidylinositol 3-kinase and identification of its binding site on p85. *Mol. Cell. Biol.* **13,** 7677–7688.

Hu, Z., Yuri, K., Ichikawa, T., and Kawata, M. (1996). Exposure of postnatal rats to glucocorticoids suppresses the development of choline acetyltransferase-immunoreactive neurons: Role of adrenal steroids in the development of forebrain cholineric neurons. *J. Chem. Neuroanatomy* **10**(1), 1–10.

Hubel, D. H., and Wiesel, T. N. (1970). The period of susceptibility to the physiological effects of unilateral eye closure in kittens. *J. Physiol.* **206,** 419–436.

Hughes, R. A., Sendtner, M., and Thoenen, H. (1993). Members of several gene families influence survival of rat motoneurons *in vitro* and *in vivo*. *J. Neurosci. Res.* **36,** 663–671.

Hughes, S. M., Lillien, L. E., Raff, M. C., Rohrer, H., and Sendtner, M. (1988). Ciliary neurotrophic factor induces type 2 astrocyte differentiation in culture. *Nature* **335,** 70–73.

Humbel, R. E. (1990). Insulin-like growth factors I and II. *Eur. J. Biochem.* **190,** 445–462.

Ibanez, C. F., Ebendal, T., Barbany, G., Murray-Rust, J., Blundell, T. L., and Persson, H. (1992). Disruption of the low affinity receptor-binding site in NGF allows neuronal survival and differentiation by binding to the trk gene product. *Cell* **69,** 329–341.

Iihara, K., Hashimoto, N., Tsukahara, T., Sakata, M., Yanamoto, H., and Taniguchi, T. (1997). Platelet-derived growth factor-BB, but not -AA, prevents delayed neuronal death after forebrain ischemia in rats. *J. Cerebral Blood Flow Metab.* **17,** 1097–1106.

Ijichi, A., Noel, F., Sakuma, S., Weil, M. M., and Tofilon, P. J. (1996). Ex vivo gene delivery of platelet-derived growth factor increases 0-2A progenitors in adult rat spinal cord. *Gene Therapy* **3**(5), 389–395.

Ikezu, T., Okamoto, T., Giambarella, U., Yokoto, T., and Nishimoto, I. (1995). In vivo coupling of insulin-like growth factor/mannose-6-phosphate receptor to heteromeric G proteins. *J. Biol. Chem.* **270,** 29224–29228.

Inoue, M., Nakayama, C., and Noguchi, H. (1996). Activating mechanism of CNTF and related cytokines. *Mol. Neurobiol.* **12,** 195–209.

Ip, N. Y., and Yancopoulos, G. P. (1992). Ciliary neurotrophic factor and its receptor complex. *Prog. Growth Factor Res.* **4,** 139–155.

Ip, N. Y., and Yancopoulos, G. D. (1996). The neurotrophins and CNTF: Two families of collaborative neurotrophic factors. *Annu. Rev. Neurosci.* **19,** 491–515.

Ip, N. Y., Li, Y., van de Stadt, I., Panayotatos, N., Alderson, R. F., and Lindsay, R. M. (1991). Ciliary neurotrophic factor enhances neuronal survival in embryonic rat hippocampal cultures. *Neuroscience* **11**(10), 3124–3134.

Ip, N. Y., Ibanez, C. F., Nye, S. H., *et al.* (1992). Mammalian neurotrophin-4: Structure, chromosomal localization, tissue distribution, and receptor specificity. *Proc. Natl. Acad. Sci. USA* **89,** 3060–3064.

Ip, N. Y., Li, Y., Yancopoulos, G. D., and Lindsay, R. M. (1993a). Cultured hippocampal neurons show responses to BDNF, NT-3, but not NGF. *J. Neurosci.* **13,** 3394–3405.

Ip, N. Y., McClain, J., Barrezueta, N. X., Aldrich, T. H., Pan, L., Li, Y., Wiegand, S. J., Friedman, B., Davis, S., and Yancopoulos, G. D. (1993b). The alpha component of the CNTF receptor is required for signaling and defines potential CNTF targets in the adult and during development. *Neuron* **10,** 89–102.

Ip, N. Y., Wiegand, S. J., Morse, J., and Rudge, J. S. (1993c). Injury-induced regulation of ciliary neurotrophic factor mRNA in the adult rat brain. *Eur. J. Neurosci.* **5,** 25–33.

Ip, N. Y., Boulton, T. G., Li, Y., Verdi, J. M., Birren, S. J., Anderson, D. J., and Yancopoulos, G. D. (1994). CNTF, FGF, and NGF collaborate to drive the terminal differentiation of MAH cells into postmitotic neurons. *Neuron* **13,** 443–445.

Irwin, R. P., Lin, S.-Z., Rogawski, M. A., Purdy, R. H., and Paul, S. M. (1994). Steroid potentiation and inhibition of N-methyl-D-asparate receptor-mediated intracellular CA responses: Structure–activity studies. *J. Phamracol. Exp. Ther.* **271,** 677–682.

Isaackson, P. J., Ullrich, A., and Bradshaw, R. A. (1984). Mouse 7S nerve growth factor: Complete sequence of a cDNA coding for the alpha-subunit precursor and its relationship to serine proteases. *Biochemistry* **23,** 5997–6002.

Ishii, D. N., Wang, C., and Li, Y. (1991). Second messengers mediating gene expression essential to neurite formation directed by insulin and insulin-like growth factors. *Adv. Exp. Med. Biol.* **293,** 361–378.

Izquierdo, I., and Medina, J. H. (1997). Memory formation: The sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. *Neurobiol. Learning Memory* **68,** 285–316. [Review]

Jackson, T. R., Blader, I. J., Hammonds-Odie, L. P., Burga, C. R., Cooke, F., Hawkins, P. T., Wolf, A. G., Heldman, K. A., and Theibert, A. B. (1996). Initiation and maintenance of NGF-stimulated neurite outgrowth requires activation of a phosphinositide 3-kinase. *J. Cell Sci.* **109,** 289–300.

Jaiswal, R. K., Moodie, S. A., Wolfman, A., and Landreth, G. E. (1994). The mitogen-activated protein kinase cascade is activated by B-Raf in response to nerve growth factor through interaction with $p21^{ras}$. *Mol. Cell. Biol.* **14,** 6944–6953.

Jensen, T. S., Gemefke, I. K., Hylderbrandt, N., Pedersen, H., Petersen, H. D., and Weile, B. (1982). Cerebral atrophy in young torture victims. *N. Engl. J. Med.* **307,** 1341.

Jing, S., Wen, D., Yu, Y., Holst, P. L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R., Louis, J. C., Hu, S., Altrock, B. W., and Fox, G. M. (1996). GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-α a novel receptor for GDNF. *Cell* **85,** 1113–1124.

Joels, M. (1997). Steroid hormones and excitability in the mammalian brain. *Frontiers Neuroendocrinol.* **18**(1), 2–48.

Joels, M., and De Kloet, E. R. (1992). Coordinative mineralocorticoid and glucocorticoid receptor-mediated control of responses to serotonin in rat hippocampus. *Neuroendocrinol.* **55,** 344–350.

Johe, K. K., Hazel, T. G., Miller, T., Dugich-Djordjevic, M. M., and McKay, R. D. (1996). Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev.* **10**(24), 3129–3140.

Johnson, D., Lanahan, A., Buck, C. R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M., and Chao, M. (1986). Expression and structure of the human NGF receptor. *Cell* **47,** 545–554.

Jones, J. I., and Clemmons, D. R. (1995). Insulin-like growth factors and their binding proteins: Biological actions. *Endocr. Rev.* **16,** 1–34.

Jones, K. R., Farinas, I., Backus, C., and Reichardt, L. F. (1994). Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. *Cell* **76,** 989–999.

Kahan, C., Julius, D., Pouyssegur, J., and Seuwon, K. (1992). Effects of 5-HT1C receptor expression on cell proliferation control in hamster fibroblasts: Serotonin fails to induce a transform phenotype. *Exp. Cell Res.* **300,** 523–527.

Kan, M., Wang, F., To, B., Gabriel, J. L., and McKeehan, W. L. (1996). Divalent cations and heparin/heparan sulfate cooperate to control assembly and activity of the fibroblast growth factor receptor complex. *J. Biol. Chem.* **271,** 26143–26148.

Kang, H., and Schuman, E. M. (1995). Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. *Science* **267,** 1658–1662.

Kaplan, D. R., and Stephens, R. M. (1994). Neurotrophin signal transduction by the Trk receptor. *J. Neurobiol.* **25,** 1404–1417.

Kaplan, D. R., Hempstead, B.L., Martin-Zanca, D., Chao, M. V., and Parada, L. F. (1991a). The trk proto-oncogene product: A signal transducing receptor for nerve growth factor. *Science* **252,** 554–558.

Kaplan, D. R., Martin-Zanca, D., and Parada, L. F. (1991b). Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. *Nature* **350,** 158–160.

Kar, S., Chabot, J. G., and Quirion, R. (1993). Quantitative autoradiographic localization of [^{125}I] insulin-like growth and factor I, [^{125}I] insulin-like growth factor II, and [^{125}I] insulin receptor binding sites in developing and adult rat brain. *J. Comp. Neurol.* **333,** 375–397.

Kar, S., Seto, D., Dore, S., Chabot, J.-G., and Quirion, R. (1997). Systemic administration of kainic acid induces selective time dependent decrease in [^{125}I] insulin-like growth factor I, [^{125}I] insulin-like growth factor II and [^{125}I] insulin receptor binding sites in adult rat hippocampal formation. *Neuroscience* **80**(4), 1041–1055.

Katoh-Semba, R., Kaisho, Y., Shintani, A., Nagahama, M., and Kato, K. (1996). Tissue distribution and immunocytochemical localization of neurotrophin-3 in the brain and peripheral tissues of rats. *J. Neurochem.* **66**(1), 330–337.

Katoh-Semba, R., Takeuchi, I.K., Semba, R., and Kato, K. (1997). Distribution of brain-derived neurotrophic factor in rats and its changes with development in the brain. *J. Neurochem.* **69**(1), 34–42.

Kawabe, T., Wen, T. C., Matsuda, S., Ishihara, K., Otsuda, H., and Sakanaka, M. (1997). Platelet-derived growth factor prevents ischemia-induced neuronal injuries in vivo. *Neurosci. Res.* **29**(4), 335–343.

Kawata, M. (1995). Roles of steroid hormones and their receptors in structural organization in the nervous system. *Neurosci. Res.* **24**(1), 1–46.

Kennedy, M. F. G., Tutton, P. J. M., and Barkala, D. H. (1983). Adrenergic factors involved in the control of crypt cell proliferation in jejunum and descending colon of mouse. *Clin. Exp. Pharmacol. Psychol.* **10,** 577–586.

Kiess, W., Yang, Y., Kessler, U., and Hoeflich, A. (1994). Insulin-like growth factor II (IGF-II) and the IGF-II/mannose-6-phosphate receptor: The myth continues. *Horm. Res.* **4**(Suppl. 2), 66–73.

Kimura, D., and Hampson, E. (1993). Neural and hormonal mechanisms mediating sex differences in cognition. *In* "Biological Approaches to the Study of Human Intelligence" (P.A. Vernon Ed.), pp. 375–397. Ablex, Norwood, N.J.

Kimura, K., Hattoris, S., Kabuyama, Y., Shizawa, Y., Takayanagi, J., Nakamura, S., Toki, S., Matsuda, Y., Onodera, K., and Fukui, Y. (1994). Neurite outgrowth of PC12 cells is suppressed by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. *J. Biol. Chem.* **269,** 18961–18967.

Kirkwood, A., Rioult, M. C., and Bear, M. F. (1996). Experience-dependent modification of synaptic plasticity in visual cortex [see comments]. *Nature* **381,** 526–528.

Klein, R., Parada, L. F., Coulier, F., and Barbacid, M. (1989). trkB, a novel tyrosine protein kinase receptor expressed during mouse neural development. *EMBO J.* **8,** 3701–3709.

Klein, R., Jing, S., Nanduri, V., O'Rourke, E., and Barbacid, M. (1991a). The trk proto-oncogene encodes a receptor for nerve growth factor. *Cell* **65,** 189–197.

Klein, R., Nanduri, V., Jing, S., Lamballe, F., Tapley, P., Bryant, S., Cordon-Cardo, C., Jones, K. R., Reichardt, L. F., and Barbacid, M. (1991b). The trk B tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. *Cell* **66,** 395–403.

Klein, R., Lamballe, F., Bryant, S., and Barbacid, M. (1992). The trkB tyrosine protein kinase is a receptor for neurotrophin-4. *Neuron* **8,** 947–956.

Klein, R., Sllos-Santiago, I., Smeyne, R. J., Lira, S. A., Brambilla, R., Bryant, S., Zhang, L., Snider, W. D., and Barbacid, M. (1994). Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements. *Nature* **368,** 249–251.

Kleinschmidt, A., Bear, M. F., and Singer, W. (1987). Blockade of "NMDA" receptors disrupts experience-dependent plasticity of kitten striate cortex. *Science* **238,** 355–358.

Klinghoffer, R. A., Duckworth, B., Valius, M., Cantley, L., and Kazlauskas, A. (1996). PDGF-dependent activation of P13 kinase is regulated by receptor binding of SH2 domain-containing proteins which influence Ras activity. *Mol. Cell. Biol.* **16,** 5905–5914.

Knight, J. B., Yamauchi, K., and Pessin, J. E. (1995). Divergent insulin and platelet-derived growth factor regulation of focal adhesion kinase (pp 125FAK) tyrosine phosphorylation, and rearrangement of actin stress fibers. *J. Biol. Chem.* **270,** 10199–10203.

Kniss, D. A., and Burry, R. W. (1988). Serum and basic fibroblast growth factor stimulates quiescent astrocytes to re-enter the cell cycle. *Brain Res.* **439,** 281–288.

Knusel, B., Michel, P. P., Schwaber, J. S., and Hefti, F. (1990). Selective and nonselective stimulation of central cholinergic and dopaminergic development in vitro by nerve growth factor, basic fibroblast growth factor, epidermal growth factor, insulin and the insulin-like growth factors I and II. *J. Neurosci.* **10,** 558–570.

Kolch, W., Heldecker, G., Kochs, G., Hummel, R., Vahldi, H., Mischak, H., Finkenzeller, G., Marme, D., and Rapp, U. R. (1993). Protein kinase Ca activates RAF-1 by direct phosphorylation. *Nature* **364,** 249–252.

Kornblum, H. I., Raymon, H. K., Morrison, R. S., Cavanaugh, K. P., Bradshaw, R. A., and Leslie, F. M. (1990). Epidermal growth factor and basic fibroblast growth factor: Effects on an overlapping population of neocortical neurons *in vitro. Brain Res.* **535,** 255–263.

Kornblum, H. I., Gall, C. M., Seroogy, K. B., and Lauterborn, J. C. (1995). A subpopulation of striatal GABAergic neurons expresses the epidermal growth factor receptor. *Neuroscience* **69,** 1025–1029.

Korte, M., Carroll, P., Wolf, E., Brem, G., Thoenen, H., and Bonhoefer, T. (1995). Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proc. Natl. Acad. Sci. USA* **92,** 8856–8860.

Kotzbauer, P., Lampe, P., Heuckeroth, R., Golden, J., Creedon, D., Johnson, E., Jr., and Milbrandt, J. (1996). Neuriturin, a relative of glial cell line derived neurotrophic factor. *Nature* **384,** 467–470.

Kouhara, H., Hadari, Y. R., Spivak-Kroizman, T., Schilling, J., Bar-Sagi, D., and Schlessinger, J. (1997). A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell* **89,** 693–702.

Kremer, N. E., D'Arcangelo, G., Thomas, S. M., DeMarco, M., Brugge, J. S., and Halegoua, S. (1991). Signal transduction by nerve growth factor and fibroblast growth factor in PC12 cells requires a sequence of Src and Ras actions. *J. Cell Biol.* **115,** 809–819.

Kuhn, H. G., Dickinson-Anson, H., and Gage, F. H. (1996). Neurogenesis in the dentate gyrus of the adult rat: Age-related decrease of neuronal progenitor proliferation. *J. Neurosci.* **16,** 2027–2033.

Kuhn, H. G., Winkler, J., Kempermann, G., Thai, L. J., and Gage, F. H. (1997). Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. *J. Neurosci.* **17**(15), 5820–5829.

Kundra, V., Escobedo, J. A., Kazlauska, A., Kim, H. K., Rhee, S. G., Williams, L. T., and Zetter, B. R. (1994). Regulation of chemotaxis by the platelet-derived growth factor receptor-beta. *Nature* **367,** 474–476.

Kuo, W.-L., Abe, M., Rhee, J., Eves, E. M., McCarthy, S. A., Yan, M., Templeton, D. J., McMahon, M., and Rosner, M. R. (1996). Raf, but not MEK or ERK, is sufficient for differentiation of hippocampal neuronal cells. *Mol. Cell. Biol.* **16**(4), 1458–1470.

Kuzis, K., Reed, S., Cherry, N. J., Woodward, W. R., and Eckenstein, F. P. (1995). Developmental time course of acidic and basic fibroblast growth factors' expression in distinct cellular populations of the rat central nervous system. *J. Comp. Biol.* **358**(1), 142–153.

Kwon, J., Eves, E. M., and Wainer, B. H. (1993). Basic fibroblast growth factor-induced differentiation in septal cell lines. *Soc. Neurosci. Abstr.* **19,** 243–240. [Abstract]

Kwon, J., Downen, M., Eves E., and Wainer B. H. (1995). Neuritogenesis in a basal forebrain cell line is mediated by catecholamines and basic fibroblast growth factor. *Soc. Neurosci. Abstr.* **21,** 18–10. [Abstract]

Kwon, J. H., Eves, E. M., Farrell, S., Segovia, J., Allan, J. T., Wainer, B. H., and Downen, M. (1996). β-Adrenergic receptor activation promotes process outgrowth in an embryonic rat basal forebrain cell line and in primary neurons. *Eur. J. Neurosci.* **8,** 2042–2055.

Kwon, J. H., Vogt Weisenhorn, D. M., Downen, M., Ruan, K., Roback, L., Joshi, H., and Wainer, B. H. (1998). β-Adrenergic and fibroblast growth factor receptors induce neuronal process outgrowth through different mechanisms. *Eur. J. Neurosci.* **10,** 2776–2789.

Lamballe, F., Klein, R., and Barbacid, M. (1991). trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. *Cell* **66,** 1–20.

Landgren, E., Blume-Jensen, P., Courtneidge, S. A., and Claesson-Welsh, L. (1995). Fibroblast growth factor receptor-1 regulation of Src family kinases. *Oncogene* **10**(10), 2027–2035.

Lankford, K. L., and Letourneau, P. C. (1989). Evidence that calcium may control neurite outgrowth by regulating the stability of actin filaments. *J. Cell Biol.* **109,** 1229–1243.

Lauder, J. M. (1993). Neurotransmitters as growth regulatory signals: Role of receptors and second messengers. *Trends Neurosci.* **16**(6), 233–240.

Lauder, J. M., and Bloom, F. E. (1974). Ontogeny of monoamine neurons in the locus coeruleus, raphe nuclei, and substantia nigra of the rat. J. Cell differentiation. *J. Comp. Neurol.* **155,** 469–481.

Lazar, L. M., and Blum, M. (1992). Regional distribution and developmental expression of epidermal growth factor and transforming growth factor-α mRNA in mouse brain by a quantitative nuclease protection assay. *J. Neurosci.* **12,** 1688–1697.

Lazar, M. A. (1993). Thyroid hormone receptors: Multiple forms, multiple possibilities. *Endocr. Rev.* **14,** 184–193.

Lee, K. A. W., and Masson, N. (1993). Transcriptional regulation by CREB and its relatives. *Biochim. Biophys. Acta* **1174,** 221–233.

Lee, K.-F., Li, E., Huber, J., Landis, S., Sharpe, A. H., Chao, M., and Jaenisch, R. (1992). Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. *Cell* **69,** 737–749.

Lee, W. H., Michels, K. M., and Bondy, C. A. (1993). Localization of insulin-like growth factor binding protein-2 messenger RNA during postnatal brain development: Correlation with insulin-like growth factors I and II. *Neuroscience* **53,** 251–265.

Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H., and Barde, Y.-A. (1989). Molecular cloning and expression of brain-derived neurotrophic factor. *Nature* **341,** 149–152.

Lenkei, Z., Palkovits, M., Corvol, P., and Llorens-Cortes, C. (1997). Expression of angiotensin type-1 (AT1) and type-2 (AT2) receptor mRNAs in the adult rat brain: A functional neuroanatomical review. *Frontiers Neuroendocrinol.* **18,** 383–439.

Lenoir, D., and Honegger, P. (1983). Insulin-like growth factor I (IGF I) stimulates DNA synthesis in fetal rat brain cell cultures. *Exp. Brain Res.* **7,** 205–213.

Leonard, D. G. B., Gorham, J. D., Cole, P., Greene, L. A., and Ziff, E. B. (1988). A nerve growth factor-regulated messenger RNA encodes a new intermediate filament protein. *J. Cell Biol.* **106,** 181–193.

Leveen, P., Pekny, M., Gebre-Medhin, S., Swolin, B., Larson, E., and Betsholtz, C. (1994). Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes Dev.* **8,** 1875–1887.

Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. *Science* **237,** 1154–1162.

Levi-Montalcini, R., and Hamburger, V. (1951). Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *J. Exp. Zool.* **116,** 321–360.

Levitt, P., and Moore, R. Y. (1979). Development of the noradrenergic innervation of neocortex. *Brain Res.* **162,** 243–259.

Levitt, P., Harvey, J. A., Friedman, E., Simansky, K., and Murphy, E. H. (1997). New evidence for neurotransmitter influences on brain development. *Trends Neurosci.* **20,** 269–274. [Review]

Lewin, G. R., and Barde, Y.-A. (1996). Physiology of the neurotrophins. *Annu. Rev. Neurosci.* **19,** 289–317.

Li, L., Oppenheim, R. W., Lei, M., and Houenou, L. J. (1994). Neurotrophic agents prevent motoneuron death following sciatic nerve section in the neonatal mouse. *J. Neurobiol.* **25,** 759–766.

Li, M., Sendtner, M., and Smith, A. (1996). Essential function of LIF receptor in motor neurons. *Nature* **378,** 724–727.

Lidow, M. S., and Rakic, P. (1994). Unique profiles of the α1-, α2-, and β-adrenergic receptors in the developing cortical plate and transient embryonic zones of the rhesus monkey. *J. Neurosci.* **14**(7), 4064–4078.

Lidow, M. S., and Rakic, P. (1995). Neurotransmitter receptors in the proliferative zones of the developing primate occipital lobe. *J. Comp. Neurol.* **360,** 393–402.

Lima, F. R., Trentin, A. G., Rosenthal, D., Chagas, C., and Moura Nea, V. (1997). Thyroid hormone induces protein secretion and morphological changes in astroglial cells with an increase in expression of glial fibrillary acidic protein. *J. Endocrinol.* **154**(1), 167–175.

Lin, L.-F. H., Mismer, D., Lile, J. D., Armes, L. G., Butler, E. T. III, Vannice, J. L., and Collins, F. (1989). Purification, cloning and expression of ciliary neurotrophic factor (CNTF). *Science* **246,** 1023–1025.

Lin, L. H., Doherty, D. H., Lile, J. D., Bektesh, S., and Collins, F. (1993). GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* **260,** 1130–1132.

Lindsay, R. M. (1995). Neuron saving schemes. *Nature* **373,** 289–290.

Lipton, S. A., and Kater, S. B. (1989). Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. *Trends Neurosci.* **12,** 265–270.

Liu, J. P., and Lauder, J. M. (1992). S-100b and insulin-like growth factor-II differentially regulate growth of developing serotonin and dopamine neurons in vitro. *J. Neurosci. Res.* **33,** 248–256.

Lloyd, H. M., Meares, J. D., and Jakobi, J. (1975). Effects of estrogen and bromocriptine on in vivo secretion and mitosis in prolactin cells. *Nature* **255,** 497–498.

Loeb, D. M., Stephens, R. M., Copeland, T., Kaplan, D. R., and Greene, L. A. (1994). A Trk nerve growth factor (NGF) receptor point mutation affecting interaction with phospholipase Cyl abolishes NGF-promoted peripherin induction but not neurite outgrowth. *J. Biol. Chem.* **269,** 8901–8910.

Louis, J.-C., Magal, E., Takayama, S., and Varon, S. (1993). CNTF protection of oligodendrocytes against natural and tumor necrosis factor-induced death. *Science* **259,** 689–692.

Lowenstein, D. H., and Arsenault, L. (1996). The effects of growth factors on the survival and differentiation of cultured dentate gyrus neurons. *J. Neurosci.* **16**(5), 1759–1769.

Luc, P.-V. T., and Wagner, J. A. (1997). Regulation of the neural-specific gene VGF in PC12 cells. *J. Mol. Neurosci.* **8,** 223–241.

Luo, Y., Gabriel, J. L., Wang, F., Zhan, X., Maciag, T., Kan, M., and McKeehan, W. L. (1996). Molecular modeling and deletion mutagenesis implicate the nuclear translocation sequence in structural integrity of fibroblast growth factor-1. *J. Biol. Chem.* **271**(43), 26876–26883.

Lustig, R. H. (1996). In vitro models for the effects of sex hormones on neurons. *Ann. N. Y. Acad. Sci.* **784,** 370–380.

Macdonald, R. L., and Olsen, R. W. (1994). GABA receptor channels. *Annu. Rev. Neurosci.* **17,** 569–602.

Magistretti, P. J., and Pellerin, L. (1997). Regulation by neurotransmitters of glial energy metabolism. *In* "Brain Plasticity" (Filogamo *et al.,* Eds.), pp. 137–142. Plenum, New York.

Mahler, M. F., Mabie, P. C., Zhang, D., and Kessler, J. A. (1997). Bone morphogenetic proteins in the nervous system. *Trends Neurosci.* **20,** 309–317.

Maiese, K., Boniece, I., DeMeo, D., and Wagner, J. A. (1993). Peptide growth factors protect against ischemia in culture by preventing nitric oxide toxicity. *J. Neurosci.* **13,** 3034–3040.

Mandel, G., Cooperman, S. S., Maue, R. A., Goodman, R. H., and Brehm, P. (1988). Selective induction of brain type II Na channels by nerve growth factor. *Proc. Natl. Acad. Sci. USA* **85,** 924–928.

Mangelsdorf, D. J., Thummel, C., Beato, C., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995). The nuclear receptor superfamily: The second decade. *Cell* **83,** 835–839.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80,** 179–185.

Martin-Zanca, D., Hughes, S. H., and Barbacid, M. (1986). A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosene kinase sequences. *Nature* **319,** 743–748.

Martin-Zanca, D., Barbacid, M., and Parada, L. F. (1989). Molecular and biochemical characterization of the human trk protooncogene. *Mol. Cell. Biol.* **9,** 24–33.

Martin-Zanca, D., Barbacid, M., and Parada, L. F. (1990). Expression of the trk proto-oncogene is restricted to the sensory cranial and spinal ganglia of neural crest origin in mouse development. *Genes Dev.* **4,** 683–694.

Martinou, J. C., Martinou, I., and Kato, A. C. (1992). Cholinergic differentiation factor (CDF/LIF) promotes survival of isolated rat embryonic motoneurons in vitro. *Neuron* **8,** 737–744.

Mascotti, F., Caceres, A., Pfenninger, K. H., and Quiroga, S. (1997). Expression and distribution of IGF-1 receptors containing a beta-subunit variant (betagc) in developing neurons. *J. Neurosci.* **17**(4), 1447–1459.

Mason, A. J., Evans, B. A., Cox, D. A., Shine, J., and Richards, R. I. (1983). Structure of mouse kallikrein gene family suggests a role in specific processing of biologically active peptides. *Nature* **300,** 300–307.

Masu, Y., Wolf, E., Holtmann, B., Sendtner, M., Brem, G., and Thoenen, H. (1993). Disruption of the CNTF gene results in motor neuron degeneration. *Nature* **365,** 27–32.

Matthews, L. S., Hammer, R. E., Behringer, R. R., D'Ercole, A. J., Bell, G. I., Brinster, R. L., and Palmiter, R. D. (1988). Growth enhancement of transgenic mice expressing human insulin-like growth factor I. *Endocrinology* **123,** 2827–2833.

Mazzoni, I. E., and Kenigsberg, R. L. (1994). Localization and characterization of epidermal growth-factor receptors in the developing rat medial septal area in culture. *Brain Res.* **656,** 115–126.

McCobb, D. P., Cohan, C. S., Connor, J. A., and Kater, S. B. (1988). Interactive effects of serotonin and acetylcholine on neurite elongation. *Neuron* **1,** 377–385.

McDonald, N. Q., Lapatto, R., Murray-Rust, J., Gunning, J., Wlodawer, A., and Blundell, T. L. (1991). New protein fold revealed by a 2.3-angstrum resolution crystal structure of nerve growth factor. *Nature* **354,** 411–450.

McEwen, B. S., Weiss, J. M., and Schwartz, L. S. (1968). Selective retention of corticosterone by limbic structures in rat brain. *Nature* **220,** 911–912.

McEwen, B. S., Alves, S. E., Bulloch, K., and Weiland, N. G. (1997). Ovarian steroids and the brain: Implications for cognition and aging. *Neurology* **48**(5, Suppl. 7), S8–S15.

McKeehan, W. L., Wang, F., and Kan, M. (1998). The heparan sulfate-fibroblast growth factor family: Diversity of structure and function. *Prog. Nucleic Acid Res. Mol. Biol.* **59,** 135–176.

McKinnon, R. D., Matsui, T., Dubois-Dalcq, M., and Aaronson, S. A. (1990). FGF modulates the PDGF-driven pathway of oligodendrocyte development. *Neuron* **5,** 603–614.

McMahon, S. B., Armanini, M. P., Ling, L. H., and Phillips, H. S. (1994). Expression and coexpression of trk receptors in subpopulations of adult primary sensory neurons projecting to identified peripheral targets. *Neuron* **12,** 1161–1171.

McMorris, F. A., and Dubois-Dalcq, M. (1988). Insulin-like growth factor I promotes cell proliferation and oligodendroglial commitment in rat glial progenitor cells developing in vitro. *Neurosci. Res.* **21,** 199–209.

Merrill, J. E., and Zimmerman, R. P. (1991). Natural and induced cytotoxicity of oligodendrocytes by microglia is inhibitable by TGF-beta. *Glia* **4,** 327–331.

Meyer, J. S., Shearman, L. P., and Collins, L. M. (1996). Monoamine transporters and the neurobehavioral teratology of cocaine. *Pharmacol. Biochem. Behav.* **55,** 585–593. [Review]

Meyer, T. E., and Habener, J. F. (1993). Cyclic adenosine 3′,5′-monophosphate response element binding protein (CREB) and related transcription-activating deoxyribonucleic acid-binding proteins. *Endocr. Rev.* **14,** 269–290.

Michler-Stuke, A., and Wolff, J. R. (1987). "Neurology & Neurobiology." A. R. Liss, New York.

Middlemas, D. S., Lindberg, R. A., and Hunter, T. (1991). trkB, a neural receptor protein tyrosine kinase: Evidence for a full-length and two truncated receptors. *Mol. Cell. Biol.* **11,** 143–153.

Migliaccio, A., Di Domenico, M., Castoria, G., de Flaco, A., Bontempo, P., Nola, E., and Auricchio, F. (1996). Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol–receptor complex in MCF-7 cells. *EMBO J.* **15**(6), 1292–1200.

Milbrandt, J. (1987). A nerve growth factor induced gene encodes a possible transcriptional regulatory factor. *Science* **238,** 797–799.

Milbrandt, J., de Sauvage, F. J., Fahrner, T. J., Baloh, R. H., Leitner, M. L., Tansey, M. G., Lampe, P. A., Heuckeroth, R. O., Kotzbauer, P. T., Simburger, K. S., Golden, J. P., Davies, J. A., Vejsada, R., Kato, A. C., Hynes, M., Sherman, D., Nishimura, M., Wang, L.-C., Vandlan, R., Moffat, B., Klein, R. D., Pousen, K., Gray, C., Garces, A., Henderson, C. E., Philips, H. S., and Johnson, E. M. Jr. (1998). Persphin, a novel neurotrophic factor related to GDNF and neuriturin. *Neuron* **20,** 245–253.

Miller, T. M., Tansey, M. G., Johnson, E. M., Jr., and Creedon, D. J. (1997). Inhibition of phosphatidylinositol 3-kinase activity blocks depolarization and insulin-like growth factor I-mediated suvival of cerebellar granule cells. *J. Biol. Chem.* **272,** 9847–9853.

Minichiello, L., and Klein, R. (1996). TrkB and trkC neurotrophin receptors cooperate in promoting survival of hippocampal and cerebellar granule neurons. *Genes Dev.* **15,** 2849–2858.

Miranda, R. C., Sohrabji, F., and Toran-Allerand, D. (1994). Interactions of estrogen with the neurotrophins and their receptors during neural development. *Horm. Behav.* **28**(4), 367–375.

Miranti, C. K., Ginty, D. D., Huang, G., Chatila, T., and Greenberg, M. E. (1995). Calcium activates serum response factor-dependent transcription by a Ras and Elk-1 independent mechanism that involves a $Ca^{2+/}$ calmodulin-dependent kinase. *Mol. Cell. Biol.* **15,** 3672–3684.

Mitchison, T., and Kirschner, M. (1984). Dynamic instability of microtubule growth. *Nature* **312,** 237–242.

Mobley, W. C., Schenker, A., and Shooter, E. M. (1976). Characterization and isolation of proteolytically modified nerve growth factor. *Biochemistry* **15,** 5543–5551.

Moore, J. B., Mobley, W. C., and Shooter, E. M. (1974). Proteolytic modification of the β nerve growth factor protein. *Biochemistry* **13,** 833–840.

Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carver-Moore, K. S., and Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. *Nature* **382,** 76–79.

Morfini, G., DiTella, M. C., Feiguin, F., Carri, N., and Caceres, A. (1994). Neurotrophin-3 enhances neurite outgrowth in cultured hippocampal pyramidal neurons. *J. Neurosci. Res.* **39,** 219–232.

Morrison, R. (1993). Epidermal growth factor: Structure, expression, and function in the central nervous system. *In* "Neurotrophic Factors" (S. E. Loughlin and J. H. Fallon, Eds.), pp. 339–357. Acedemic Press, San Diego.

Morrison, R. S., de Vellis, J., Lee, Y. L., Bradshaw, R. A., and Eng, L. F. (1985). Hormones and growth factors induce the synthesis of glial fibrillary acidic protein in rat brain astrocytes. *Neurosci. Res.* **14,** 167–176.

Morrison, R. S., Keating, R. F., and Moskal, J. R. (1988). Basic fibroblast growth factor and epidermal growth factor exert differential trophic effects on CNS neurons. *J. Neurosci. Res.* **21,** 71–79.

Muller, Y., Rocchi, E., Lazaro, J. B., and Clos, J. (1995). Thyroid hormone promotes BCL-2 expression and prevents apoptosis of early differentiating cerebellar granule neurons. *Int. J. Dev. Neurosci.* **13**(8), 871–885.

Nakaki, T., Nukayama, M., Yamamoto, S., and Kato, R. (1989). α1 adrenergic stimulation and β2-adrenergic inhibition of DNA synthesis in vascular smooth muscle cells. *Mol. Pharmacol.* **37,** 30–36.

Nawa, H., Bessho, Y., Carnahan, J., Nakanishi, S., and Mizuno, K. (1993). Regulation of neuropeptide expression in cultured cerebral cortical neurons by brain-derived neurotrophic factor. *J. Neurochem.* **60,** 772–775.

Neveu, I., and Arenas, E. (1996). Neurotrophins promote the survival and development of neurons in the cerebellum of hypothyroid rats in vivo. *J. Cell. Biol.* **133**(3), 631–646.

Nguyen, T. T., Scimeca, J.-C., Filloux, C., Peraldi, P., Carpentier, J.-L., and Van Obberghen, E. (1993). Co-regulation of the mitogen-activated protein kinase, extracellular signal-regulated kinase 1 and the 90 kDa ribosomal S6 kinase in PC12 cells. *J. Biol. Chem.* **268,** 9803–9810.

Nikkhah, G., Odin, P., Smith, A., Tingstrom, A., Othberg, A., Brundin, P., Funa, K., and Lindvall, O. (1993). Platelet-derived growth factor promotes survival of rat and human mesencephalic dopaminergic neurons in culture. *Exp. Brain Res.* **92,** 516–523.

Nishizuka, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase c. *Science* **258,** 607–614.

Nordling, K., Sundstrom, E., and Bondesson, L. (1992). Inhibiting effects of serotonin antagonists on the proliferation of mercuric chloride stimulated human peripheral blood T lymphocytes. *Int. Arch. Allergy Immunol.* **97,** 105–108.

Obermeier, A., Halfter, H., Wiesmuller, K. H., Jung, G., Schlessinger, J., and Ullrich, A. (1993a). Tyrosine 785 is a major determinant of Trk-substrate interaction. *EMBO J.* **12,** 933–941.

Obermeier, A., Lammers, R., Wiesmuller, K., Jung, G., Schlessinger, J., and Ullrich, A. (1993b). Identification of trk binding sites for SHC and phosphatidylinositol 3′-kinase and formation of a multimeric signaling complex. *J. Biol. Chem.* **268,** 22963–22966.

Ohiwa, M., Murakami, H., Iwashita, T., Asai, N., Iwata, Y., Imai, T., Funahashi, H., Takagi, H., and Takahashi, M. (1997). Characterization of Ret-Shc-Grb2 complex induced by GDNF, MEN 2A, and MEN 2B mutations. *Biochem. Biophys. Res. Commun.* **237,** 747–751.

Ohmichi, M., Decker, S., and Saltiel, A. (1992). Activation of phosphatidylinositol 3-kinase by nerve growth factor involves indirect coupling of the trk proto-oncogne with src homology 2 domains. *Neuron* **9,** 769–777.

Ohmichi, M., Matuoka, K., Takenawa, T., and Saltiel, A. R. (1994). Growth factors differentially stimulate the phosphorylation of Shc proteins and their association with Grb2 in PC-12 pheochromocytoma cells. *J. Biol. Chem.* **269,** 1143–1148.

Oppenheim, R. W., Houenou, L. J., Johnson, J. E., Lin, L.-F. H., Li, L., Lo, A. C., Newsome, A. L., Prevette, D. M., and Wang, S. (1995). Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. *Nature* **373,** 344–346.

Oumesmar, B. N., Vignais, L., and Evercooren, B.-V. (1997). Developmental expression of platelet-derived growth factor α-receptor and glial cells of the mouse CNS. *J. Neurosci.* **17**(1), 125–139.

Ozawa, K., Urono, T., Miyakawa, K., Seo, M., and Imamura, T. (1996). Expression of the fibroblast growth factor family and their receptor family genes during mouse brain development. *Brain Res. Mol. Brain Res.* **41**(1–2), 279–288.

Palmer, T. D., Ray, J., and Gage, F. H. (1995). PGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. *Mol. Cell. Neurosci.* **6**(5), 474–486.

Park, O. K., Schaefer, T. S., and Nathans, D. (1996). In vitro activation of Stat3 by epidermal growth factor receptor kinase. *Proc. Natl. Acad. Sci. USA* **93**(24), 13704–13708.

Park, T. H., and Mytilineou, C. (1992). Protection from 1-methyl-4-phenylpyridinium (MPP+) toxicity and stimulation of regrowth of MPP (+)-damaged dopaminergic fibers by treatment of mesencephalic cultures with EGF and basic FGF. *Brain Res.* **599,** 83–97.

Partanen, J., Vainikka, S., Korhonen, J., Armstrong, E., and Alitalo, K. (1995). "Diverse Receptors for Fibroblast Growth Factors." Cancer Biology Laboratory, University of Helsinki.

Pasini, B., Ceccherini, I., and Romeo, G. (1996). Ret mutations in human disease. *Trends Genet.* **12,** 138–144.

Patterson, S. L., Abel, T., Deuel, T. A. S., Martin, K. C., Rose, J. C., and Kandel, E. R. (1996). Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron* **16,** 1137–1145.

Peles, E., Ben-Levy, R., Tzahar, E., Liu, N., Wen, D., and Yarden, Y. (1993). Cell-type specific interaction of Neu differentiation factor (NDF hergulin) with Neu HER-2 suggest complex ligand–receptor relationships. *EMBO J.* **12,** 961–971.

Perez, J., and Kelley, D. B. (1996). Trophic effects of androgen: Receptor expression and the survival of laryngeal motor neurons after axotomy. *J. Neurosci.* **16**(21), 6625–6633.

Perez, J., and Kelley, D. B. (1997). Androgen mitigates axotomy-induced decreases in calbindin expression in motor neurons. *J. Neurosci.* **1**(19), 7396–7403.

Pfaff, D. W., and Keiner, M. (1973). Atlas of estradiol concentrating cells in the central nervous system of the adult female rat. *J. Comp. Neurol.* **151,** 121–158.

Pichel, J. G., Shen, L., Hui, S. Z., Granholm, A.-C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J., Sariola, H., and Westphal, H. (1996). Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature* **382,** 73–76.

Pillay, T. S., Sasaoka, T., and Olefsky, J. M. (1995). Insulin stimulates the tyrosine dephosphorylation of ppl25 focal adhesion kinase. *J. Biol. Chem.* **270**(3), 991–994.

Pinkas-Kramarski, R., Eilam, R., Spiegler, O., Lavi, S., Liu, N., Chang, D., Wen, D., Schwartz, M., and Yarden, Y. (1994). Brain neurons and glial cells express Neu differentiation factor heregulin: A survival factor for astrocytes. *Proc. Natl. Acad. Sci. USA* **91,** 9387–9391.

Plowman, G. D., Green, J. M., Culouscou, J. M., Carlton, G. W., Rothwell, V. M., and Buckley, S. (1993). Heregulin induces tyrosine phosphorylation of HER4p 180erbB4. *Nature* **366,** 473–475.

Poddar, R., Paul, S., Chaudhury, S., and Sarkar, P. K. (1996). Regulation of actin and tubulin gene expression by thyroid hormone during rat brain development. *Brain Res. Mol. Brain Res.* **35**(1–2), 111–118.

Porter, J. T., and McCarthy, K. D. (1997). Astrocytic neurotransmitter receptors in situ and in vivo. *Prog. Neurobiol.* **51,** 439–455. [Review]

Porterfield, S. P., and Hendrich, C. E. (1993). The role of thyroid hormones in prenatal and neonatal neurological development—Current perspectives. *Endocr. Rev.* **14,** 94–106.

Poulsen, K. T., Armanini, M. P., Klein, R. D., Hynes, M. A., Phillips, H. S., and Rosenthal, A. (1994). TGF beta 2 and TGF beta 3 are potent survival factors for midbrain dopaminergic neurons. *Neuron* **13,** 1245–1252.

Prehn, J. H. M., Bindokas, V. P., Marcuccilli, C. J., Krajewski, S., Reed, J. C., and Miller, R. J. (1994). Regulation of neuronal bcl-2 protein expression and calcium homeostasis by transforming growth factor type beta confers wide-range protection on rat hippocampal neurons. *Proc. Natl. Acad. Sci. USA* **91,** 12599–12603.

Pringle, N. P., and Richardson, W. D. (1993). A singularity of PDGF alpha-receptor expression in the dorsoventral axis of the neural tube may define the origin of the oligodendrocyte lineage. *Development* **117,** 525–533.

Qiu, M.-S., and Green, S. H. (1992). PC12 cell neuronal differentiation is associated with prolonged $p21^{ras}$ activity and consequent prolonged EFK activity. *Neuron* **9,** 705–717.

Rabin, S. J., Cleghon, V., and Kaplan, D. R. (1993). SNT, a differentiation-specific target of neurotrophic factor-induced tyrosine kinase activity in neurons and PC12 cells. *Mol. Cell. Biol.* **13,** 2203–2213.

Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A., and Shooter, E. M. (1987). Gene transfer and molecular cloning of the rat NGF receptor. *Nature* **325,** 593–597.

Raff, M. C. (1989). Glial cell diversification in the rat optic nerve. *Science* **243,** 1450–1455.

Raff, M. C., Lillien, L. E., Richardson, W. D., Burne, J. F., and Noble, M. D. (1988). Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. *Nature* **333**(6173), 562–565.

Raffioni, S., and Bradshaw, R. A. (1992). Activation of phosphatidylinositol 3-kinase by epidermal growth factor, basic fibroblast growth factor, and nerve growth factor in PC12 pheochromocytoma cells. *Proc. Natl. Acad. Sci. USA* **89,** 9121–9125.

Ray, J., Baird, A., and Gage, F. H. (1997). A 10-amino acid sequence of fibroblast growth factor 2 is sufficient for its mitogenic activity on neural progenitor cells. *Proc. Natl. Acad. Sci. USA* **94**(13), 7047–7052.

Rechler, M. M., and Brown, A. L. (1992). Insulin-like growth factor binding proteins: Gene structure and expression. *Growth Regul.* **2,** 55–68.

Reilly, J. F., Maher, P. A., and Kumari, V. G. (1998). Regulation of astrocyte GFAP expression by TGF-beta 1 and FGF-2. *Glia* **22**(2), 202–210.

Relton, J. K., and Rothwell, N. J. (1992). Interleukin-1 receptor antagonist inhibits ischemic and excitotoxic neuronal damage in the rat. *Brain Res. Bull.* **29,** 243–246.

Renaud, F., Desset, S., Oliver, L., Gimenez-Gallego, G., Van Obberghen, E., Courtois, Y., and Laurent, M. (1996). The neurotrophic activity of fibroblast growth factor 1 (FGF1) depends on endogenous FGF1 expression and is independent of the mitogen-activated protein kinase cascade pathway. *J. Biol. Chem.* **271**(5), 1801–2811.

Reuss, B., Dermietzel, R., and Unsicker, K. (1998). Fibroblast growth factor 2 (FGF-2) differentially regulates connexin (cx) 43 expression and function in astroglial cells from distinct brain regions. *Glia* **22**(1), 19–30.

Reynolds, B. A., and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255,** 1707–1710.

Reynolds, B. A., and Weiss, S. (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev. Biol.* **175**(1), 1–13.

Richardson, W. D., Pringle, N., Mosley, M. J., Westermark, B., and Dubois-Dalcq, M. (1988). A role for platelet-derived growth factor in normal gliogenesis in the central nervous system. *Cell* **53**(2), 309–319.

Robbins, T. W. (1997). Arousal systems and attentional processes. *Biol. Psychol.* **45,** 57–71. [Review]

Robson, S., and Burgoyne, R. (1988). Differential levels of tyrosinated, detyrosinated and acetylated α-tubulins in neurites and growth cones of dorsal root ganglion neurons. *Cell Motil. Cytoskel.* **12,** 273–282.

Rochlin, M. W., Wickline, K. M., and Bridgman, P. C. (1996). Microtubule stability decreases axon elongation but not axoplasm production. *J. Neurosci.* **16,** 3236–3246.

Rodriguez-Tebar, A., Dechant, G., and Barde, Y.-A. (1990). Binding of brain-derived neurotrophic factor to the nerve growth factor receptor. *Neuron* **4,** 487–492.

Rodriguez-Tebar, A., Dechant, G., Gotz, R., and Barbe, Y. A. (1992). Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. *EMBO J.* **11,** 917–922.

Rosnet, O., Marchetto, S., and deLapeyriere, O. (1991). Murine *Flt3,* a gene encoding a novel tyrosine kinase receptor of the PDGFR/csfir family. *Oncogene* **6,** 1641–1650.

Ross, R., Glomet, J., Kariya, B., and Harker, L. (1974). A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. *Proc. Natl. Acad. Sci. USA* **71,** 1207–1210.

Ross, R., Raines, E. W., and Bowen-Pope, D. F. (1986). The biology of platelet-derived growth factor. *Cell* **49,** 155–169.

Rothwell, N. J., Luheshi, G., and Toulmond, S. (1996). Cytokines and their receptors in the central nervous system: Physiology, pharmacology, and pathology. *Pharmacol. Ther.* **69,** 85–95.

Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P. G., and Pawson, T. (1992). Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature* **360,** 689–692.

Sadowski, H. B., Shuai, K., Darnell, J. E., Jr., and Gilman, M. Z. (1993). A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. *Science* **261,** 1739–1744.

Sakata, M., Yanamoto, H., Hashimoto, N., Iithara, K., Tsukahara, T., Taniguchi, T., and Kikuchi, H. (1998). Induction of infant tolerance by platelet-derived growth factor against reversible focal ischemia. *Brain Res.* **784**(1–2), 250–255.

Sanchez, M. P., Silos-Santiago, I., Frisen, J., He, B., Lira, S. A., and Barbacid, M. (1996). Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature* **382,** 70–72.

Sariola, H., and Saino, K. (1997). The tip-top branching ureter. *Curr. Opin. Cell Biol.* **9,** 877–884.

Sasahara, M., Fries, J. W. U., Raines, E. W., Gown, A. M., Westrum, L. E., Frosch, M. P., Bonthron, D. T., Ross, R., and Collins, T. (1991). PDGF B-chain in neurons of the central nervous system, posterior pituitary, and in a transgenic model. *Cell* **64,** 217–227.

Sassone-Corsi, P. (1995). Transcription factors responsive to cAMP. *Annu. Rev. Cell Biol.* **11,** 355–377.

Sassone-Corsi, P., Der, C. J., and Verma, I. M. (1989). *ras*-Induced neuronal differentiation of PC12 cells: Possible involvement of *fos* and *Jun*. *Mol. Cell. Biol.* **9,** 3174–3183.

Savage, C. R., Jr., Inagami, T., and Cohen, S. (1972). The primary structure of epidermal growth factor. *J. Biol. Chem.* **247,** 7612–7621.

Schatteman, G. C., Morrison-Graham, K., Van Koppen, A., Weston, J. A., and Bowen-Pope, D. F. (1992). Regulation and role of the PDGF receptor α-subunit expression during embryogenesis. *Development* **115,** 123–131.

Schnell, L., Schneider, R., Kolbech, R., Barde, Y.-A., and Schwab, M. E. (1994). Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. *Nature* **367,** 170–173.

Schooltinck, H., Stoyan, T., Roeb, E., Heinrich, P. C., and Rose-John, S. (1992). Ciliary neurotrophic factor induces acute-phase protein expression in hepatocytes. *FEBS Lett.* **314,** 280–284.

Schreiber, G., Aldred, A. R., Jaworowski, A., Nilsson, C., Achen, M. G., and Segal, M. B. (1990). Thyroxine transport from blood to brain via transthyretin synthesis in chromed plexus. *Am. J. Physiol.* **258,** R338–R345.

Schuchard, M., Landers, J. P., Punkay Sandhu, N., and Spelsberg, T. C. (1993). Stroid hormone regulation of nuclear proto-oncogenes. *Endocr. Rev.* **14,** 659–669.

Schwartz, J. P., and Mishler, K. (1990). β-Adrenergic receptor regulation, through cyclic AMP, of nerve growth factor expression in rat cortical and cerebellar astrocytes. *Cell. Mol. Neurobiol.* **10**(3), 447–457.

Scott, J., Selby, M., Urdea, M., Quiroga, M., Bell, G. I., and Rutter, W. J. (1983). Isolation and nucleotide sequence of a cDNA encoding the precursor of mouse nerve growth factor. *Nature* **302,** 538–540.

Segal, R. A., and Greenberg, M. E. (1996). Intracellular signaling pathways activated by neurotrophic factors. *Annu. Rev. Neurosci.* **19,** 463–489.

Segarra, A. C., and McEwen, B. S. (1991). Estrogen increases spine density in ventromedial hypothalamic neurons of periopubertal rats. *Neuroendocrinology* **54,** 365–372.

Sei, Y., Vitkovic, L., and Yokoyama, M. M. (1995). Cytokines in the central nervous system: Regulatory roles in neuronal function, cell death and repair. *Neuroimmunomodulation* **2,** 121–133.

Selby, M. J., Edwards, R., Sharp, F., and Rutter, W. J. (1976). Mouse nerve growth factor gene: Structure and expression. *Mol. Cell. Biol.* **7,** 3057–3064.

Sendtner, M., Kreutzberg, G. W., and Thoenen, H. (1992a). Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. *Nature* **345,** 4401.

Sendtner, M., Schmalbruch, H., Stockli, K. A., Carroll, P., Kreutzberg, G. W., and Thoenen, H. (1992b) Ciliary neurotrophic factor prevents degeneration of motor neurons in mouse mutant progressive motor neuronopathy. *Nature* **358,** 502–504.

Seroogy, K. B., Gall, C. M., Lee, D. C., and Kornblum, H. I. (1995). Proliferative zones of postnatal rat brain express epidermal growth factor receptor mRNA. *Brain Res.* **670,** 157–164.

Setalo, G., Jr., Singh, M., Warren, M. F., and Toran-Allerand, D. (1997). Direct association of heat shock protein HSP90 and the extracellular signal regulated kinases, ERK1/2. A possible link between estrogen and neurotrophin signaling. *Soc. Neurosci. Abstr.* **23**(Part 2), 670.15.

Seuwon, K., Magnaldo, I., and Pouyssegur, J. (1988). Serotonin stimulates DNA synthesis in fibroblasts acting through 5-HT1B receptors coupled to Ci-protein. *Nature* **335,** 254–256.

Shaoul, E., Reich-Slotky, R., Berman, B., and Ron, D. (1995). Fibroblast growth factor receptors display both common and distinct signaling pathways. *Oncogene* **10**(8), 1553–1561.

Sher, E. S., Xu, X. M., Adams, P. M., Craft, C. M., and Stein, S. A. (1998). The effects of thyroid hormone level and action in developing brain: Are these targets for the actions of polychlorinated biphenyls and dioxins? *Toxicol. Ind. Health* **14**(1–2), 121–158.

Shihabuddin, L. S., Ray, J., and Gage, F. H. (1997). FGF-2 is sufficient to isolate progenitors found in the adult mammalian spinal cord. *Exp. Neurol.* **148**(2), 173–176.

Shimasaki, S., and Ling, N. (1992). Identification and molecular characterization of insulin-like growth factor binding proteins (IGFBP-1, -2, -3, -4, -5 and -6). *Prog. Growth Factor Res.* **3,** 243–266.

Shing, Y., Christofori, G., Hanahan, D., Ono, Y., Sasada, R., Igarashi, K., and Folkman, J. (1993). Betacellulin: A mitogen from pancreatic β cell tumors. *Science* **259,** 1604–1607.

Shitaka, Y., Matsuki, N., Saito, H., and Katsuki, H. (1996). Basic fibroblast growth factor increases functional L-type Ca^{2+} channels in fetal rat hippocampal neurons: Implications for neurite morphogenesis in vitro. *J. Neurosci.* **16**(20), 6476–6489.

Shuai, K., Ziemiecki, A., Wilks, A. F., Harpur, A. G., Sadowski, H. B., Gilman, M. Z., and Darnell, J. E. (1993). Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins. *Nature* **366,** 580–583.

Shuai, K., Horvath, C. M., Huang, L. H. T., Quareshi, S. A., Cowburn, D., and Darnell, J. E., Jr. (1994). Interferon activation of the transcription factor Star91 involves dimerization through SH2-phosphytyrosyl peptide interactions. *Cell* **76,** 821–828.

Sieber-Blum, M. (1991). Role of the neurotrophic factors BDNF and NGF in the commitment of pluripotent neural crest cells. *Neuron* **6,** 949–955.

Silva, J. E., Leonard, J. I., Cranz, F. R., and Larsen, P. R. (1982). Evidence for two tissue-specific pathways for in vivo thyroxine 5′-deiodination in the rat. *J. Clin. Invest.* **69,** 1176–1184.

Simerly, R. B., Chang, C., Muramatsu, M., and Swanson, L. W. (1990). Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: An in situ hybridization study. *J. Comp. Neurol.* **294,** 76–95.

Simmonds, M. A. (1991). Modulation of the GABAs receptor by steroids. *Sem. Neurosci.* **3,** 231–239.

Singer, C. A., Rogers, K. L., Strickland, T. M., and Dorsa, D. M. (1996). Estrogen protects primary cortical neurons from glutamate toxicity. *Neurosci. Lett.* **212**(1), 13–16.

Singh, M., Warren, M. F., Setalo, G., Jr., and Toran-Allerand, D. (1997). The estrogen receptor exists in a multimeric complex consisting of at least B-RAF, MEK and HSP-90 in explants of the cerebral cortex. *Soc. Neurosci. Abstr.* **23**(Part 2), 670.14.

Singleton, J. R., Dixit, V. M., and Feldman, E. L. (1996). Type I insulin-like growth factor receptor activation regulates apoptotic proteins. *J. Biol. Chem.* **271,** 31791–31794.

Slotkin, T. A., and Bartolome, J. (1986). Role of ornithine decarboxylase and polyamines in nervous system development. *Brain Res.* **17,** 307–320.

Smallwood, P. M., Munoz-Sanjuan, I., Tong, P., Macke, J. P., Hendry, S. H. C., Gilbert, D. J., Copeland, N. G., Jenkins, N. A., and Nathans, J. (1996). Fibroblast growth factor (FGF) homologous factors: New members of the FGF family implicated in nervous system development. *Proc. Natl. Acad. Sci. USA* **93,** 9850–9857.

Smeyne, R. J., Klein, R., Schnapp, A., Long, L. K., Bryant, S., Lewin, A., Lira, S. A., and Barbacid, M. (1994). Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. *Nature* **368,** 246–249.

Smits, A., Kato, M., Westermark, B., Nister, M., Heldin, C. H., and Funa, K. (1991). Neurotrophic activity of platelet-derived growth factor (PDGF): Rat neuronal cells possess functional PDGF β-type receptors and respond to PDGF. *Proc. Natl. Acad. Sci. USA* **88,** 8159–8163.

Smits, A., Ballagi, E., and Funa, K. (1993). PDGF-BB exerts trophic activity on cultured GABA interneurons from the newborn rat cerebellum. *Eur. J. Neurosci.* **5,** 986–994.

Sohrabji, F., Greene, L. A., Miranda, R. C., and Toran-Allerand, C. D. (1994). Reciprocal regulation of asdrogen and NGF receptors by their ligands in PC12 cells. *J. Neurobiol.* **25**(8), 974–988.

Soltoff, S. P., and Cantley, L. C. (1996). p120cbl is a cytosolic adapter protein that associates with phosphoinositide 3-kinase in response to epidermal growth factor in PC12 and other cells. *J. Biol. Chem.* **271,** 563–567.

Soltoff, S. P., Carraway, K. L., Prigent, S. A., Gullick, W. G., and Cantley, L. C. (1994). ErbB3 is involved in activation of phosphatidylinositol 3-kinase in response to epidermal growth factor. *Mol. Cell. Biol.* **14,** 3550–3558.

Soppet, D., Escandon, E., Maragos, J., *et al.* (1991). The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. *Cell* **65,** 895–903.

Soriano, P. (1994). Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes Dev.* **8,** 1888–1896.

Spindler, K. D. (1997). Interactions between steroid hormones and the nervous system. *Neurotoxicology* **18**(3), 745–754.

Spoerri, P. E. (1988). Neurotrophic effects of GABA in cultures of embryonic chick brain and retina. *Syna* **2,** 11–22.

Spranger, M., Lindholm, D., Bandtlow, C., Heumann, R., Gnahn, H., Na;u;her-Noe;a;, M., and Thoenen, H. (1990). Regulation of nerve growth factor (NGF) synthesis in the rat central nervous system: Comparison between the effects of interleukin-1 and various growth factors in axtrocyte cultures and *in vivo*. *Eur. J. Neurosci.* **2,** 69–76.

Squinto, S. P., Aldrich, T. H., Lindsay, R. M., Morrissey, D. M., Panayotatos, N., *et al.* (1990). Identification of functional receptors for ciliary neurotrophic factor on neuronal cell lines and primary neurons. *Neuron* **5,** 757–766.

Squinto, S. P., Stitt, T. N., Aldrich, T. H., Davis, S., Bianco, S. M., Radziejewski, C., Glass, D. J., Masiakowski, P., Furth, M. E., Valenzuela, D. M., DiStefano, P. S., and Yancopoulos, G. D. (1991). trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. *Cell* **65,** 885–893.

Stachowiak, M. K., Moffett, J., Maher, P., Tucholski, J., and Stachowiak, E. K. (1997). Growth factor regulation of cell growth and proliferation in the nervous system. A new intracrine nuclear mechanism. *Mol. Neurobiol.* **15**(3), 257–283.

Stahl, N., Boulton, T. G., Farruggella, T., Ip, N. Y., Davis, S., Witthuhn, B. A., Quelle, F. W., Silennoinen, O., Barbieri, G., Pelegrini, S., *et al.* (1994). Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. *Science* **263,** 92–95.

Stahl, N., Farruggella, T. J., Boulton, T. G., Zhong, Z., Darnell, J. E., Jr., and Yancopoulos, G. D. (1995). Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science* **267,** 1349–1353.

Stephens, R. D., Loeb, D., Copeland, T., Pawson, T., Greene, L. A., and Kaplan, D. (1994). Trk receptors use redundant signal transduction pathways involving SHC and PLC gamma 1 to mediate NGF responses. *Neuron* **12,** 691–705.

Stewart, C. E., and Rotwein, P. (1996). Growth, differentiation, and survival: Multiple physiological functions for insulin-like growth factors. *Physiol. Rev.* **76,** 1005–1026.

Stylianopoulou, F., Efstratiadis, A., Herbert, J., and Pintar, J. (1988a). Pattern of the insulin-like growth factor II gene expression during rat embryogenesis. *Development* **103,** 497–506.

Stylianopoulou, F., Herbert, J., Soares, M. B., and Efstratiadis, A. (1988b). Expression of the insulin-like growth factor II gene in the choroid plexus and the leptomeninges of the adult rat central nervous system. *Proc. Natl. Acad. Sci. USA* **85,** 141–144.

Suen, K., Bustelo, X. R., Pawson, T., and Barbacid, M. (1993). Molecular cloning of the mouse *grb2* gene: Differential interaction of the Grb2 adaptor protein with epidermal growth factor and nerve growth factor receptors. *Mol. Cell. Biol.* **13,** 5500–5512.

Symes, A. J., Rajan, P., Corpus, L., and Fink, S. (1995). C/EBP-related sites in addition to a STAT site are necessary for ciliary neurotrophic factor-leukemia inhibitory factor-dependent transcriptional activation by the vasoactive intestinal peptide cytokine response element. *J. Biol. Chem.* **270,** 8068–8075.

Szeberenyi, J., Cai, H., and Cooper, G. M. (1990). Effect of a dominant inhibitory Ha-*ras* mutation on neuronal differentiation of PC12 cells. *Mol. Cell. Biol.* **10,** 5324–5332.

Szeberenyi, J., Erhardt, P., Cai, H., and Cooper, G. M. (1992). Role of ras in signal transduction from the nerve growth factor receptor: Relationship to protein kinase C, calcium and cyclic AMP. *Oncogene* **7,** 2105–2113.

Tanaka, E. M., and Kirschner, M. W. (1991). Microtubule behavior in the growth cones of living neurons during axon elongation. *J. Cell Biol.* **115,** 345–363.

Tao, Y., Black, I. B., and DiCicco-Bloom, E. (1997). In vivo neurogenesis is inhibited by neutralizing antibodies to basic fibroblast growth factor. *J. Neurobiol.* **33**(3), 289–296.

Thaler, C. D., Suhr, L., Ip, N. Y., and Katz, D. M. (1944). Leukemia inhibitory factor and neurotrophins support overlapping populations of rat nodose sensory neurons in culture. *Dev. Biol.* **161,** 338–344.

Timpe, L. C., and Fantl, W. J. (1994). Modulation of a voltage-activated potassium channel by peptide growth factor receptors. *J. Neurosci.* **13**(3, Pt. 1), 1195–1201.

Tobet, S. A., and Hanna, I. K. (1997). Ontogeny of sex differences in the mammalian hypothalamus and preoptic area. *Cell. Mol. Neurobiol.* **17**(6), 565–601.

Todd, R. D. (1992). Neural development is regulated by classical neurotransmitters: Dopamine D_2 receptor stimulation enhances neurite outgrowth. *Biol. Psych.* **31,** 794–807.

Toker, A., Meyer, M., Reddy, K. K., Falck, J. R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M., and Cantley, L. C. (1994). Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3, 4ZP2 and PtdIns-3,4,5-P3. *J. Biol. Chem.* **269,** 32358–32367.

Toran-Allerand, C. D., Hashimoto, K., Greenough, W. T., and Saltarelli, M. (1983). Sex steroids and the development of the newborn mouse hypothalamus and preoptic *in vitro*. III. Effects of estogen on dendritic differentiation. *Dev. Brain Res.* **7,** 97–101.

Toran-Allerand, C. D., Miranda, R. C., Bentham, W. D., Sohrabji, F., Brown, T. J., Hochberg, R. B., and MacLusky, N. J. (1992). Estrogen receptors colocalize with low-affinity nerve growth factor receptors in cholinergic neurons of the basal forebrain. *Proc. Natl. Acad. Sci. USA* **89**(10), 4668–4672.

Treanor, J. J. S., Goodman, L., deSauvage, F., Stone, D. M., Poulsen, K. T., Beck, C. D., Gray, C., Armanini, M. P., Pollock, R. A., Hefti, F., Phillips, H. S., Goddard, A., Moore, M. W., Buj-Bello, A., Davies, A. M., Asai, N., Takahashi, M., Vandlen, R., Henderson, C. E., and Rosenthal, A. (1996). Characterization of a multicomponent receptor for GDNF. *Nature* **382,** 80–87.

Trentin, A. G., Rosenthal, D., and Maura Neto, V. (1995). Thyroid hormone and conditioned medium effects on astroglial cells from hypothyroid and normal rat brain: Factor secretion, cell differentiation, and proliferation. *J. Neurosci. Res.* **41**(3), 409–417.

Trupp, M., Arenas, E., Fainzilber, M., Nilsson, A.-S., Sieber, B.-A., Grigoriou, M., Kilkenny, C., Salazar-Grueso, E., Pachnis, V., Arumae, U., Sariola, H., Saarma, M., and Ibanez, C. F. (1996). Functional receptor for GDNF encoded by the c-ret proto-oncogene. *Nature* **381,** 785–789.

Tucker, M. S., Khan, I., Fuchs-Young, R., Price, S., Steininger, T. L., Greene, G., Wainer, B. H., and Rosner, M. R. (1993). Localization of immunoreactive epidermal growth factor receptor in neonatal and adult rat hippocampus. *Brain Res.* **631,** 65–71.

Ullrich, A., and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell* **61,** 203–212.

Uno, H., Eisele, S., Sakai, A., Shelton, S., Baker, E., DeJesus, O., and Holdens, J. (1994). Neurotoxicity of glucocorticoids in the primates brain. *Horm. Behav.* **28,** 336–348.

Valenzuela, C. F., Kazkauskas, A., Brozowski, S. J., Weiner, J. L., DeMali, K. A., McDonald, B. J., Moss, S. J., Dunwiddie, T. V., and Harris, R. A. (1995). Platelet-derived growth factor receptor is a novel modulator of type A γ-aminobutyric acid-gated ion channels. *Mol. Pharmacol.* **48,** 1099–1107.

Valenzuela, C. F., Xiong, Z., MacDonald, J. F., Weiner, J. L., Frazier, C. J., Dunwiddlie, T. V., and Harris, R. A. (1996). Platelet-derived growth factor induces a long-term inhibition of N-methyl-D-aspartate receptor function. *J. Biol. Chem.* **271,** 16151–16159.

Valenzuela, C. F., Kazlauskas, A., and Weiner, J. L. (1997). Roles of platelet-derived growth factor in the developing and mature nervous systems. *Brain Res. Rev.* **24,** 77–89.

van der Geer, P., Hunter, T., and Lindberg, R. A. (1994). Receptor protein–tyrosine kinases and their signal transduction pathways. *Annu. Rev. Cell Biol.* **10,** 251–337.

VanRenterghem, B., Brownin, M. D., and Maller, J. L. (1994). Regulation of mitogen-activated protein kinase activation by protein kinases A and C in a cell-free system. *J. Biol. Chem.* **269,** 24666–24672.

Varon, S., Nomura, J., and Shooter, E. M. (1967a). Subunit structure of a high-molecular-weight form of the nerve growth factor from mouse submaxillary gland. *Proc. Natl. Acad. Sci. USA* **57,** 1782–1789.

Varon, S., Normura, J., and Shooter, E. M. (1967b). The isolation of the mouse nerve growth factor protein in a high molecular weight form. *Biochemistry* **6,** 2202–2209.

Vescovi, A. L., Reynolds, B. A., Fraser, D. D., and Weiss, S. (1993). bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. *Neuron* **11,** 951–966.

Vetter, M. L., Martin-Zanca, D., Parada, L. F., Bishop, J. M., and Kaplan, D. R. (1991). Nerve growth factor rapidly stimulates tyrosine phosphorylation of phospholipase C-γ 1 by a kinase activity associated with the product of the *trk* protooncogene. *Proc. Natl. Acad. Sci. USA* **88,** 5650–5654.

Vignais, L., Oumesmar, B. N., and Baron-Van Evercooren, A. (1995). PDGF-α receptor is expressed by mature neurones of the central nervous system. *NeuroReport* **6,** 1993–1996.

Visser, T. L., Leonard, J. L., Kaplan, M. M., and Larsen, P. R. (1982). Kinetic evidence suggesting two mechanisms for iodothyronine 5′-deiodination in rat cerebral cortex. *Proc. Natl. Acad. Sci. USA* **79,** 5080–5084.

Vogt Weisenhorn, D. M., Lehner, A. N., Roback, L. J., and Wainer, B. H. (1997). Differential effects of growth factors and neurotransmitters on posttranslational modifications of microtubules in developing neurons in vitro. *Soc. Neurosci. Abstr.* **23,** 599.

Vogt Weisenhorn, D. M., Roback, L., and Wainer, B. H. (1998). Signalling pathways involved in two distinct forms of neuritogenesis in a neuronal progenitor cell line. *Soc. Neurosci.* **24,** 1282.

Vossler, M. R., Yao, H., York, R. D., Pan, M.-G., Rim, C. S., and Stork, P. J. S. (1997). cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. *Cell* **89,** 73–82.

Walter, I. B. (1996). Triiodothyronine exerts a trophic action on rat sensory neuron survival and neurite outgrowth through different pathways. *Eur. J. Neurosci.* **8**(3), 455–466.

Wanaka, A., Milbrandt, J., and Johnson, F. M. (1991). Expression of FGF receptor gene in rat development. *Development* **111,** 455–468.

Wang, J. K., Gao, G., and Goldfarb, M. (1994). Fibroblast growth factor receptors have different signaling and mitogenic potentials. *Mol. Cell. Biol.* **14**(1), 181–188.

Wang, T. W., Li, B. Y., Danielson, P. D., Shah, P. C., Rockwell, S., Lechleider, R. J., Martin, J., Manganaro, T., and Donahoe, P. K. (1996). The immunophilin FKBP12 functions as a common inhibitor of the TGF beta family type I receptors. *Cell* **86,** 435–444.

Weise, B., and Grothe, C. (1993). Localization of bFGF and FGF-receptor in the developing nervous system of the embryonic and newborn rat. *J. Neurosci. Res.* **34,** 442–453.

Westermark, B., and Wasteson, A. (1976). A platelet factor stimulating human normal glial cells. *Exp. Cell Res.* **98,** 170–174.

Whitaker-Azmitia, P. M. (1991). Role of serotonin and other neurotransmitter receptors in brain development: Basis for developmental pharmacology. *Pharmacol. Rev.* **43**(4), 553–561.

Wickelgren, I. (1997). Estrogen stakes claim to cognition. *Science* **276,** 675–678.

Widenfalk, J., Nosrat, C., Tomac, A., Westphal, H., Hoffer, B., and Olson, L. (1997). Neuriturin and glial derived neurotrophic factor receptor beta, novel proteins related to GDNF and

GDNFR-alpha with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. *J. Neurosci.* **17,** 8506–8519.

Williams, B. P., Park, J. K., Alberta, J. A., Muhlebach, S. G., Hwang, G. Y., Roberts, T. M., and Stiles, C. D. (1997). A PDGF-regulated immediate early gene response initiates neuronal differentiation in ventricular zone progenitor cells. *Neuron* **18**(4), 553–562.

Wolswijk, G., Riddle, P. N., and Noble, M. (1991). Platelet-derived growth factor is mitogenic for 0-2A adult progenitor cells. *Glia* **4**(5), 495–503.

Wong, M., and Moss, R. L. (1994). Patch-clamp analysis of direct steroidal modulation of glutamate receptor-channels. *Neuroendocrinology* **6,** 347–355.

Wood, K. W., Sarnecki, C., Roberts, T. M., and Blenis, J. (1992). ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MPA kinase, Raf-1, and RSK. *Cell* **68,** 1041–1050.

Wood, K. W., Qi, H., D'Arcangelo, G., Armstrong, R. C., Roberts, T. M., and Halegoua, S. (1993). The cytoplasmic raf oncogene induces a neuronal phenotype in PC12 cells: A potential role for cellular raf kinases in neuronal growth factor signal transduction. *Proc. Natl. Acad. Sci. USA* **90,** 5016–5020.

Wood, T. L., Streck, R. D., and Pintar, J. E. (1992). Expression of the IGFBP-2 gene in post-implantation rat embryos. *Development* **114,** 59–66.

Wooley, C. S., and McEwen, B. S. (1993). Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. *J. Comp. Neurol.* **336,** 291–306.

Woolsey, T. A., and Wann, J. R. (1976). Areal changes in mouse cortical barrels following vibrissal damage at different postnatal ages. *J. Comp. Neurol.* **170,** 53–66.

Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994). Mechanism of activation of the TGF beta receptor. *Nature* **370,** 341–347.

Xing, J., Ginty, D. D., and Greenberg, M. E. (1996). Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* **273,** 959–963.

Yamada, K., Spooner, B., and Wassells, N. (1971). Ultrastructure and function of growth cones and axons in cultured nerve cells. *J. Cell Biol.* **49,** 614–635.

Yamada, M., Enokido, Y., Ikeuchi, T., and Hatanaka, H. (1995). Epidermal growth factor prevents oxygen-triggered apoptosis and induces sustained signalling in cultured rat cerebral cortical neurons. *Eur. J. Neurosci.* **7,** 2130–2138.

Yamada, M., Ikeuchi, T., Aimoto, S., and Hatanaka, H. (1996). EGF-induced sustained tyrosine phospholylation and decreased rate of down-regulation of EGF receptor in PC12h-R cells which show neuronal differentiation in response to EGF. *Neurochem. Res.* **21,** 821–828.

Yamagata, K., Sanders, L. K., Kaufmann, W. E., Yee, W., Barnes, C. A., Nasthans, D., and Worley, P. F. (1994). *rheb,* a growth factor- and synaptic activity-regulated gene, encodes a novel ras-related protein. *J. Biol. Chem.* **269,** 16333–16339.

Yamashita, H., Ten Dijke, P., Heldin, C. H., and Miyazono, K. (1996). Bone morphogenetic protein receptors. *Bone* **19,** 569–574.

Yan, G.-Z., and Ziff, E. B. (1995). NGF regulates the PC12 cell cycle machinery through specific inhibition of the Cdk kinases and induction of cyclin D1. *J. Neurosci.* **15,** 6200–6212.

Yan, G.-Z., and Ziff, E. B. (1997). Nerve growth factor induces transcription of the p21 WAF1/CIP1 and cyclin D1 genes in PC12 cells by activating the Sp1 transcription factor. *J. Neurosci.* **17,** 6122–6132.

Yan, Q., Elliott, J. L., Matheson, C., Sun, J., Zhang, L., Mu, X. M., Rex, K. L., and Snider, W. D. (1993). Influences of neurotrophins on mammalian motoneurons in vivo. *J. Neurobiol.* **24,** 1555–1577.

Yan, Q., Matheson, C., and Lopez, O. T. (1995). *In vivo* neurotrophic effects of GDNF on neonatal and adult facial motor neurons. *Nature* **373,** 341–344.

Yan, Q., Radeke, M. J., Matheson, C. R., Talvenheimo, J., Welcher, A. A., and Einstein, S. C. (1997). Immunocytochemical localization of TrkB in the central nervous system of

the adult rat (published erratum appears in *J. Comp. Neurol.* 1997 Jun 16: **382**(4), 546–547). *J. Comp. Neurol.* **378**(1), 546–571.

Yao, R., and Cooper, G. (1995). Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* **267,** 2003–2006.

Yee, W. M., and Worley, P. F. (1997). Rheb interacts with raf-1 kinase and may function to integrate growth factor- and protein kinase A-dependent signals. *Mol. Cell. Biol.* **17,** 921–933.

Yeh, H. J., Ruit, K. G., Wang, Y. X., Parks, W. C., Snider, W. D., and Deuel, T. F. (1991). PDGF A-chain gene is expressed by mammalian neurons during development and maturity. *Cell* **64,** 209–216.

Yokoyama, M., Morrison, R. S., Black, I. B., and Dreyfus, C. F. (1994). Septal neuron cholinergic and GABAergic functions: Differential regulation by basic fibroblast growth factor and epidermal growth factor. *Dev. Brain Res.* **78,** 201–209.

Young, S. W., Dickens, M., and Tavare, J. M. (1994). Differentiation of PC12 cells in response to a cAMP analogue is accompanied by sustained activation of mitogen-activated protein kinase. Comparison with the effects of insulin, growth factors and phorbol esters. *FEBS Lett.* **338,** 212–216.

Zackenfels, K., Oppenheim, R. W., and Rohrer, H. (1995). Evidence for an important role of IGF-I and IGF-II for the early development of chick sympatheric neurons. *Neuron* **14,** 731–741.

Zhang, F. X., and Hutchins, J. B. (1996). Expression of platelet-derived growth factor (PDGF) receptor alpha-subunit in mouse brain: Comparison of patch mutants and normal littermates. *Cell. Mol. Neurobiol.* **16**(4), 479–487.

Zheng, J. Q., Felder, M., Connor, J. A., and Poo, M. (1994). Turning of nerve growth cones induced by neurotransmitters. *Nature* **368,** 140–144.

Zheng, J. Q., Wan, J.-j., and Poo, M.-m. (1996). Essential role of filopodia in chemotrypic turning of nerve growth cone induced by a glutamate gradient. *J. Neurosci.* **16,** 1140–1149.

Zhong, Z., Wen, Z., and Darnell, J. E., Jr. (1994). Stat2: A STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* **264,** 95–98.

Zhu, X., Komiya, H., Chirino, A., Faham, S., Fox, G. M., Arakawa, T., Hsu, B. T., and Rees, D. C. (1991). Three dimensional structures of acidic and basic fibroblast growth factors. *Science* **251,** 90–93.

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Conformational Changes of Contractile Proteins and Their Role in Muscle Contraction

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The review summarizes the results of studies on conformational changes in contractile proteins that occur during muscle contraction. Polarized fluorescence of tryptophan residues in actin and of fluorescent probes bound specifically to different sites on actin, myosin, or tropomyosin in muscle fibers was measured. The results show that the transition of actomyosin complex from the weak to the strong-binding state is accompanied by a change in the orientation of F-actin subunits with the C and N termini moving opposite to a large part of the subunit. Myosin light chains and some areas in the 20-kDa domain of myosin head move in the same direction as the C- and N-terminal regions of actin. It is established that troponin, caldesmon, calponin, and myosin systems of regulation of muscle contraction modify intramolecular actomyosin rearrangements in a Ca^{2+}-dependent manner. The role of intramolecular movements of contractile proteins in muscle contraction is discussed.

KEY WORDS: Actin, Myosin, Tropomyosin, Actin–myosin interaction, Muscle contraction, Polarized fluorescence.

I. Introduction

There are several types of molecular motors in eukaryotic cell which can convert chemical energy into mechanical movement. Actomyosin, the complex of myosin and actin, is the most widely distributed of these molecular motors and works in skeletal, cardiac, and smooth muscle. The basic mechanism of force production is very similar in all these muscle types.

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Muscle contraction has been known to be based on interactions of myosin filaments with actin filaments and ATP (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954). There are sufficient grounds to believe that in the force-generation process, the myosin cross-bridges go through several stages, the most significant of which are the so-called "strong" and "weak" forms of myosin binding to actin (Lymn and Taylor, 1971; Stein *et al.,* 1979; Geeves, 1992). The strong binding (the AM and AM–ADP stages of the cycle of ATP hydrolysis with actomyosin) is characterized by a high myosin affinity for actin (Marston and Weber, 1975; Margossian and Lowey, 1978; Greene and Eisenberg, 1980a), a slow kinetics of myosin head attachment to and detachment from actin filaments (White and Taylor, 1976; Marston, 1982), and a capability of regulated actin for cooperative activation (Bremel and Weber, 1972; Greene and Eisenberg, 1980b).

On the contrary, the weak binding (the AM–ATP and AM–ADP–P_i stages of ATP hydrolysis cycle) is characterized by a low affinity of myosin to actin (Stein *et al.,* 1979; Chalovich *et al.,* 1981), a fast kinetics of the myosin head attachment to and detachment from actin (Lymn and Taylor, 1971; Stein *et al.,* 1979; Chalovich *et al.,* 1981), and a lack of capability for the cooperative activation of regulated actin filaments (Chalovich *et al.,* 1983).

Based on these differences, it was suggested that the structure of the "weakly" associated actomyosin complex differed substantially from the structure of the "strongly" associated actomyosin complex and that force generation resulted from structural alterations produced by a transition from weak to strong binding in the actomyosin complex (White and Taylor, 1976; Eisenberg and Hill, 1985; Chalovich, 1992).

Participation of the structural changes of myosin cross-bridges in the force-generation mechanisms has been reliably demonstrated (Tokunaga *et al.,* 1991; Cooke, 1995). On the other hand, some facts also indicate participation of actin conformational changes in the mechanisms of muscle contraction. Involvement of changes in actin filament flexibility in force generation was suggested based on *in vitro* experiments more than 20 years ago (Oosawa *et al.,* 1972). Subsequently it was possible to show that the interaction of the myosin head with actin, apart from changes in actin filament flexibility (Oosawa, 1983; Egelman, 1985), produced a shift of some areas within the actin subunit, particularly movement of the small domain (Popp *et al.,* 1991). Actin conformational changes are of a clearly cooperative character (Oosawa *et al.,* 1972; Orlova *et al.,* 1995; Egelman and Orlova, 1995a,b; Orlova and Egelman, 1993, 1997).

This review summarizes the data obtained by polarized fluorescence measurements both in UV and in visible light that indicate an important role of intramolecular movements of actomyosin polypeptide chains in mechanisms of muscle contraction.

II. Polarized Fluorescence of Muscle Fibers

Among various optical approaches for investigation of the structure and properties of macromolecules, the study of their polarized fluorescence is important. The polarized fluorescence analysis allows the determination of volume, shape, and rigidity of macromolecules and the study of processes of intramolecular energy transfer, the effect of the microenvironment on the source of emission, the mechanisms of intra- and intermolecular interaction, conformational changes in molecules, etc. Usually the polarization techniques deal with isotropic systems such as solutions of dyes and proteins. However, there are some studies indicating that the polarized fluorescence technique can also be used for solving many problems regarding studying the structure and properties of macromolecules in such anisotropic systems as muscle fiber. A particular advantage of this approach is that information may be obtained without disrupting the integrity of the object studied.

A. Analysis of Polarized Fluorescence of Muscle Fibers

Fluorescent probes located in thin or thick filaments of a muscle fiber repeat a strictly ordered spatial arrangement of the contractile proteins and of the corresponding amino acid residues to which they are bound. The anisotropic arrangement of the fluorophores gives rise to the polarized fluorescence of the muscle fiber. From an analysis of the polarized fluorescence, the arrangement and motility of the fluorescent probes in an average molecule of the protein can be determined. Changes in the polarized fluorescence are considered to be an indication of conformational changes in the proteins containing the fluorescent probe.

The conformational changes of proteins in a muscle fiber are usually described using mathematical modeling of experimental data. For this purpose, a model-dependent system of analysis is used (Kaulin, 1968a; Rozanov *et al.,* 1971; Tregear and Mendelson, 1975; Yanagida and Oosawa, 1978; Wilson and Mendelson, 1983; Morales, 1984; Andreev *et al.,* 1995; Irving, 1996). The muscle fiber is assumed to be a cylindrical system with the symmetry axis along the fiber. The polarized fluorescence of the fiber is well described by a model, suggesting the presence of two populations of fluorophores—oriented and chaotically arranged. The oscillators of the oriented fluorophores are thought to be arranged along the forming surface of the cone, whose axis coincides with the fiber axis. Absorption and emission of light are operated by linear (i.e., completely anisotropic) oscillators. The orientation of the oscillators relative to the fluorophores is constant.

Let us accept that the fluorescence-exciting light passes along the OY axis (Fig. 1a). The symbols $\parallel$ and $\perp$ designate parallel and perpendicular components of this light. The fiber is arranged along the OZ axis, and the thin filament lies parallel the OW axis. $\Theta_{1/2}$ is the angle between the fiber axis and thin filament axis. Because the thin filament is flexible (Fujime and Ishiwata, 1971; Oosawa *et al.*, 1972; Yanagida and Oosawa, 1978; Oosawa, 1980), $\Theta_{1/2}$ is not equal to zero. In the coordinate system UVW, oscillators are located along the forming contour of the cone whose axis coincides with the axis OW (Fig. 1b). Oscillators of absorption (A) and emission (E) form the angles Φ_A and Φ_E, respectively, at the cone apex. γ is the angle between the oscillators A and E. Next, the angle values Φ_A, Φ_E, and $\Theta_{1/2}$ and the relative amount of the chaotically arranged fluorophores (N) may be calculated from measurements of intensities of four components of the fiber polarized fluorescence when the fiber is oriented parallel ($_{\parallel}I_{\parallel}$, $_{\parallel}I_{\perp}$) and perpendicular ($_{\perp}I_{\perp}$, $_{\perp}I_{\parallel}$) to the polarization plane of the exciting light (Tregear and Mendelson, 1975; Yanagida and Oosawa, 1978; Kakol *et al.*, 1987).

Changes in the parameters of polarized fluorescence (Φ_A, Φ_E, $\Theta_{1/2}$, and N) provide information about the alteration in the protein conformation. Changes in the angles Φ_A and Φ_E reflect rearrangement of the region of the polypeptide chain containing fluorophore. Changes in $\Theta_{1/2}$ and N characterize the thin filament flexibility [described by the module of elastic-

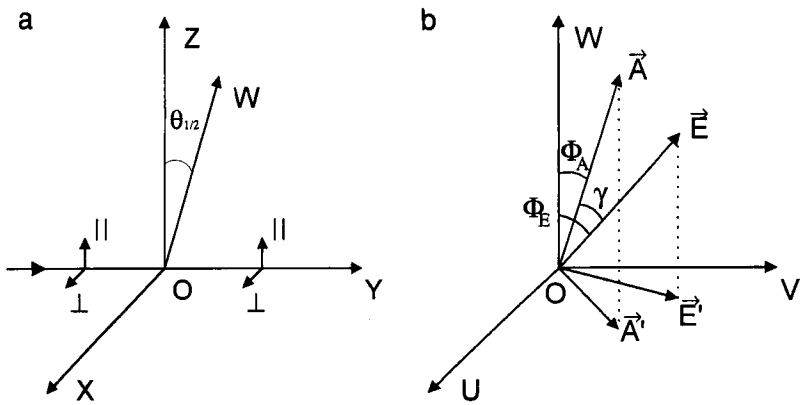

FIG. 1 Diagram of angle coordinates of thin filament (a) and dipoles of absorption (OA) and emission (OE) of the fluorophore (b). The light of excitation and fluorescence spreads along the OY axis. The actin filament axis (OW) deviates from the long axis of the muscle fiber (OZ) by the angle $\Theta_{1/2}$. Oscillators of absorption (A) and emission (E) are arranged along the forming cone surface, whose axis coincides with the axis OW and forms at the apex of the cones angles Φ_A and Φ_E, respectively. γ, angle between axes of the absorption oscillator and emission oscillator.

ity of the thin filament (Yanagida and Oosawa, 1978)] and flexibility of the area of the polypeptide chain containing fluorophore, respectively. Because the character of changes in Φ_A usually does not differ from the corresponding changes in Φ_E, the Φ_A values are not presented in this review.

B. Simulation of Different Stages of the ATP Hydrolysis Cycle in Polarized Fluorescence Investigations

In many works, muscle fibers free of myosin and the regulatory proteins (ghost muscle fibers) were used to study conformational changes in actin. Thin filaments of these fibers were intact (Borovikov and Gusev, 1983) or reconstructed from G-actin modified preliminarily with a fluorescent dye (Borovikov *et al.,* 1988d; Khoroshev *et al.,* 1989; Khoroshev and Borovikov, 1991; Khoroshev, 1992).

Two different states of the actomyosin complex in the fiber, the weak and the strong binding, were simulated when decorating actin filaments with myosin heads [myosin subfragment 1 (S1) or heavy meromyosin (HMM)] that were modified by a bifunctional reagent, pPDM (Borovikov *et al.,* 1981b, 1986a, 1990c). The conditions were chosen when contacts occurred between two of the three reactive myosin SH-groups, namely, between Cys707 and Cys697 and between Cys697 and Cys540. In the first case, an intermediate stage of the ATPase reaction, the actomyosin–ATP complex (AM–ATP or AM–ADP–P_i), was modeled. The myosin head acquired the capability to bind to actin in the so-called "weak-binding" form (Chalovich *et al.,* 1983). In the second case, an intermediate stage of the ATPase reaction of AM–ADP was modeled, and the myosin heads acquired the capability to form the "strong-binding" form with actin (Chaussepied *et al.,* 1988).

In some experiments, the strong-binding form was also modeled by formation of the so-called rigor complex (stage AM) between myosin head and actin. Actin filaments were decorated in the absence of nucleotide or in the presence of ADP by heads of myosin whose Cys707 was modified or not modified by NEM (Borovikov *et al.,* 1986a, 1996a).

C. Application of the Polarized Fluorescence Technique for Studying Muscle Contraction

From the first studies of polarized fluorescence of muscle fibers that were performed both in UV (Barsky *et al.,* 1968; Aronson and Morales 1969; Borovikov *et al.,* 1971) and in visible light (Kaulin, 1968a,b), this method was shown to be promising for the study of the molecular mechanisms of

muscle contraction. Subsequent measurements of polarized intrinsic tryptophan fluorescence of contractile proteins of rabbit skeletal muscle fiber (Dos Remedios *et al.,* 1972), thin and thick filaments of the giant muscle fiber of *Balanus rostratus* (Borovikov *et al.,* 1972, 1974; Borovikov and Chernogriadskaia, 1979; Borovikov, 1980), and fluorescent dyes located in myosin heads (Kaulin and Golfand, 1970; Nichei *et al.,* 1974; Tregear and Mendelson, 1975; Borejdo and Putnam, 1977; Borejdo *et al.,* 1979) or in F-actin (Yanagida and Oosawa, 1978, 1980; Borovikov *et al.,* 1982; Prochniewicz-Nakayama *et al.,* 1983) of skeletal muscle fiber have revealed that conformational changes of these proteins occur at different functional states of the fiber.

Studies of the fluorescence polarization of dyes dealt mainly with elucidation of myosin cross-bridge capability for changing their orientation during muscle contraction. Changes in spatial arrangement and motility of myosin cross-bridges were shown to occur in transition from relaxation to rigor or contraction (Nichei *et al.,* 1974; Tregear and Mendelson, 1975; Borejdo and Putnam, 1977; Borejdo *et al.,* 1979; Wilson and Mendelson, 1983). However, there were no dramatic changes in the orientation of myosin heads (Yanagida, 1981, 1984, 1985).

Analysis of polarized tryptophan fluorescence in isotropic discs of *B. rostratus* giant sarcomeres (Borovikov *et al.,* 1972, 1974; Borovikov and Chernogriadskaia, 1979; Borovikov, 1980) and in muscle fibers that contain mainly actin filaments (ghost muscle fibers) revealed cooperative conformational changes in F-actin. These changes were shown to play an important role in force generation and mechanisms of regulation of the actin–myosin interaction (Borovikov *et al.,* 1981b, 1982, 1986a,b; Borovikov and Gusev, 1983; Borovikov and Karandashov, 1983; Borovikov, 1984a,b, 1985; Borovikov and Levitsky, 1984, 1985; Borovikov and Lebedeva, 1987; Levitsky *et al.,* 1990).

Interesting information on molecular mechanisms of muscle contraction was obtained recently by studying polarized fluorescence of probes located in different areas of the actin subunit (Borovikov *et al.,* 1988d, 1991a,b; Nowak *et al.,* 1991; Borovikov and Kakol, 1991; Borovikov, 1992; Khoroshev, 1992), myosin heavy chains (Miyanishi and Borejdo, 1989; Ajtai and Burghardt, 1987, 1989, 1995; Ajtai *et al.,* 1994; Borovikov *et al.,* 1991b; Tanner *et al.,* 1992; Andreev and Borejdo, 1992, 1995; Andreev *et al.,* 1993, 1995; Burghardt and Ajtai, 1994; Berger *et al.,* 1995, 1996), myosin light alkali chains (Borovikov *et al.,* 1991b; Levitsky *et al.,* 1991), and regulatory chains (Ling *et al.,* 1996; Allen *et al.,* 1996) as well as in tropomyosin (Borovikov *et al.,* 1989b, 1990b, 1993; Szczesna *et al.,* 1989).

Thus, it has been shown that when the conformational state of actomyosin complex shifts from weak to strong binding a marked change in orientation

of the subdomain-1 area, including C- and N-terminal regions, relative to other areas of the actin subunit takes place (Borovikov *et al.*, 1988d, 1991a,b; Borovikov and Kirillina, 1991, 1992; Borovikov and Kakol, 1991; Borovikov, 1992; Khoroshev, 1992). Spatial rearrangements of the actin polypeptide chain are accompanied by intramolecular movements in the heavy and light chains of myosin. Regulatory proteins control actomyosin intramolecular movements in a Ca^{2+}-dependent manner (Nowak *et al.*, 1989, 1991; Borovikov *et al.*, 1990a, 1993, 1996a,b; Borovikov and Kakol, 1991; Borovikov, 1992; Khoroshev, 1992; Khoroshev *et al.*, 1995). These and other facts that will be considered later support the view that intramolecular movements of actomyosin polypeptide chains play an essential role in the molecular mechanisms of muscle contraction.

III. Changes in Actin Conformation Initiated by the Actin–Myosin Interaction

It was shown that modeling of the strong and weak binding of myosin heads to actin in muscle fiber was accompanied by changes in polarized fluorescence of both actin tryptophan residues and fluorescent probes (ε-ADP, rhodamine–phalloidin, FITC, rhodamine–maleimide, fluorescein–maleimide, 1,5-IAEDANS, 5-IAF, and NBD-Cl) that were specifically bound to different areas of this protein. The type of changes in the fluorescent parameters depended on the fluorophore location and the structural–functional state of the actomyosin complex (Yanagida and Oosawa, 1978; Borovikov *et al.*, 1981b, 1982, 1986a, 1990c, 1991a,b, 1996a,b; Borovikov and Gusev, 1983; Borovikov, 1985, 1992; Borovikov and Kakol, 1991; Borovikov and Kirillina, 1991, 1992; Khoroshev, 1992; Khoroshev *et al.*, 1995). The evidence indicated that the actin–myosin interaction was accompanied by alterations of actin structure that took place in different areas of the actin subunit.

A. Movements of the Polypeptide Chain in the Central Part of the Actin Subunit

Movements of actin polypeptide chain were studied in ghost muscle fibers, with their thin filaments intact or modified by fluorescent probes (see Section II). The value of the angle Φ_E between emission oscillator and the thin filament axis, the value of the angle $\Theta_{1/2}$ between the fiber axis and the thin filament axis, and the relative amount of the chaotically arranged

fluorophores (N) localized in actin were found to change markedly when modeling different stages of the ATP hydrolysis cycle.

The following regularity was noticed: In the case of tryptophan, rhodamine–phalloidin, ε-ADP, NBD-C1, and FITC [i.e., the fluorophores located in the central part (core) of the thin filament], formation of the strong-binding state of actomyosin complex (the AM or AM–ADP stages) was accompanied by a decrease in the angles between the emission oscillators of the probes and the filament axis, i.e., by a decrease in Φ_E values (Tables I and II) and by an increase in the actin filament flexibility as the value of the angle $\Theta_{1/2}$ increased (Table II). On the contrary, the weak binding of myosin to actin (stages AM–ATP or AM–ADP–P_i) resulted in an increase in the angle Φ_E of the same fluorophores and a decrease of the thin filament flexibility; th angle $\Theta_{1/2}$ decreased (Table II).

Hence, in the strong binding of actin to myosin (at the AM or AM–ADP stages), flexibility of the thin filament is higher, whereas the angles of the fluorophore orientation are lower than the corresponding values of the parameters characteristic of the weak binding (of the AM–ATP or AM–ADP–P_i stages). In other words, actin subunits can be present in the actomyosin complex in at least two structural–functional states that differ from each other in the orientation of various areas of the actin polypeptide chain and in rigidity of actin–myosin bonds.

The previously mentioned fluorescent probes are located at different sites in actin. Thus, FITC is bound to Lys61 (Miki, 1989, 1991), i.e., it is located in the subdomain 2 (Kabsch and Holmes, 1995); ε-ADP is located

TABLE I

Effect of Strong Binding between S1 and F-Actin on Fluorescence Polarization Parameters of Probes Located in Different Areas of the Actin Subunit

Fluorophore	Site	$\Delta\Phi_E$[a]	ΔN[a]
Tryptophan	Try340, Try356 (subdomain 1)	−1.5	0.24
Rhodamine–phalloidin	Between three adjacent subunits	−1.6	0.06
ε-ADP	Interdomain cleft	−2.1	0.06
FITC	Lys61 (subdomain 2)	−1.2	0.03
1,5-IAEDANS	Cys374 (subdomain 1)	7.4	−0.15
Rhodamine–maleimide	Cys374 (subdomain 1)	2.6	−0.13
Fluorescein–maleimide	Cys10 (subdomain 1)	6.3	−0.21
5-IAF	Cys10 (subdomain 1)	11.6	−0.14
DASM	Cys374 (subdomain 1)	8.6	−0.36
NBD-C1	Lys373 (subdomain 1)	1.0	−0.13

[a] $\Delta\Phi_E$ and ΔN are calculated as the difference between the corresponding values after and before actin decoration by S1.

TABLE II

Effect of the Strong or Weak Binding between Myosin Subfragment 1 (S1) or Heavy Meromyosin (HMM) and Action on Fluorescence Polarization Parameters of Rhodamine–Phalloidin and 1,5-IAEDANS Bound to Actin

	State of actomyosin			Fluorophores: Rhodamine–phalloidin-labeled actin		Fluorophores: 1.5-IAEDANS-labeled actin			
F-actin decorated by	AM-ATP-Pi	AM-ADP	AM	Φ_E	$\Theta_{1/2}$	Φ_E	N	β_1	Reference
S1				40.1	13.4	54.4	0.28	14.3	Borovikov *et al.* (1990c)
	+			41.3	12.5	53.2	0.39	12.9	
		+		39.8	14.3	55.9	0.16	16.1	
			+	39.3	15.5	57.3	0.10	18.0	
HMM				42.0	13.6	53.7	0.33	11.7	Borovikov *et al.* (1991a),
	+			42.6	12.7	53.3	0.49	10.7	Borovikov and Kirillina
		+		40.8	14.4	54.5	0.25	13.7	(1992)
			+	40.5	15.3	56.6	0.08	16.1	

in the cleft between the two protein domains (Holmes *et al.*, 1993), whereas rhodamine–phalloidin is most likely located near the filament axis and bound to the sites of contacts between three adjacent subunits (Lorenz *et al.*, 1993). Hence, at the actin–myosin interaction, the cleft and the areas of actin–actin contacts, including the loop between residues 61 and 65 (loop 61–65), shift in the same direction.

With respect to tryptophan residues of actin, there are no definite data indicating which of them are fluorescent in F-actin. Two of the four tryptophan residues (Try340 and Try356) are supposed to be fluorescent (Kuznetsova *et al.*, 1996). These residues have a high motility (Kuznetsova *et al.*, 1996) and are located in the hydrophobic microenvironment (Vedenkina *et al.*, 1972; Kuznetsova *et al.*, 1996) in subdomain-1 (Kabsch and Holmes, 1995). If the previously mentioned tryptophan residues are fluorescent in thin filaments, it may be that in the strong binding, the area of the subdomain-1 polypeptide chain, which contains these tryptophan residues, moves in the same direction as the other regions of actin discussed previously.

Because the changes in the disposition of actin subunit sites located at a significant distance from each other seem to be similar, it may be assumed that the transition from the weak binding of myosin heads to actin to strong binding is accompanied by changes in orientation of the entire actin subunit or of its larger relative to the thin filament axes. The results of recent electron microscopic and X-ray diffraction studies can be interpreted as supporting the previous assumption (Orlova and Egelman, 1993, 1997; Popp *et al.*, 1991).

It was shown that the actin subunit seems to have the polypeptide chain areas whose changes in spatial organization, which are initiated by the actin–myosin interaction, differ from those described previously. This concerns, first of all, the C and N termini of the actin polypeptide chain.

B. Movements of the C- and N-Terminal Areas of the Actin Subunit

It is established that the angle Φ_E for the fluorescent probes located in the C- or N-terminal areas of the actin polypeptide chain and bound specifically to Cys374 (1,5-IAEDANS, rhodamine–maleimide, and fluorescein–maleimide), Lys373 (NBD-C1), or Cys10 (fluorescein–maleimide and 5-IAF) increases at the strong binding (Table I) and decreases at the weak binding (Table II). These changes of the angle Φ_E are opposite those observed in the corresponding conditions for tryptophan, ε-ADP, FITC, or rhodamine–phalloidin located in the actin filament core. This indicates

that movements of the C- and N-terminal regions of the actin polypeptide chain do not seem to coincide with the movements of the larger part of the actin subunit (Borovikov *et al.*, 1991b; Borovikov, 1992; Khoroshev, 1992).

Indeed, the angle β_1 between oscillators of the fluorophores located in the C-terminal area and oscillators of the fluorophores located in other actin regions changes significantly during modeling of different stages of the ATP hydrolysis cycle, especially the angle between the emission oscillators of the 1,5-IAEDANS bound to Cys374 and rhodamine–phalloidin (Borovikov *et al.*, 1990c, 1991a,b; Borovikov and Kirillina, 1991, 1992; Borovikov, 1992).

The following regularity is observed: The β_1 value is higher in modeling of the AM stage and lower in modeling of the AM–ATP stage. In the AM–ADP stage, the β_1 value is lower than that in the AM stage but higher than that in the AM–ATP stage (Table II). Hence, the transition from weak to strong binding is accompanied by a movement of the larger part of the actin subunit relative to the thin filament axes, with this shift being opposite to that of subdomain-1.

The shift of the C- and N-terminal areas of the actin subunit can be greater than the value from the data referred to previously, if one assumes that the changes in the β_1 value are a result of the movement of subdomain-1 or its part in the plane perpendicular to the thin filament. Changes in the spatial arrangement of the actin C terminus are postulated in the literature (Miki, 1991; Ostap and Thomas, 1991; Orlova and Egelman, 1995).

The C terminus is likely to be located in close vicinity to the myosin strong-binding site on actin (Reisler, 1993; Dos Remedios and Moens, 1995; Milligan, 1996). Thus, the subdomain-1 shift initiated by the actin–myosin interaction may occur due to conformational changes of some superficial areas of the myosin-binding site on actin as a result of the "placement" of the myosin head in these actin areas. The data obtained in studying the motility of the fluorescent probes located in the actin C-terminal area do not contradict this suggestion. It was shown that the fluorophore motility (estimated from the N value) in the protein C-terminus area (e.g., the motility of 1,5-IAEDANS connected with Cys374) was essentially lower in the strong rather than in the weak binding of myosin with actin (Table II).

Hence, in the strong binding, the myosin head occupies a position on actin which restricts freedom of displacement of the protein C-terminal area; this is not observed in the weak binding of myosin with actin.

C. Flexibility of Different Areas of the Actin Polypeptide Chain

The flexibility of different areas of the actin subunit in thin filament of muscle fiber seems to be different. This is indicated by marked differences

in the N values for the fluorophores located at different regions in actin. The following regularity is observed: Fluorophores in the central part (the core) of the thin filament are characterized by a relatively low motility and small changes in orientation in binding with myosin compared with the corresponding changes observed for fluorophores in the peripheral thin filament areas. Thus, the relatively low motility and small orientation changes initiated by interaction with myosin were revealed for ε-ADP (the fluorophore was located in the cleft of the actin subunit) and for rhodamine–phalloidin (the probe was most probably located quite near the thin filament axis). On the contrary, high motility and pronounced orientation changes were shown for the actin C- and N-terminal areas (the probe was bound to Cys374, Lys373, or Cys10) and for the loop 61–65 (the label was bound to Lys61), with the flexibility of C- and N-terminal areas being higher than that of the loop 61–65 (Borovikov *et al.,* 1991b; Borovikov, 1992; Khoroshev, 1992). Hence, different areas of the actin subunit in the thin filament differ from each other in their motility. The peripheral subunit areas, subdomain-1 and subdomain-2, have the highest motility. The shift in the S1 seems to result from the intramolecular movements of the heavy chains in myosin head (Botts *et al.,* 1989; Johnson *et al.,* 1991).

IV. Conformational Changes in Myosin Initiated by Its Interaction with Actin and Nucleotide

Myosin interaction with actin and nucleotide was shown to induce the conformational changes in myosin heavy chains (Miyanishi and Borejdo, 1989; Ajtai and Burghardt, 1987, 1989, 1995; Ajtai *et al.,* 1994; Borovikov *et al.,* 1991b; Tanner *et al.,* 1992; Andreev and Borejdo, 1992, 1995; Andreev *et al.,* 1993, 1995; Burghardt and Ajtai, 1994; Berger *et al.,* 1995, 1996), light alkali chains (Borovikov *et al.,* 1991b; Levitsky *et al.,* 1991), and regulatory chains (Ling *et al.,* 1996; Allen *et al.,* 1996).

A. Movements in Myosin Heavy Chain

Changes in the structural state of the myosin head in the actomyosin complex of the muscle fiber, which are initiated by the transition of actomyosin complex from the AM to the AM–ADP stage, are accompanied by a decrease in the angle Φ_E of the fluorophores bound specifically to Cys707 or Cys697. The angle β_2, formed by emission oscillators of 1,5-IAEDANS

bound to myosin Cys707 or Cys697 and of rhodamine–phalloidin located in actin, decreases in the presence of Mg–ADP (Table III).

In a similar way, changes also occur in angle β_2 in transition of the glycerinated muscle fiber from the rigor to the relaxed state or in its contraction, and the range of β_2 changes correlate with the tension developed by the muscle fiber. Large changes in β_2 in the course of fiber contraction correspond to the high force developed by the fiber (Borovikov *et al.,* 1991a; Borovikov and Kakol, 1991).

B. Movements of Myosin Light Chains

Actin–myosin interaction was shown to be accompanied by marked changes of polarization parameters of fluorophores that were located at a significant distance from actin, for instance, those located in myosin alkali light chains A1 (the fluorophore was connected with Cys177) or in A2 (the fluorophore was bound to Cys136) (Borovikov *et al.,* 1991b; Borovikov, 1992). In this case, the transition of the actomyosin complex from the AM–ADP to the AM stage was also accompanied by a decrease in angles Φ_E and β_2 (Table III).

Recently, movements of regulatory light chains of myosin at muscle contraction have also been revealed (Irving *et al.,* 1995; Allen *et al.,* 1996; Berger *et al.,* 1996; Ling *et al.,* 1996). In these experiments the value of angle Φ_E was shown to be higher in stage AM than in stage AM–ATP

TABLE III

Effect of the Strong Binding between Myosin Subfragment 1 (S1) and F-Actin on Fluorescence Polarization Parameters of 1,5-IAEDANS Bound Specifically to the Heavy (Cys707 or Cys697) and Alkali Light Chains A_1 (Cys177) or A_2 (Cys136) of Myosin

State of actomyosin						
AM-ADP	AM	Site	Φ_E	N	β_2	Reference
+		Cys707	41.8	0.12	1.9	Borovikov *et al.* (1991a,b)
	+	Cys707	43.5	0.08	4.4	
+		Cys697	48.1	0.24	8.2	
	+	Cys697	50.0	0.20	10.9	
+		Cys177	58.0	0.16	18.1	Levitsky *et al.* (1991),
	+	Cys177	59.6	0.12	20.5	Borovikov *et al.*
+		Cys136	58.9	0.19	19.0	(1991a,b)
	+	Cys136	60.0	0.16	20.9	

(Ling *et al.,* 1996). Hence, the shift of the C- and N-terminal areas of actin subunit due to its interaction with myosin is accompanied by movement of some sites substantially different from those directly participating in the actin–myosin interaction. These sites include alkali and regulatory light chains and some areas in myosin heavy chain.

C. Flexibility of Myosin Polypeptide Chains

Flexibility of different areas of polypeptide chains in the myosin head seems to be different. This is indicated by different N values for the fluorophores located at different myosin sites. Thus, it was shown that in strong binding, the N value is different for the fluorophores located in myosin heavy and light chains. A relatively small flexibility was characteristic of fluorophores located in the light chains of myosin, A_1 and A_2 (the fluorophores were connected with Cys177 and Cys136, respectively), and in the 20-kDa domain of the heavy chain (the fluorophores were connected with Cys707 or Cys697; Table III). On the other hand, the myosin regulatory light chain had a high flexibility (Irving *et al.,* 1995; Allen *et al.,* 1996; Berger *et al.,* 1996; Ling *et al.,* 1996). It is interesting that the flexibility of A_2 was higher than that of A_1 (Table III). The lower A_1 flexibility compared with that of A_2 seemed to be due to restrictions of the A_1 movements in its "anchoring" on actin filaments at the AM stage (Levitsky *et al.,* 1991).

The transition of the actomyosin complex from the AM to the AM–ADP (Table III) or the AM–ADP–P_i stage is accompanied by a pronounced rise in the mobility of the fluorophores located in the myosin head. This is accounted for, first of all, by a high flexibility of the actin–myosin bonds at these stages of the ATP hydrolysis cycle (Borovikov *et al.,* 1991a).

Thus, myosin in muscle fiber can be in at least two different structural–functional states: the non-force-productive state (weak binding) and the force-productive state (strong binding) (Geeves, 1992). These states differ in spatial arrangement and flexibility of different areas of myosin heavy and light chains and in the character of myosin interaction with actin.

V. Regulation of Conformational Changes of Actomyosin by Thin and Thick Filament-Associated Regulatory Proteins

The conformational changes in myosin and actin can be modified in a Ca^{2+}-dependent manner by phosphorylation of myosin regulatory light chains (Borovikov *et al.,* 1982, 1986b, 1987, 1988a, 1989a; Borovikov and Karandashov, 1983; Borovikov and Levitsky, 1984, 1985; Kakol *et al.,* 1987; Szczesna

et al., 1987, 1989; Wrotek *et al.,* 1989; Borovikov and Kakol, 1991; Efimova, 1992; Efimova and Borovikov, 1995; Stepkowski *et al.,* 1995) and by special regulatory proteins of the thin filament (Borovikov *et al.,* 1976, 1988b,c,d, 1990a,b, 1996a,b; Galazkiewicz *et al.,* 1987; Dobrowolski *et al.,* 1988; Nowak *et al.,* 1989, 1991; Borovikov and Gusev, 1993; Solovyova *et al.,* 1995; Avrova *et al.,* 1996; Avrova, 1998).

A. Regulation of Actomyosin Conformation by Thin Filament Regulatory Proteins

It is believed that the thin filament proteins tropomyosin, troponin, caldesmon, and calponin produce conformational changes in actin that lead to activation or inhibition of strong binding between myosin and actin and enhance or restrict the movements of polypeptide chains in actin and myosin (Galazkiewicz *et al.,* 1987; Dobrowolski *et al.,* 1988; Nowak *et al.,* 1989, 1991; Borovikov *et al.,* 1996a,b; Solovyova *et al.,* 1995; Avrova *et al.,* 1996; Avrova, 1998).

Thus, it was shown that binding of the previously mentioned proteins to actin has a significant effect on spatial arrangement and motility of some areas in the actin polypeptide chain. It was established that angle β_1 between the emission oscillators of 1,5-IAEDANS specifically bound to the actin C terminus and the emission oscillator of rhodamine–phalloidin located near the F-actin axis markedly changes when the thin filaments contain the regulatory proteins. Calponin, caldesmon, and troponin-I were shown to increase angle β_1, whereas tropomyosin diminished it. In the presence of tropomyosin, β_1 changes ($\Delta\beta_1$) induced by caldesmon or troponin-I increased, whereas changes of this angle initiated by calponin decreased (Table IV).

Both of the dyes used were bound to the amino acid residues that were located in or close to the myosin-binding site of actin (Reisler, 1993; Dos Remedios and Moens, 1995; Milligan, 1996). Thus, the changes in angle β_1 can be taken as evidence of alterations in the configuration of this site, initiated by the regulatory proteins with tropomyosin-induced rearrangements of the site appearing to differ from those induced by caldesmon, calponin, and troponin-I (Nowak *et al.,* 1991).

Changes in the myosin site configuration significantly affect the character of the myosin head interaction with actin. Indeed, as indicated in Table IV, tropomyosin (from both skeletal and smooth muscles) activates strong binding (stage AM) and increases the relative amount of subunits that are switched on in the thin filament (Galazkiewicz *et al.,* 1987; Dobrowolski *et al.,* 1988; Nowak *et al.,* 1991). The activation is accompanied by changes in the values of β_1 (the angle between the emission oscillators of 1,5-

TABLE IV

Effect of Skeletal Muscle Tropomyosin, Smooth Muscle Tropomyosin, Troponin-I, Caldesmon, or Calponin on the Changes in the Relative Orientation of Fluorophores Attached to Myosin and/or Actin Induced by Strong Binding of S1 to F-Actin

Regulatory protein added	$\Delta\beta_1$[a]	$\Delta\beta_2$[a]	Reference
None	3.7	6.1	Nowak *et al.* (1989, 1991)
TN-I	2.9		
TMs	4.1	7.5	
TMs–TN-I	1.7		
CD	1.7	5.4	
TMg	5.1	7.5	
TMg–CD	1.1	4.6	
CN	0.5	4.1	Borovikov *et al.* (1996a,b)
TMg–CN	2.1	5.1	

Note. Abbreviations used: TMs, skeletal muscle tropomyosin; TMg, smooth muscle tropomyosin; TN-I, troponin-I; CD, caldesmon; CN, calponin.

[a] $\Delta\beta_1$ and $\Delta\beta_2$ are calculated as the difference between the corresponding values after and before thin filaments were decorated with S1.

IAEDANS and rhodamine–phalloidin attached to actin) and β_2 (the angle between the emission oscillators of 1,5-IAEDANS and rhodamine–phalloidin attached to myosin and actin, respectively). The changes in the values of β_1 and β_2 are larger in the presence of tropomyosin than in its absence (Table IV). Hence, the activation of strong binding is accompanied by enhancement in intramolecular movements in actin and myosin.

On the contrary, troponin, caldesmon, or calponin in the absence of Ca^{2+} (pCa $\geq$ 8) and calmodulin substantially diminish changes in angles β_1 and β_2, which are initiated by formation of the AM stage (Table IV).

The observed effects indicate that the regulatory proteins inhibit the formation of force-producing strong binding between actin and myosin (the AM stage), thereby restricting interdependent movements of some areas of the polypeptide chains both in actin and in myosin. Instead of strong binding, the myosin heads form weak or near weak binding in muscle fiber and switch actin monomers off (Nowak *et al.*, 1991; Borovikov *et al.*, 1996a,b). This effect is reversible and is easily eliminated in the presence of calcium (pCa $\leqq$ 6) and the corresponding Ca^{2+}-binding proteins. In the case of troponin-I, troponin-C is required, whereas in the case of caldesmon or calponin calmodulin is required. Tropomyosin diminishes the effect of calponin (Borovikov *et al.*, 1996a), whereas the effects of troponin-I and caldesmon are enhanced in the presence of this protein (Table IV; Nowak *et al.*, 1991; Borovikov *et al.*, 1996b).

Formation of the non-force-producing weak binding between actin and myosin is also strongly affected by the regulatory proteins (Borovikov *et*

al., 1996a,b). In this case, AM–ATP or AM–ADP–P_i stage formation in the presence of caldesmon and calponin leads to a marked fall in the β_1 value. This indicates an increase in a relative amount of switched-off actin subunits in the thin filaments. Thus, thin filament-associated regulatory proteins can modulate the character of the actin–myosin interaction and the intramolecular movements of actomyosin polypeptide chains. The effect of tropomyosin is substantially different from that of troponin-I, caldesmon, and calponin.

B. Myosin-Associated Regulation of the Actin Conformational State

The changes of actin conformation can also be modulated by changes in myosin structural state. Thus, in studying mechanisms of myosin-associated regulation of contraction in smooth (Solovyova *et al.,* 1995; Avrova *et al.,* 1996; Avrova, 1998) and skeletal muscles (Borovikov *et al.,* 1982, 1989a; Borovikov and Karandashov, 1983; Borovikov and Levitsky, 1984; Kakol *et al.,* 1987; Wrotek *et al.,* 1989; Borovikov, Kakol, 1991; Efimova, 1992; Efimova and Borovikov, 1995; Stepkowski *et al.,* 1995), the effect of myosin regulatory light chain phosphorylation and Ca^{2+} was revealed. These factors affect the character of intramolecular actin movements caused by the formation of strong and weak binding (the AM and AM–ADP–P_i stages, respectively).

When modeling the AM stage with smooth muscle myosin, dephosphorylation of regulatory light chains almost completely inhibits strong binding and thereby restricts the movements of the C-terminal area of the actin polypeptide chain relative to the rest of the molecule. This effect is reversible because the inhibition is eliminated by phosphorylation of the regulatory light chain in the presence of Ca^{2+} (Borovikov *et al.,* 1996a,b; Solovyova *et al.,* 1995; Avrova *et al.,* 1996).

In skeletal muscle, the effect of myosin light chain phosphorylation and the action of Ca^{2+} on actin intramolecular movements were also present. However, these effects were not pronounced. Dephosphorylation of the light chains only diminished the shift of the C-terminal area of actin polypeptide chain at low Ca^{2+} concentration, whereas phosphorylation of these chains, at the same concentration of Ca^{2+}, activated the movement of this area (Borovikov and Kakol, 1991).

VI. Tropomyosin Conformational Changes Initiated by the Actin–Myosin Interaction

Studies on polarized fluorescence of the probes bound to skeletal or smooth muscle tropomyosin have shown the actin–myosin interaction to be accom-

panied by conformational changes of tropomyosin (Borovikov *et al.*, 1989b, 1990b, 1993). These changes were modulated in a Ca^{2+}-dependent manner by thin filament regulatory proteins troponin and caldesmon (Borovikov *et al.*, 1993) and by phosphorylation of myosin regulatory light chains (Borovikov *et al.*, 1989a; Szczesna *et al.*, 1989).

A. Tropomyosin Movement Relative to Actin

It was shown that binding of myosin S1 to regulated actin filaments decreased Φ_E and N values (Table V). Hence, the actin–myosin interaction at the AM stage initiated conformational tropomyosin changes that produced changes in flexibility and spatial arrangement of this protein in a muscle fiber.

Regulatory proteins of the thin filament modulate tropomyosin conformational changes in a Ca^{2+}-dependent manner. In the absence of Ca^{2+} ($pCa \geq 8$), caldesmon and troponin markedly inhibit the tropomyosin conformational changes and associated movements of this protein because changes of the fluorescent parameters are decreased or even reversed in the presence of the regulatory proteins. The effect of the regulatory proteins is eliminated in the presence of Ca^{2+} and Ca^{2+}-binding proteins (Table V).

It is interesting to note that under the influence of Ca^{2+} and the regulatory proteins, tropomyosin shifts relative to some actin regions, whereas its position relative to other actin areas remains unchanged. Thus, angle β_3

TABLE V

Effect of Ca^{2+} and S1 on Fluorescence Polarization Parameters of 1,5-IAEDANS Bound Specifically to Skeletal (TMs) or Smooth Tropomyosin (TMg)

Addition	Ca^{2+}	S1	Φ_E	N	β_3	Reference
TMs	+, −	−	58.4	0.45	4.3	Borovikov *et al.* (1993)
TMs–TN	−	−	57.8	0.32	3.6	
TMs–TN	+	−	57.0	0.08	0.5	
TMs–TN	−	+	56.4	0.12	1.8	
TMs–TN	+	+	55.8	0.03	−1.8	
TMg	+, −	−	57.8	0.21	5.3	
TMg–CD	−	−	58.3	0.31	4.9	
TMg–CD–CM	+	−	57.7	0.25	3.0	
TMg–CD	−	+	58.0	0.19	2.3	
TMg–CD–CM	+	+	57.3	0.08	0.4	

Note. Abbreviations used: TN, troponin; CD, caldesmon; CM, calmodulin. β_3, angle between the emission oscillator of 1,5-IAEDANS bound to tropomyosin and the corresponding emission oscillator of rhodamine–phalloidin bound to actin.

between the emission oscillators of 1,5-IAEDANS bound to tropomyosin and rhodamine–phalloidin bound to actin essentially did not change in all experiments. This suggests that the effect of Ca^{2+} did not markedly change the spatial arrangement of tropomyosin relative to actin areas that contained the fluorescent probe rhodamine–phalloidin (Borovikov *et al.,* 1993).

Also, there was no substantial Ca^{2+} effect present on the interposition of the actin subdomain-2 and tropomyosin. The change in Ca^{2+} concentration from pCa 5 to pCa 7 had no significant effect on angle β_3, formed by the emission oscillators of FITC bound to actin Lys61 and of 1,5-IAEDANS bound to tropomyosin (Khoroshev, 1992).

In contrast, angle β_3, formed by the emission oscillators of the fluorescent probes bound specifically to the actin C terminus and to tropomyosin, changes markedly (Table V). This indicates that under the effect of Ca^{2+} and corresponding calcium-binding proteins (troponin-C or calmodulin) a shift of tropomyosin relative to the actin subdomain-1 (or at least of a tropomyosin area containing a fluorescent probe relative to the C terminus of actin) may take place in both skeletal and smooth muscle contraction.

Because the site of myosin strong binding is located in close vicinity to the C-terminal area of the actin subunit (Reisler, 1993; Dos Remedios and Moens, 1995; Milligan, 1996), the Ca^{2+}-dependent tropomyosin movement relative to this actin region can be considered as support for the hypothesis of the steric regulation of muscle contraction (Parry and Squire, 1973; Lehman *et al.,* 1995).

B. Change of Tropomyosin Flexibility in Thin Filaments

The shift of tropomyosin in regulated actin filaments is accompanied by a dramatic change in its flexibility. This is indicated by marked changes in the N value (Table V). Thus, in the presence of Ca^{2+} ($pCa \leq 6$), the N value decreases for skeletal muscle tropomyosin by more than nine times compared with the corresponding value in the absence of Ca^{2+} ($pCa \geq 6$).

Taking into account the higher (compared with F-actin) tropomyosin flexibility (Ishii and Lehrer, 1985) and the presence of bonds between actin and tropomyosin (Stewart and McLachlan, 1975), it is suggested that in the presence of Ca^{2+} such changes in tropomyosin conformation occur which are accompanied by an increase in the relative number of bonds between tropomyosin and actin. As a result, tropomyosin is switched on (Lednev, 1980; Chalovich, 1992) and forms the so-called strong binding with actin. In the absence of Ca^{2+}, however, tropomyosin is switched off (Lednev, 1980; Chalovich, 1992), the number of actin–tropomyosin bonds decreases, and weak binding is formed between tropomyosin and actin. With this weak binding, tropomyosin seems to shift to a position that

diminishes the probability of formation of strong binding between myosin and actin.

It is interesting to note that myosin S1 diminishes whereas caldesmon and troponin-I increase the *N* value (Table V). Hence, S1 activates whereas caldesmon and troponin-I inhibit the formation of strong binding between tropomyosin and actin.

C. The Effect of Myosin Light Chain Phosphorylation on Tropomyosin Conformation

The conformational changes in tropomyosin accompanying the actin–myosin interaction depend on the myosin structural state. This conclusion resulted from the study of the myosin-associated regulatory system of skeletal muscle (Borovikov *et al.,* 1989b; Szczesna *et al.,* 1989). Phosphorylation of myosin regulatory light chains (LC_2) at low Ca^{2+} concentrations activates the switching on of tropomyosin and the formation of strong binding between actin and tropomyosin. This is indicated by a decrease in angle Φ_E and the *N* value in the formation of the AM stage between the phosphorylated heavy meromyosin and actin. Hence, LC_2 phosphorylation activates strong binding between tropomyosin and actin, i.e., switches on tropomyosin.

In contrast, an increase in the Ca^{2+} concentration and LC_2 dephosphorylation eliminate this effect. Angle Φ_E and *N* values increase (Szczesna *et al.,* 1989). Weak binding seems to take place between tropomyosin and actin. Hence, the change in the myosin structural state in the actomyosin complex affects the tropomyosin conformation.

Thus, tropomyosin in the muscle fiber can be present in at least two different structural–functional states: the switched on and switched off states, which differ in their spatial arrangement and the character of interaction with actin.

VII. Modification of Actin Structure Inhibiting Changes in Actin Conformation Induced by Myosin

Several works using the polarized fluorescence technique studied the effects of actin structure modification on the character of actin conformational changes initiated by its interaction with myosin. The "freezing" of the actin structure by glutaraldehyde (Prochniewicz-Nakayama and Yanagida, 1982; Borovikov, 1984a,b) and phalloidin (Borovikov, 1984a,b; Borovikov *et al.,* 1984a), and actin cleavage by subtilisin and its alteration due to develop-

ment of denervational atrophy in muscle (Borovikov *et al.*, 1981a, 1983, 1984b, 1992; Borovikov, 1985; Kirillina *et al.*, 1992; Szczepanowska *et al.*, 1987, 1991, 1995, 1998) inhibit the conformational changes of actin initiated by the actin–myosin interaction.

A. Inhibition of Actin Conformational Changes by Phalloidin and Glutaraldehyde

Modification of the actin structure by phalloidin or glutaraldehyde was shown to inhibit conformational changes of the protein induced by formation of the strong-binding actomyosin complex. Thus, after actin modification by 0.05 or 0.5% glutaraldehyde, decoration of actin filaments by HMM or S1 (modeling the AM stage of the ATPase cycle) resulted in a slight or no change in the Φ_E and $\Theta_{1/2}$ parameters. Hence, glutaraldehyde inhibits the ability of actin to bind strongly to myosin (Borovikov, 1984a,b).

After actin modification by glutaraldehyde, the fibers reconstructed from the corresponding proteins change their length more slowly during their isometric contraction and develop only 30–50% of the isotonic tension compared with the untreated reconstructed fibers (Borovikov, 1985). Hence, inhibition of the actin ability to bind strongly to myosin correlates with the inhibition of the muscle fiber motor activity.

Similar conclusions were drawn after studying the effect of phalloidin on the character of the actin–myosin interaction and on tension developed by muscle fiber at the transition from relaxation to rigor (Borovikov, 1984a,b; Borovikov *et al.*, 1984a). In these experiments, inhibition of changes in the fluorescent parameters also correlated with a decrease in tension developed by the muscle fiber.

B. Subtilisin Cleavage of the DNAase-I Binding Loop Inhibits Intramolecular Movements in the Actin Subunit

Actin cleavage by subtilisin inhibits actin intramolecular movements (Borovikov *et al.*, 1998). Thin filaments were reconstructed from intact G-actin and G-actin cleaved by subtilisin at the DNAase-I binding loop (loop 38–52). It was shown that angle β_1, formed by the emission oscillators of the two dyes located at the periphery and in the core of actin filament (1,5-IAEDANS bound to Cys374 and rhodamine–phalloidin, respectively), differed significantly in the filaments, reconstructed from the intact and cleaved actin (Table VI).

Because the 38–52 loop is involved in the actin–actin contacts in thin filament and because this region of the subunit as well as the C terminus

TABLE VI

Effect of Subtilisin or Denervational Atrophy on Changes in the Relative Orientation of Fluorophores Bound to Actin Induced by Strong Binding of Myosin Subfragment 1 (S1) to F-Actin

Type of experiment	Addition of S1	$\Delta\beta_1$	Reference
Control[a]	None	12.0	Borovikov *et al.* (1998)
	+	19.7	
Subtilisin cleavage	None	6.8	
	+	8.4	
Control[b]	None	11.5	Szczepanowska *et al.* (1998)
	+	16.4	
Denervation (4 days)	None	9.8	
	+	8.0	

[a] The study was carried out on ghost fibers prepared from rabbit m. psoas.
[b] The study was carried out on ghost fibers prepared from mice m. soleus.

is close to the myosin binding site (Lorenz *et al.*, 1993; Mossakowska *et al.*, 1993; Tirion *et al.*, 1995), it may be concluded that the change in β_1 value reflects modification of the myosin site configuration by subtilisin (Borovikov *et al.*, 1998).

The configuration of this site seems to determine the character of actin–myosin interaction and the range of intramolecular movements in actin. As indicated in Table VI, actin modification by subtilisin leads to a dramatic inhibition of the β_1 changes, induced by myosin head binding to actin (i.e., formation of the AM stage). Hence, subtilisin cleavage of the loop 38–52 is accompanied by inhibition of actin intramolecular movements, resulting from the inhibition of strong binding between actin and myosin.

In addition, subtilisin cleavage of the loop 38–52 leads to a marked decrease in the affinity of myosin to actin, inhibits sliding of actin filaments over the proteolytic myosin fragment heavy meromyosin (Schwyter *et al.*, 1989, 1990), and decreases (by more than 60–70%) actin-activated Mg-ATPase activity of myosin S1 (Borovikov *et al.*, 1998). Thin filaments containing subtilisin-treated actin are more rigid than the intact filaments and the flexibility of the actin C terminus in the modified filaments is lower than that in controls (Borovikov *et al.*, 1998).

Thus, subtilisin cleavage of the loop 38–52 induces conformational changes in actin that inhibit the force-producing strong binding between actin and myosin and intramolecular movements in actin. Inhibition of the actin intramolecular movements is accompanied by a decrease in the actin-activated ATPase activity of myosin.

C. The Effect of Denervational Atrophy on Actin Intramolecular Movements

Development of denervational atrophy leads to both inhibition of the intramolecular movements in actin subunits during their interaction with myosin and a decrease in tension developed by the muscle fiber.

Thus, angle β_1, formed by emission oscillators of the fluorophores, rhodamine–phalloidin, and 1,5-IAEDANS, bound to actin has been shown to increase markedly in decoration of intact thin filaments by S1 (Table VI). Hence, the C-terminal area is shifted relative to other parts of the actin subunit. This effect is significantly inhibited in denervational atrophy development (4–14 days after denervation). Instead of an increase in angle β_1, a decrease occurs (Szczepanowska *et al.,* 1998; Table VI).

Denervational atrophy is also accompanied by a weakening in the muscle fiber contractile function. Such fibers develop only 50–70% of the isometric tension developed by the intact muscle fibers (Borovikov, 1985). Reinnervation leads to restoration of the fiber contractile properties. It is accompanied by recovery of actin's ability to change its conformation at strong binding (Borovikov *et al.,* 1992; Szczepanowska *et al.,* 1987, 1995, 1998). Hence, the restriction of the intramolecular movements in actin subunit initiated by an alteration of actin conformation at denervational atrophy are accompanied by the inhibition of the contractile function of the muscle fibers.

VIII. Concluding Remarks

Fluorescence polarization studies have shown that, in the ATP hydrolysis cycle, the actomyosin complex can be in at least two different structural–functional states referred to as non-force producing (weak binding of myosin to actin) and force producing (strong binding of myosin to actin). These states differ in the mode of actin–myosin interaction and in spatial orientation and flexibility of different areas in polypeptide chains of myosin head and actin subunit.

The actomyosin complex transition from weak to the strong binding is accompanied by a change in orientation of the actin subunit, including the area of the interdomain cleft (the site for ADP location), the loop between residues 61–65 in S2, and hydrophobic areas of S1, including Trp340 and Trp356. Peripheral C- and N-terminal areas of S1 move in the opposite direction relative to the core of the thin filament. The shift can be rather large given that the movements revealed by polarized fluorescence analysis

are projections of subdomain-1 movements on a plane perpendicular to the thin filament.

Change in mode of actin–myosin interaction in the course of ATP hydrolysis cycle is accompanied by spatial rearrangement of a number of regions in the myosin head. These rearrangements involve both heavy and light chains of this protein. It is interesting to note that areas of the myosin 20-kDa domain, including Cys707 and Cys697, and the myosin light chains (both alkali and regulatory) move in the same direction as do the actin C and N termini. The range of the shift of these parts in the actin subunit and myosin head correlates with the tension developed by the muscle fiber. The larger shift of the polypeptide chains corresponds to the larger force developed by the muscle fiber.

The freezing of actin and myosin structures by chemical reagents, actin cleavage by subtilisin, or an alteration of actin due to the development of denervational atrophy in the muscle fibers inhibit the intramolecular movements in actomyosin and thereby decrease or completely inhibit the capability of the muscle fiber to generate tension. The previous data indicate that it is possible that tension generation by muscle fiber is accompanied by changes in the interposition of at least some central and peripheral areas in actomyosin, including some regions of the myosin heavy chain, particularly those of the 20-kDa domain, and changes in the actin polypeptide chain, such as the formation of the interdomain cleft, the subdomain-2 loop between residues 61 and 65, the hydrophobic regions in subdomain-1 including Trp340 and Trp356, and C- and N-terminal areas of subdomain-1. Further studies are necessary to reveal the role of movement of each of the previously mentioned regions of actomyosin polypeptide chains in the mechanism of force production in muscle fiber. However, the movement of myosin domains and/or some areas of the polypeptide chains of the myosin head and the shift of the C- and N-terminal regions of the actin subunit relative to the actin core play an important role in this mechanism.

It is necessary to note that communication between different actomyosin areas can have an important role in force generation by muscle fiber. This conclusion is based on analysis of the results of experiments with subtilisin. Subtilisin cleavage of the DNAase-I binding loop inhibits shifting of actin S1 in actin interaction with myosin. It is accompanied by a decrease in myosin affinity to actin, actomyosin ATPase activity, and the velocity of thin filaments sliding over myosin. Hence, actomyosin polypeptide chain movement and myosin head turning in ATP hydrolysis cycle are possible only if there is communication between C and N termini located in subdomain-1 and the DNAase-I binding loop of subdomain-2.

Significant changes in flexibility of the actin filament on the whole and of different areas in the actomyosin polypeptide chains take place in ATP hydrolysis cycle. Thin filament flexibility was shown to increase sharply at the transition from weak to strong binding between myosin and actin. On the contrary, the rigidity of the C and N termini and of the bond between the myosin head and actin sharply increases. The high rigidity may be essential for force generation because it can play an important role in tension transmission from the periphery of the actin monomer to the head of myosin and then to its tail.

It should be emphasized that the thin filament becomes flexible in the force-producing strong-binding state. This observation, in my opinion, makes the proposed role of thin filament as a bearing for the cross-bridge, producing tension by a turn of the myosin head, less probable. It is more likely that only some areas of the actin subunit can be candidates for serving as a bearing at this stage. These are, for example, peripheral areas of subdomain-1 that, according to polarized fluorescence evidence, become much less flexible at AM and AM–ADP stages of the ATP hydrolysis cycle. Thin filament becomes rigid in the non-force-producing weak-binding state (AM–ATP stage). High rigidity of the thin filament and weak binding between actin and myosin seem to allow cross-bridges to slide along actin filaments at this stage of the ATP hydrolysis cycle.

The conformational changes of actomyosin in ATP hydrolysis cycle can take place in the following order: In the presence of ATP, weak binding is formed between the myosin head and actin. High flexibility of C- and N-terminal areas facilitates myosin head binding to actin. The formation of the complex AM–ATP (of the non-force-producing state) is accompanied by actomyosin conformational changes that bring about a change in the configuration of the areas participating in the interaction between actin and myosin (i.e., configuration of the sites of actin–myosin interaction changes). As a result, the myosin binding site on actin becomes capable of activating ATP hydrolysis. Phosphate release from ATP initiates strong binding (the force-producing state) and induces intramolecular movements of the polypeptide chains in actomyosin that provide movement of peripheral parts of the actin subunit and some areas of the myosin heavy chain, probably the whole myosin head, along the plane perpendicular to the thin filament.

Data indicate that the spatial organization of the C and N termini of actin and of some other peripheral actin areas determines the mode of actin interaction with myosin. The change in configuration of these areas seems to involve the regulatory mechanisms of muscle contraction. Indeed, it has been shown that thin filament-associated regulatory proteins tropomyosin, troponin, caldesmon, and calponin, in their interaction with actin,

induce a shift of C- and N-terminal areas relative to the central regions of the actin subunit. Because actin C- and N-terminal areas are involved in the myosin binding site or located in close proximity to it, the shift of subdomain-1 peripheral areas can be considered an indication of a change in configuration of this site.

Much biochemical evidence indicates that the regulatory proteins of thin filaments diminish myosin affinity for actin (Chalovich, 1992). Taking into account both these observations and fluorescent microscopy evidence, it is possible that one reason for the diminished myosin affinity for actin in the presence of regulatory proteins is a change of myosin binding site configuration on actin. In other words, the change of myosin binding site configuration hinders myosin heads from binding to actin.

Changes in configuration of the myosin binding site have been shown to modify actomyosin functional properties and affect the mode and range of the movement of actomyosin polypeptide chains in the transition from weak to strong binding. Thus, caldesmon, calponin, and troponin inhibit movement in the actin subunit and the shifts of the C and N termini initiated by the actin–myosin interaction. This is accompanied by inhibition of actomyosin ATPase activity, thin filament sliding velocity over myosin, and tension developed by muscle fibers (Chalovich, 1992). Hence, regulatory proteins are capable of affecting actomyosin contractile properties by inhibiting force-producing strong binding between actin and myosin.

Tropomyosin modulates the effects of the regulatory proteins. Thus, tropomyosin enhances the effects of caldesmon and troponin but diminishes those of calponin. Tropomyosin and other regulatory proteins changed the spatial arrangement of subdomain-1 peripheral areas and affected intramolecular movements of actomyosin polypeptide chains. Consequently, actomyosin contractile properties can be modified by regulatory proteins by changing myosin binding site configuration, in particular spatial arrangement of actin C- and N-terminal areas. Such modification of the myosin binding site results in inhibition of the force-producing strong binding between actin and myosin.

A similar mechanism seems to be employed in the myosin-associated system of regulation of muscle contraction. In this case regulation is also carried out by inhibiting strong binding formation. However, in this system myosin regulatory light chains play the key role. Thus, it was shown that change of conformation of these myosin chains, induced by their dephosphorylation and/or exchange of Ca^{2+} for Mg^{2+}, may lead to changes in actin binding site configuration that result in the formation of the nonforce-producing state instead of the force-producing state. This is accompanied by inhibition of intramolecular movements both in actin and in myosin and weakening of actomyosin contractile capacity, as it was observed under

the influence of the thin filament-associated regulatory proteins. Hence, molecular mechanisms of regulation of muscle contraction associated with different regulatory systems have much in common. In all cases, muscle contraction is regulated by inhibiting the strong-binding state (stage AM of the ATP hydrolysis cycle) in the muscle fiber.

Analyses of polarized fluorescence have also shown that both skeletal and smooth muscle tropomyosin can occur in muscle fiber in at least two structural–functional states that differ from each other in flexibility and interposition of tropomyosin and myosin binding site on actin. The thin filament-associated protein troponin, the caldesmon–calmodulin complex, and myosin regulatory light chains drive the conformational state of tropomyosin in a Ca^{2+}-dependent manner.

Thus, troponin makes the skeletal tropomyosin molecule rigid in the presence of Ca^{2+} ($pCa \leq 5$), whereas Ca^{2+} removal induces a tropomyosin shift relative to the myosin binding site and sharply increases the flexibility of the molecule. This sharp rise of skeletal tropomyosin flexibility, in my opinion, makes the proposed mechanical blocking of myosin attachment to actin by tropomyosin less probable. Indeed, it seems unlikely that the very flexible tropomyosin molecule could mechanically block myosin binding sites on actin. At low Ca^{2+} concentration, the troponin–tropomyosin complex induces conformational changes in actin that result in a change of myosin binding site configuration on actin. Such rearrangements, in my opinion, can not only diminish myosin affinity for actin but also change the mode of interaction of these proteins.

A slight shift of smooth muscle tropomyosin relative to actin and a sharp change in its flexibility occur under the effect of caldesmon. As with skeletal tropomyosin, caldesmon-initiated conformational changes in smooth muscle tropomyosin are accompanied by a change in spatial arrangement of actin C and N termini, i.e., by a change in myosin binding site configuration. This seems to lead to inhibition of strong binding between actin and myosin (AM stage) and weakening of actomyosin contractile functions. Hence, caldesmon-initiated tropomyosin conformational changes can inhibit muscle contraction by changing myosin binding site configuration on actin.

Thus, evidence obtained by polarized fluorescence technique suggests that in the process of muscle contraction intramolecular movements in contractile proteins occur which play an important role in the mechanisms of muscle contraction.

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References

Ajtai, K., and Burghardt, T. P. (1987). Probe studies of the Mg-ADP state of muscle cross-bridges: Microscopic and wavelength-dependent fluorescence polarization from 1,5-IAEDANS-labeled myosin subfragment-1 decorating muscle fibers. *Biochemistry* **26,** 4517–4523.

Ajtai, K., and Burghardt, T. P. (1989). Fluorescent modification and orientation of myosin sulfhydryl 2 in skeletal muscle fibers. *Biochemistry* **28,** 2204–2210.

Ajtai, K., and Burghardt, T. P. (1995). Conformation of xanthene dyes in the sulfhydryl 1 binding site of myosin 2. *Biochemistry* **34,** 15943–15952.

Ajtai, K., Toft, D. J., and Burghardt, T. P. (1994). Path and extent of cross-bridge rotation during muscle contraction. *Biochemistry* **33,** 5382–5391.

Allen, T. S., Ling, N., Irving, M., and Goldman, Y. E. (1996). Orientation changes in myosin regulatory light chains following photorelease of ATP in skinned muscle fibers. *Biophys. J.* **70,** 1847–1862.

Andreev, O. A., and Borejdo, J. (1992). Two different acto-S1 complexes. *J. Muscle Res. Cell Motil.* **13,** 532–533.

Andreev, O. A., and Borejdo, J. (1995). Binding of heavy-chain and essential light-chain 1 of S1 to actin depends on the degree of saturation of F-actin filaments with S1. *Biochemistry* **34,** 14829–14833.

Andreev, O. A., Andreeva, A. A., and Borejdo, J. (1993). Polarization of fluorescently labeled myosin subfragment-1 fully or partially decorating muscle fibers and myofibrils. *Biophys. J.* **63,** 1027–1038.

Andreev, O. A., Takashi, R., and Borejdo, J. (1995). Fluorescence polarization study of the rigor complexes formed at different degrees of saturation of actin filaments with myosin subfragment-1. *J. Muscle Res. Cell Motil.* **16,** 353–367.

Aronson, J. F., and Morales, M. F. (1969). Polarization of tryptophan fluorescence in muscle. *Biochemistry* **8,** 4517–4522.

Avrova, S. V. (1998). Regulation of actin–myosin interaction in smooth muscle by calponin. PhD thesis, Institute of Cytology, St. Petersburg.

Avrova, S. V., Borovikov, Yu. S., Horiuchi, K. Y., and Chacko, S. (1996). Conformational changes induced by phosphorylated and dephosphorylated heavy meromyosin in actin in "ghost" fiber are modulated by calcium. *Biophys. J.* **70,** A381.

Barsky, I. Ya., Rozanov, Yu. M., Chernogriadskaya, N. A., Shiffers, L. A., and Shudel, M. S. (1968). Polarized fluorescence of microstructures of biological objects. *Izvestia USSR Acad. Sci.* **32,** 1546–1549.

Berger, C. L., Craik, J. S., Trentham, D. R., Corrie, J. E., and Goldman, Y. E. (1995). Fluorescence polarization from isomers of tetramethylrhodamine at SH-1 in rabbit psoas muscle fibers. *Biophys. J.* **68,** 78S–80S.

Berger, C. L., Craik, J. S., Trentham, D. R., Corrie, J. E. T., and Goldman, Y. E. (1996). Fluorescence polarization of skeletal muscle fibers labeled with rhodamine isomers on the myosin heavy chain. *Biophys. J.* **71,** 3330–3343.

Borejdo, J., and Putnam, S. (1977). Polarization of fluorescence from single skinned glycerinated rabbit psoas fibers in rigor and relaxation. *Biochem. Biophys. Acta* **459,** 578–595.

Borejdo, J., Putnam, S., and Morales, M. F. (1979). Fluctuations in polarized fluorescence: Evidence that muscle cross-bridges rotate repetitively during contraction. *Proc. Natl. Acad. Sci. USA* **76,** 6346–6350.

Borovikov, Yu. S. (1980). Studies on structural changes of F-actin and myosin in living, intact and damaged muscle fibers by means of polarized ultraviolet fluorescence microscopy. *Microsc. Acta* **82,** 379–388.

Borovikov, Yu. S. (1984a). A study on structural changes in muscle contractile proteins by polarized ultraviolet fluorescent microscopy. VIII. Effect of phalloidin and glutaraldehyde on conformation of F-actin. *Tsitologiya* **26,** 1262–1266.

Borovikov, Yu. S. (1984b). The effect of glutaraldehyde and phalloidin on the conformation of F-actin. *Gen. Physiol. Biophys.* **3,** 513–516.

Borovikov, Yu. S. (1985). Polarizational–microfluorimetric study of muscle contraction. DSc thesis, Institute of Cytology, Leningrad.

Borovikov, Yu. S. (1992). The changes in disposition of various regions of actin monomer and myosin head during ATP hydrolysis cycle. *In* "Muscle as a Machine. Energy Transduction in the Contractile System" (M. Morales, ed.), pp. 30–32. National Institutes of Health, Bethesda, MD.

Borovikov, Yu. S., and Chernogriadskaia, N. A. (1979). Studies on conformational changes in F-actin of glycerinated muscle fibers during relaxation by means of polarized ultraviolet fluorescence microscopy. *Microsc. Acta* **81,** 383–392.

Borovikov, Yu. S., and Gusev, N. B. (1983). Effect of troponin–tropomyosin complex and Ca^{2+} on conformational changes in F-actin induced by myosin subfragment-1. *Eur. J. Biochem.* **136,** 363–369.

Borovikov, Yu. S., and Kakol, I. (1991). Conformational changes of contractile proteins accompanying modulation of skeletal muscle contraction. Polarized microfluorimetry investigations. *Gen. Physiol. Biophys.* **10,** 245–264.

Borovikov, Yu. S., and Karandashov, E. A. (1983). Effect of Ca^{2+} on conformation of actin in the F-actin-heavy meromyosin complex. *Biochem. Int.* **7,** 319–328.

Borovikov, Yu. S., and Kirillina, V. P. (1991). Effect of Mg-ADP on the structure of actin in the F-actin–myosin-subfragment 1 complex. *Tsitologiya* **33,** 68–75.

Borovikov, Yu. S., and Kirillina, V. P. (1992). Effect of Mg-ADP on structure of actin in F-actin–myosin subfragment 1 complex. *Basic Appl. Myol.* **2,** 169–174.

Borovikov, Yu. S., and Lebedeva, N. N. (1987). Amplitude of tension developed by glycerinated muscle fibers at rigorization depends on the structural changes in F-actin induced by formation of actomyosin complex. *Tsitologiya* **29,** 1192–1195.

Borovikov, Yu. S., and Levitsky, D. I. (1984). Conformational changes of F-actin induced by myosin subfragment-1: High sensitivity to Ca^{2+}. *Biochemistry (Moscow)* **49,** 767–771.

Borovikov, Yu. S., and Levitsky, D. I. (1985). Fluorescence polarization study on Ca^{2+}-sensitivity of conformational changes in F-actin induced by the formation of F-actin–subfragment-1 complex. *Gen. Physiol. Biophys.* **4,** 457–463.

Borovikov, Yu. S., Shudel, M. S., Chernogriadskaya, N. A. Rozanov, Yu. M., and Barsky, I. Ya. (1971). On changes in azimuth characteristics of ultraviolet polarized fluorescence of individual muscle fibers in stretching and contraction. *Doklady USSR Acad. Sci.* **196,** 962–964.

Borovikov, Yu. S., Rozanov, Yu. M., Barsky, I. Ya., Shudel, M. S., and Chernogriadskaya, N. A. (1972). A study of the polarized ultraviolet fluorescence of giant muscle fibers of Balanus rostratus. *Tsitologiya* **14,** 957–960.

Borovikov, Yu. S., Chernogriadskaya, N. A., and Rozanov, Yu. M. (1974). A study of structural changes in myosin and actin filaments of muscle fibre by the polarized ultraviolet fluorescence microscopy. *Tsitologiya* **16,** 977–982.

Borovikov, Yu. S., Bogdanova, M. S., Chernogriadskaya, N. A., Rozanov, Yu. M., and Kirillina, V. P. (1976). A study on structural changes in muscle contractile proteins by the polarized ultraviolet fluorescent microscopy. II. Effect of the troponin–tropomyosin complex on F-actin conformation in muscle fiber. *Tsitologiya* **18,** 1502–1505.

Borovikov, Yu. S., Kirillina, V. P., Moskalenko, I. E., and Strankfeld, I. G. (1981a). Changes of F-actin structure during muscle denervation. *Biophysica* **26,** 754–755.

Borovikov, Yu. S., Levitsky, D. I., Kirillina, V. P., and Poglazov, B. F. (1981b). DTNB-myosin light chain operates F-actin conformational reconstructions induced by myosin subfragment-1. *Doklady USSR Acad. Sci.* **259,** 732–735.

Borovikov, Yu. S., Levitsky, D. I., Kirillina, V. P., and Poglazov, E. F. (1982). Effect of Ca^{2+} binding to 5,5′-dithiobis (2-nitrobenzoic acid) light chains on conformational changes of F-actin caused by myosin subfragment-1. *Eur. J. Biochem.* **125,** 343–347.

Borovikov, Yu. S., Moskalenko, I. E., Kirillina, V. P., and Strankfeld, I. G. (1983). Study of the state of F-actin in denervated muscle fibre by polarized UV fluorescence microscopy. *Gen. Physiol. Biophys.* **2,** 1–11.

Borovikov, Yu. S., Bukatina, A. E., and Sonkin, B. Ja. (1984a). The effect of phallotoxins on the structure of F-actin in myosin-free ghost muscle fibres of rabbit. *FEBS Lett.* **176,** 441–443.

Borovikov, Yu. S., Moskalenko, I. E., Kirillina, V. P., and Strankfeld, I. G. (1984b). The detection of denervation-induced structural changes in actin by phalloidin. *Gen. Physiol. Biophys.* **3,** 277–280.

Borovikov, Yu. S., Kakol, I., Szczesna, D., Kirillina, V. P., and Levitsky, D. I. (1986a). Cross-linking of SH-groups in myosin heads changes character of F-actin conformational rearrangements induced by myosin subfragment-1 or heavy meromyosin. *Doklady USSR Acad. Sci.* **281,** 216–219.

Borovikov, Yu. S., Kakol, I., Szczesna, D., Kirillina, V. P., and Levitsky, D. I. (1986b). Effect of phosphorylation of rabbit skeletal muscle myosin light chains on the nature of conformational changes of F-actin induced by heavy meromyosin. *Biochemistry (Moscow)* **51,** 691–694.

Borovikov, Yu. S., Wrotek, M., Aksenova, N. B., Lebedeva, N. N., and Kakol, I. (1987). Influence of Mg^{2+} and Ca^{2+} bound to 1,5-IAEDANS-labeled phosphorylated and dephosphorylated heavy meromyosin complexed with F-actin on polarized fluorescence of the fluorophore. *FEBS Lett.* **223,** 409–412.

Borovikov, Yu. S., Wrotek, M., Aksenova, N. B., Lebedeva, N. N., and Kakol, I. (1988a). Effect of Ca^{2+} on myosin head motility in the F-actin–heavy meromyosin complex. *Biochemistry (Moscow)* **53,** 97–100.

Borovikov, Yu. S., Galazkiewicz, B., and Dabrowska, R. (1988b). Effect of caldesmon on the type of conformational changes in F-actin induced by myosin subfragment-1 binding. *Biochemistry (Moscow)* **53,** 179–182.

Borovikov, Yu. S., Dobrowolsky, Z., Aksenova, N. B., and Dabrowska, R. (1988c). Tropomyosin from the smooth and skeletal muscles initiates various conformational changes in skeletal F-actin. *Biochemistry (Moscow)* **53,** 1817–1820.

Borovikov, Yu. S., Dobrowolsky, Z., and Dabrowska, R. (1988b). Tropomyosin and myosin subfragment-1 induce in thin muscle fiber filaments differing conformational changes in the C-terminal portion of the polypeptide chain of actin. *Tsitologiya* **30,** 1014–1017.

Borovikov, Yu. S., Wrotek, M., Lebedeva, N. N., and Kakol, I. (1989a). Effect of phosphorylated myosin light chains and Ca^{2+} on F-actin conformation during skeletal muscle contraction. *Biochemistry (Moscow)* **54,** 161–166.

Borovikov, Yu. S., Szczesna, D., and Kakol, I. (1989b). Effect of phosphorylation of myosin light chains on the structural state of tropomyosin in thin filaments decorated with heavy meromyosin. *Biochemistry (Moscow)* **54,** 1041–1045.

Borovikov, Yu. S., Nowak, E., and Dabrowska, R. (1990a). Effects of caldesmon and smooth muscle tropomyosin on the motility of myosin heads in ghost muscle fibers. *Biochemistry (Moscow)* **55,** 1498–1502.

Borovikov, Yu. S., Nowak, E., Khoroshev, M. I., and Dabrowska, R. (1990b). The effect of caldesmon and subfragment-1 of myosin on the structural state of tropomyosin modified with 1.5-IAEDANS in ghost fibers. *Biochemistry (Moscow)* **55,** 1294–1298.

Borovikov, Yu. S., Szczesna, D., Khoroshev, M. I., and Konkol, I. (1990c). "Freezing" of heavy meromyosin with a bifunctional reagent inhibits dependence of the actin–myosin interaction on phosphorylation of myosin light chains. *Tsitologiya* **32,** 481–488.

Borovikov, Yu. S., Kuleva, N. V., and Khoroshev, M. I. (1991a). Polarization microfluorimetry study of interaction between myosin head and F-actin in muscle fibers. *Gen. Physiol. Biophys.* **10,** 441–459.

Borovikov, Yu. S., Vdovina, I. B., Khoroshev, M. I., and Kirillina, V. P. (1991b). The orientation of fluorescent probes attached to actin and myosin subfragment-1 at the strong and the weak binding of these proteins in ghost fiber. *J. Muscle Res. Cell Motil.* **12,** 104.

Borovikov, Yu. S., Kirillina, V. P., Szczepanowska, J., and Carraro, U. (1992). Polarized microfluorimetry investigation of conformational changes of F-actin in ghost fibers of fast (EDL) and slow (SOL) rat muscle induced by functional electrostimulation. *Basic Appl. Myol.* **2,** 139–144.

Borovikov, Yu. S., Nowak, E., Khoroshev, M. I., and Dabrowska, R. (1993). The effect of Ca^{2+} on the conformation of tropomyosin and actin in regulated actin filaments with or without bound myosin subfragment 1. *Biochim. Biophys. Acta* **1163,** 280–286.

Borovikov, Yu. S., Horiuchi, K. Y., Avrova, S. V., and Chacko, S. (1996a). Modulation of actin conformation and inhibition of actin filament velocity by calponin. *Biochemistry* **35,** 13849–13857.

Borovikov, Yu. S., Khoroshev, M. I., and Chacko, S. (1996b). Comparison of the effects of calponin and a 38-kda caldesmon fragment on formation of the strong-binding state in ghost muscle fibers. *Biochem. Biophys. Res. Commun.* **223,** 240–244.

Borovikov, Yu. S., Moraczewska, I., Khoroshev, M. I., and Strzelecka-Golaszewska, H. (1998). Subtilisin cleavage of actin inhibits formation of strong binding between actin and myosin in a ghost muscle fiber. Submitted for publication.

Botts, J., Thomason, J. F., and Morales, M. F. (1989). On the origin and transmission of force in actomyosin subfrsagment-1. *Proc. Natl. Acad. Sci. USA* **86,** 2204–2208.

Bremel, R. D., and Weber, A. (1972). Cooperation within actin filament in vertebrate skeletal muscle. *Nature* **238,** 97–101.

Burghardt, T. P., and Ajtai, K. (1994). Following the rotational trajectory of the principal hydrodynamic frame of a protein using multiple probes. *Biochemistry* **33,** 5376–5381.

Chalovich, J. M. (1992). Actin mediated regulation of muscle contraction. *Pharmacol. Ther.* **55,** 95–148.

Chalovich, J. M., Chock, P. B., and Eisenberg, E. (1981). Mechanism of action of troponin and tropomyosin. *J. Biol. Chem.* **256,** 575–578.

Chalovich, J. M., Greene, L. E., and Eisenberg, E. (1983). Crosslinked myosin subfragment I: A stable analog of the subfragment-1–ATP complex. *Proc. Natl. Acad. Sci. USA* **80,** 4909–4913.

Chaussepied, P., Morales, M. F., and Kassab, R. (1988). The myosin SH_2-50-kilodalton fragment crossbond: Location and consequences. *Biochemistry* **27,** 1778–1785.

Cooke, R. (1995). The actomyosin engine. *FASEB J.* **9,** 636–642.

Dobrowolski, Z., Borovikov, Yu. S., Nowak, E., Galazkiewicz, B., and Dabrowska, R. (1988). Comparison of Ca^{2+}-dependent effects of caldesmon–tropomyosin–calmodulin and troponin–tropomyosin complexes on the structure of F-actin in ghost fibers and its interaction with myosin heads. *Biochim. Biophys. Acta* **956,** 140–150.

Dos Remedios, C. G., and Moens, P. D. (1995). Actin and the actomyosin interface. *Biochim. Biophys. Acta* **1228,** 99–124.

Dos Remedios, C. G., Millikan, R. G. C., and Morales, M. F. (1972). Polarization of tryptophan fluorescence from single striated muscle fibers. A molecular probe of contraction. *J. Gen. Physiol.* **59,** 103–120.

Efimova, N. N. (1992). Study of effect of phosphorylation of LC-2 myosin on character of interaction of actin and myosin in the vertebrate skeletal musculature by the method of polarized microfluorimetry. PhD thesis, Institute of Cytology, St. Petersburg.

Efimova, N. N., and Borovikov, Yu. S. (1995). Effect of phosphorylation of myosin light chains and ion strength on actin–myosin interaction in the relaxed skeletal muscle fiber. *Biochemistry (Moscow)* **60,** 1799–1802.

Egelman, E. H. (1985). The structure of F-actin. *J. Muscle Res. Cell Motil.* **6,** 129–151.

Egelman, E. H., and Orlova, A. (1995a). Allostery, cooperativity, and different structural states in F-actin. *J. Struct. Biol.* **115,** 159–162.

Egelman, E. H., and Orlova, A. (1995b). New insights into actin filament dynamics. *Curr. Opin. Struct. Biol.* **5,** 172–180.

Eisenberg, E., and Hill, T. L. (1985). Muscular contraction and free energy transduction in biological systems. *Science* **227,** 999–1006.

Fujime, S., and Ishiwata, S. (1971). Dynamic study of F-actin by quasielastic scattering of laser light. *J. Mol. Biol.* **62,** 251–265.

Galazkiewicz, B., Borovikov, Yu. S., and Dabrowska, R. (1987). The effect of caldesmon on actin–myosin interaction in skeletal muscle fibers. *Biochim. Biophys. Acta* **916,** 368–375.

Geeves, M. A. (1992). The actomyosin ATPase: A two-state system. *Philos. Trans. R. Soc. London* **336,** 63–70.

Greene, L. E., and Eisenberg, E. (1980a). Dissociation of the actin subfragment-one complex by adenyl-5′-yl imidodiphosphate, ADP, and PP. *J. Biol. Chem.* **255,** 543–548.

Greene, L. E., and Eisenberg, E. (1980b). Cooperative binding of myosin subfragment-I to the actin–troponin–tropomyosin complex. *Proc. Natl. Acad. Sci. USA* **77,** 2616–2620.

Holmes, K. C., Tirion, M., Popp, D., Lorenz, M., Kabsch, W., and Milligan, R. A. (1993). A comparison of the atomic model of F-actin with cryo-electron micrographs of actin and decorated actin. *Adv. Exp. Med. Biol.* **332,** 15–22.

Huxley, A. F., and Niedergerke, R. (1954). Structural changes in muscle during contraction. Interference microscopy of living muscle fibres. *Nature (London)* **173,** 971–973.

Huxley, H. E., and Hanson, J. (1954). Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. *Nature (London)* **173,** 973–976.

Irving, M. (1996). Steady-state polarization from cylindrically symmetric fluorophores undergoing rapid restricted motion. *Biophys. J.* **70,** 1830–1835.

Irving, M., St.-Claire-Allen, T., Sabido-David, C., Craik, J. S., Brandmeier, B., Kendrick-Jones, J., Corrie, J. E., Trentham, D. R., and Goldman, Y. E. (1995). Tilting of the light-chain region of myosin during step length changes and active force generation in skeletal muscle. *Nature* **375,** 688–691.

Ishii, Y., and Lehrer, S. S. (1985). Fluorescence studies of the conformation of pyrene-labeled tropomyosin: Effects of F-actin and myosin subfragment 1. *Biochemistry* **24,** 6631–6638.

Johnson, W. C., Bivin, D. B., Ue, K., and Morales, M. F. (1991). A search for protein structural changes accompanying the contractile interaction. *Proc. Natl. Acad. Sci. USA* **88,** 9748–9750.

Kabsch, W., and Holmes, K. C. (1995). The actin fold. *FASEB J.* **9,** 167–174.

Kakol, I., Borovikov, Yu, S., Szczesna, D., Kirillina, V. P., and Levitsky, D. I. (1987). Conformational changes of F-actin in myosin-free ghost single fibre induced by either phosphorylated or dephosphorylated heavy meromyosin. *Biochim. Biophys. Acta* **913,** 1–9.

Kaulin, A. B. (1968a). Polarizational fluorescence of stained muscle fibers. I. Polarizational fluorescence of acridine orange in muscle fibers in norm and in injury. *Tsitologiya* **10,** 123–125.

Kaulin, A. B. (1968b). Polarizational fluorescence of stained muscle fibers. II. Rotational depolarization of fluorescence. *Tsitologiya* **10,** 184–191.

Kaulin, A. B., and Golfand, K. A. (1970). Polarizational fluorescence of stained muscle fibers. IV. Change of the acridine orange orientation in glycerolized fibers under effect of ATP. *Tsitologiya* **12,** 172–177.

Khoroshev, M. I. (1992). Study of conformational changes of F-actin in its interaction with myosin molecule head by the method of polarized microfluorimetry. PhD thesis, Institute of Cytology, St. Petersburg.

Khoroshev, M. I., and Borovikov, Yu. S. (1991). Ghost muscle fiber with thin filaments reconstructed from non-muscle actin: A model to study cytoskeleton, using polarizational microfluorimetry. *Tsitologiya* **33,** 76–79.

Khoroshev, M. I., Kuleva, N. B., and Borovikov, Yu. S. (1989). Effect of myosin subfragment-1 on structure of actin from skeletal muscles and platelets. *Tsitologiya* **31,** 1133–1137.

Khoroshev, M. I., Borovikov, Yu. S., Avrova, S. V., Horiuchi, K., Kirillina, V. P., and Chacko, S. (1995). Effect of caldesmon fragment with molecular mass 38 kDa and calponin on the actin ability to form the "strong" form of binding with myosin heads. *Tsitologiya* **38,** 346–350.

Kirillina, V. P., Borovikov, Yu. S., Szczepanowskaya, I., and Carraro, I. (1992). Effect of functional electrostimulation of rat fast and slow muscles on the tropomyosin and actin functional state in regulated thin filaments of the ghost muscle fiber. *Tsitologiya* **34,** 74–79.

Kuznetsova, I., Antropova, O., Turoverov, K. K., and Khaitlina, S. (1996). Conformational changes in subdomain 1 of actin induced by proteolytic cleavage within the DNase I-binding loop: Energy transfer from tryptophan to AEDANS. *FEBS Lett.* **383,** 105–108.

Lednev, V. V. (1980). Some aspects of regulation of the muscle contraction (an analytical review). *In* "Structural Basis and Regulation of the Biological Motility," pp. 221–270. Nauka, Moscow.

Lehman, W., Vibert, P., Uman, P., and Craig, R. (1995). Steric-blocking by tropomyosin visualized in relaxed vertebrate muscle thin filaments. *J. Mol. Biol.* **251,** 191–196.

Levitsky, D. I., Borovikov, Yu. S., Nikolaeva, O. P., Golitsyna, N. L., and Poglazov, B. F. (1990). Effect of myosin alkaline light chains on interaction of myosin subfragment-1 with actin in solution and in ghost muscle fiber. *Biochemistry (Moscow)* **55,** 1690–1699.

Levitsky, D. I., Golitsyna, N. L., Nikolaeva, O. P., and Borovikov, Yu. S. (1991). Interaction of isoforms of myosin subfragment-1 containing fluorescein-labeled alkaline light chains with muscle fiber actin. *Biochemistry (Moscow)* **56,** 639–647.

Ling, N., Shrimpton, C., Sleep, J., Kendrick-Jones, J., and Irving, M. (1996). Fluorescent probes of the orientation of myosin regulatory light chains in relaxed, rigor and contracting muscle. *Biophys. J.* **70,** 1836–1846.

Lorenz, M., Popp, D., and Holmes, K. C. (1993). Refinement of the F-actin model against X-ray fiber diffraction data by the use of a directed mutation algorithm. *J. Mol. Biol.* **234,** 826–836.

Lymn, R. W., and Taylor, E. W. (1971). Mechanism of adenosine triphosphate hydrolysis by actomyosin. *Biochemistry* **10,** 4617–4624.

Margossian, S. S., and Lowey, S. (1978). Interaction of myosin subfragments with F-actin. *Biochemistry* **17,** 5431–5439.

Marston, S. B. (1982). The rates of formation and dissociation of actin–myosin complexes. *Biochem. J.* **230,** 453–460.

Marston, S. B., and Weber, A. (1975). The dissociation constant of the actin–heavy meromyosin subfragment-I complex. *Biochemistry* **14,** 3868–3873.

Miki, M. (1989). Interaction of Lys-61 labeled actin with myosin subfragment-1 and the regulatory proteins. *J. Biochem.* **106,** 651–655.

Miki, M. (1991). Detection of conformational changes in actin by fluorescence resonance energy transfer between tyrosine-69 and cysteine-374. *Biochemistry* **30,** 10878–10884.

Milligan, R. A. (1996). Protein–protein interactions in the rigor actomyosin complex. *Proc. Natl. Acad. Sci. USA* **93,** 21–26.

Miyanishi, T., and Borejdo, S. (1989). Differential behavior of two residues of the myosin head in muscle fibres. *Biochemistry* **28,** 1287–1294.

Morales, M. F. (1984). Calculation of the polarized fluorescence from a labeled fiber. *Proc. Natl. Acad. Sci. USA* **81,** 8145–8156.

Mossakowska, M., Moraczewska, J., Khaitlina, S., and Strzelecka-Golaszewska, H. (1993). Proteolytic removal of three C-terminal residues of actin alters the monomer–monomer interactions. *Biochem. J.* **289,** 897–902X.

Nichei, T., Mendelson, R., and Botts, J. (1974). Use of fluorescence polarization to observe changes in attitude of S-1 moieties in muscle fibres. *Biophys. J.* **14,** 236–242.

Nowak, E., Borovikov, Yu. S., and Dabrowska, R. (1989). Caldesmon weakens the binding between myosin heads and actin in ghost fibers. *Biochim. Biophys. Acta* **999,** 289–292.

Nowak, E., Borovikov, Yu. S., Khoroshev, M. I., and Dabrowska, R. (1991). Troponin I and caldesmon restrict alterations in actin structure occurring on binding of myosin subfragment 1. *FEBS Lett.* **281,** 51–54.

Oosawa, F. (1980). The flexibility of F-actin. *Biophys. Chem.* **11,** 443–446.

Oosawa, F. (1983). Macromolecular assembly of actin. *In* "Muscle and Non-muscle Motility" (A. Stracher, ed.), pp. 151–216. Academic Press, New York.

Oosawa, F., Fujime, S., Ishiwata, S., and Mihashi, K. (1972). Dynamic property of F-actin and thin filament. *Cold Spring Harbor Symp. Quant. Biol.* **37,** 277–285.

Orlova, A., and Egelman, E. H. (1993). A conformational change in the actin subunit can change the flexibility of the actin filament. *J. Mol. Biol.* **232,** 334–341.

Orlova, A., and Egelman, E. H. (1995). Structural dynamics of F-actin: I. Changes in the C-terminus. *J. Mol. Biol.* **245,** 582–597.

Orlova, A., and Egelman, E. H. (1997). Cooperative rigor binding of myosin to actin is a function of F-actin structure. *J. Mol. Biol.* **265,** 469–474.

Orlova, A., Prochniewicz, E., and Egelman, E. H. (1995). Structural dynamics of F-actin: II. Cooperativity in structural transitions. *J. Mol. Biol.* **245,** 598–607.

Ostap, E. M., and Thomas, D. D. (1991). Rotational dynamics of spin-labeled F-actin during activation of myosin S1 ATPase using caged ATP. *Biophys. J.* **59,** 1235–1241.

Parry, D. A. D., and Squire, J. M. (1973). Structural role of tropomyosin in muscle regulation: Analysis of the X-ray diffraction patterns from relaxed and contracting muscle. *J. Mol. Biol.* **75,** 33–35.

Popp, D., Maeda, Y., Stewart, A. A., and Holmes, K. C. (1991). X-ray diffraction studies on muscle regulation. *Adv. Biophys.* **27,** 89–103.

Prochniewicz-Nakayama, E., and Yanagida, T. (1982). The effect of crossbonding of thin filament with glutaraldehyde on the contractility of muscle fiber. *J. Biochem.* **92,** 1269–1277.

Prochniewicz-Nakayama, E., Yanagida, T., and Oosawa, F. (1983). Studies on conformation of F-actin in muscle fibers in the relaxed state, rigor, and during contraction using fluorescent phalloidin. *J. Cell Biol.* **97,** 1663–1667.

Rozanov, Yu. M., Chernogriadskaya, N. A., Barsky, I. Ya., Borovikov, Yu. S., and Shudel, M. S. (1971). Ultraviolet polarization fluorescence of muscle fibers and some other anisotropic cytological objects. *Tsitologiya* **13,** 190–200.

Schwyter, D., Phillips, M., and Reisler, E. (1989). Subtilisin-cleaved actin: Polymerization and interaction with myosin subfragment-1. *Biochemistry* **28,** 5889–5895.

Schwyter, D. H., Kron, S. J., Toyoshima, Y. Y., Spudich, J. A., and Reisler, E. (1990). Subtilisin cleavage of actin inhibits in vitro sliding movement of actin filaments over myosin. *J. Cell Biol.* **111,** 465–470.

Solovyova, S. V., Horiuchi, K. Y., Efimova, N. N., Borovikov, Yu. S., and Chacko, S. (1995). Effect of phosphorylation of myosin regulatory light chain on actin–heavy meromyosin interaction. *J. Muscle Res. Cell Motil.* **16,** 159.

Stein, L. A., Schwarz, R. P., Chock, P. B., and Eisenberg, E. (1979). Mechanism of actomyosin adenosine triphosphatase. Evidence that adenosine 5′-triphosphate hydrolysis can occur without dissociation of the actomyosin complex. *Biochemistry* **18,** 3895–3909.

Stepkowski, D., Szczesna, D., Babiychuk, E. B., Borovikov, Yu. S., and Kakol, I. (1995). Significance of the N-terminal fragment of myosin regulatory light chain for myosin–actin interaction. *Biochem. Mol. Biol. Int.* **35,** 677–684.

Stewart, M., and McLachlan, A. D. (1975). Fourteen actin-binding sites on tropomyosin? *Nature (London)* **257,** 331–333.

Szczepanowska, J., Borovikov, Yu. S., and Jakubiec-Puka, A. (1987). Effect of denervation, reinnervation and hypertrophy on the state of actin filaments in skeletal muscle fibres. *Eur. J. Cell Biol.* **43,** 394–402.

Szczepanowska, J., Borovikov, Yu. S., and Jakubiec-Puka, A. (1991). Effect of innervation on the acto–myosin interaction. *J. Muscle Res. Cell Motil.* **12,** 84.

Szczepanowska, J., Borovikov, Yu. S., and Jakubiec-Puka, A. (1995). Influence of denervation and muscle inactivity on organization of F-actin. *J. Muscle Res. Cell Motil.* **16,** 155–156.

Szczepanowska, J., Borovikov, Yu. S., and Jakubiec-Puka, A. (1998). Effects of denervation and muscle inactivity on the organization of F-actin. *Muscle Nerve* **21,** 309–317.

Szczesna, D., Borovikov, Yu. S., Lebedeva, N. N., and Kakol, I. (1987). Effect of phosphorylation of myosin light chains on interaction of heavy meromyosin with regulated F-actin in ghost fibers. *Experientia* **43,** 194–196.

Szczesna, D., Borovikov, Yu. S., Kakol, I., and Sobieszek, A. (1989). Interaction of tropomyosin with F-actin–heavy meromyosin complex. *Biol. Chem. Hoppe-Seyler* **370,** 399–407.

Tanner, J. W., Thomas, D. D., and Goldman, Y. E. (1992). Transients in orientation of fluorescent cross-bridge probe following photolysis of caged nucleotides in skeletal muscle fibers. *J. Mol. Biol.* **223,** 185–203.

Tirion, M. M., ben-Avraham, D., Lorenz, M., and Holmes, K. C. (1995). Normal modes as refinement parameters for the F-actin model. *Biophys. J.* **68,** 5–12.

Tokunaga, M., Sutoh, K., and Wakabayashi, T. (1991). Structure and structural change of the myosin head. *Adv. Biophys.* **27,** 157–167.

Tregear, R., and Mendelson, R. (1975). Polarization from a helix of fluorophores and its relation to that obtained from muscle. *Biophys. J.* **15,** 455–467.

Vedenkina, N. S., Burshtein, E. A., and Gotberg, M. I. (1972). Study of actin by the method of fluorescence. *In* "Mechanisms of Muscle Contraction," pp. 4–9. Nauka, Moscow.

White, H. D., and Taylor, E. W. (1976). Energetics and mechanism of actomyosin adenosine triphosphatase. *Biochemistry* **15,** 5818–5826.

Wilson, M. G. A., and Mendelson, R. A. (1983). A comparison of order and orientation of cross-bridges in rigor and relaxed muscle fibres using fluorescence polarization. *J. Muscle Res. Cell Motil.* **4,** 671–693.

Wrotek, M., Borovikov, Yu, S., Lebedeva, N. N., and Kakol, I. (1989). Some properties of glycerinated skeletal muscle fibers containing phosphorylated myosin. *Gen. Physiol. Biophys.* **8,** 569–578.

Yanagida, T. (1981). Angles of nucleotides bound to crossbridges in glycerinated muscle fiber at various concentrations of epsilon ATP epsilon ADP and epsilon AMPPNP detected by polarized fluorescence. *J. Mol. Biol.* **146,** 539–560.

Yanagida, T. (1984). Angles of fluorescently labelled myosin heads and actin monomers in contracting and rigor stained muscle fiber. *In* "Contractile Mechanisms in Muscle" (G. H. Pollak and H. Sugi, eds.). Plenum, New York.

Yanagida, T. (1985). Angle of active site of myosin heads in contracting muscle during sudden length changes. *J. Muscle Res. Cell Motil.* **6,** 43–52.

Yanagida, T., and Oosawa, F. (1978). Polarized fluorescence from ε-ADP incorporated into F-actin in a myosin-free single fibre: Conformation of F-action and changes induced in it by heavy meromyosin. *J. Mol. Biol.* **126,** 507–524.

Yanagida, T., and Oosawa, F. (1980). Conformational changes of F-actin–ε-ADP in thin filaments in myosin-free muscle fibres induced by Ca^{2+}. *J. Mol. Biol.* **128,** 313–320.

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INDEX

A

B

C

D

E

F

G

H

I

K

M

N

O

P

R

S

T

V