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Front cover photograph: An Example of plant microtubules observed during cell cycle progression. (For more details, see Chapter 4, Fig. 1.)

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Transcription Factors in Cardiogenesis: The Combinations That Unlock the Mysteries of the Heart

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Heart formation is one of the first signs of organogenesis within the developing embryo and this process is conserved from flies to man. Completing the genetic roadmap of the molecular mechanisms that control the cell specification and differentiation of cells that form the developing heart has been an exciting and fast-moving area of research in the fields of molecular and developmental biology. At the core of these studies is an interest in the transcription factors that are responsible for initiation of a pluripotent cell to become programmed to the cardiac lineage and the subsequent transcription factors that implement the instructions set up by the cells commitment decision. To gain a better understanding of these pathways, cardiac-expressed transcription factors have been identified, cloned, overexpressed, and mutated to try to determine function. Although results vary depending on the gene in question, it is clear that there is a striking evolutionary conservation of the cardiogenic program among species. As we move up the evolutionary ladder toward man, we encounter cases of functional redundancy and combinatorial interactions that reflect the complex networks of gene expression that orchestrate heart development. This review focuses on what is known about the transcription factors implicated in heart formation and the role they play in this intricate genetic program.

KEY WORDS: Heart development, Cardiogenesis, Transcription factors, Promoter analysis, Knockout mice, Cell specification, Cell differentiation.

I. Introduction

Since the earliest records in human history, the heart has fascinated mankind. The heart was theorized to hold the soul and is an icon of love. Functionally, the heart is a pump and is present in some form in all complex organisms ranging from flatworms to man. In vertebrates the heart is the first visible organ that forms and provides the first evidence of an organism's left–right asymmetry. From seminal studies done on skeletal muscle, it became clear that skeletal muscle cell specification and differentiation are controlled by the expression of a family of proteins—the MyoD family of basic helix–loop–helix (bHLH) transcription factors (Buckingham, 1994a; Olson and Klein, 1994). The four members—MyoD, myogenin, Myf5, and Mrf4—are capable of inducing a variety of fibroblast cell lines to express skeletal muscle-specific genes (Braun *et al.,* 1989; Davis *et al.,* 1987; Edmondson and Olson, 1989; Miner and Wold, 1990; Tapscott *et al.,* 1988; Weintraub *et al.,* 1989; Wright *et al.,* 1989). All four of the genes have been inactivated via homologous recombination, and the results of studies using these mice have been fundamental to the understanding of myogenesis. It is these and subsequent studies using the skeletal muscle system that have established the paradigm of cell specification and differentiation. The development of this model system created a template into which other cell types could be plugged and for which the transcription factors that control the process need only be filled in.

Recently, a large body of experimental evidence has revealed a greater understanding of the molecular mechanisms that control cell specification and terminal differentiation of the developing heart and the conservation of this molecular program throughout evolution is quite striking. This pathway begins with external inductive signals from overlying tissues that instruct a specific subset of transcription factors within the signaled cell to coordinate a complex array of gene expression. Just as an orchestra is composed of a variety of instruments, a variety of transcription factors combine to specify the cardiomyocytes, endocardium, pericardium, and cells of cardiac conductive system. These committed cells receive further communication through signaling, which results in cardiac morphogenesis and terminal differentiation analogous to the various instruments in an orchestra playing their individual parts, adding layer after layer of depth and function to produce a finished symphony. The transcription factors that respond to cardiac induction signals are the instruments in this orchestra. In place of sections of woodwinds, brass, and percussion there are sections of homeobox, MADS box, Zinc Finger and bHLH proteins, which we overexpress, mutate, and knock out trying to figure out the tune.

Thus, we provide an overview of the current knowledge of cardiac inductive signals. We also discuss what is known about the function and position of the transcription factors that lie within the molecular pathways controlling cell specification and differentiation of the cells that form the heart. We will also emphasize

functional redundancies and combinatorial interactions of the various transcription factors in heart development and point out the gaps in our understanding of this dynamic and complex process.

II. Cell Specification and Differentiation and Morphogenesis of the Cardiac Lineage

A. Cell Specification and Differentiation

The initiation of gastrulation starts complex movement of epiblast cells through the primitive streak that forms the endodermal, mesodermal, and ectodermal cell layers that take part in forming various structures of the developing embryo. Cells that are destined to form the vertebrate heart move through the primitive streak to a position that is anterior and lateral to the forming neural tube (Fig. 1, see color insert). The process of cardiogenesis involves a complex series of events that consist of (i) specification of mesodermal- and neural crest-derived cells to become programmed to the cardiac lineage, (ii) the growth and differentiation of these cells to cardiomyocytes, and (iii) their migration and morphogenic patterning into the mature heart. Abnormalities that occur at each step often lead to congenital heart disease, which occurs at a frequency of 1 in 1000 live births, making this the most common cause of human congenital defect (Hoffman, 1987). The precardiac mesoderm located in the anterior lateral regions of the embryo first becomes specified to form cardiac tissue well before the appearance of any recognizable heart structures (Yutzey and Bader, 1995). The programmed cardiac cells form two bilateral heart tubes, which migrate centrally at the midline of the embryo, fusing into a linear heart tube. The linear tube begins to beat and then undergoes rightward looping, and subsequent septation leads to the formation of a four-chambered heart (Fishman and Chien, 1997; Olson and Srivastava, 1996) (Fig. 1). Concurrently, neural crest cells migrate from the neural folds and populate the areas that will become the cardiac outflow tract and aortic arches (Kirby and Waldo, 1995). Although the fundamental process of cardiogenesis is well understood, less is known about the transcription factors that control and implement the specification and differentiation of heart formation.

The early stages of heart development are nearly identical in all vertebrate species and the differences that occur in the later stages of septation, chamber, and outflow tract formation result from different uses of the lungs (Fishman and Chien, 1997). The cardiac field represents the total area of mesodermal precursors that can be signaled to become cardiomyocytes. Experiments that address induction of the cells within this field into the cardiac lineage indicate that anterior endoderm can convert mesoderm to cardiac fate (Schultheiss *et al.,* 1995). The cardiac-inducing ability of the anterior endoderm can be mimicked by bone morphogenic protein-2

(BMP-2) and an unknown endodermal factor (Schultheiss *et al.,* 1997). The inductive proprieties of BMP-2 can be inhibited by the secreted protein noggin, which binds to BMPs and antagonizes BMP activity, indicating that BMP activity is required for myocardial specification of this tissue (Schultheiss *et al.,* 1997). BMP-4, which is expressed in the ectoderm that surrounds the cardiac fated mesoderm, also plays a role in cell specification. Experiments in chick using recombinant proteins of both BMP-2 and -4 show induction of cardiac gene expression. A set of experiments that demonstrates the role of BMPs in cardiac specification involves the use of the BMP antagonist noggin. Addition of noggin to early chick embryos blocks cardiac development (Schultheiss *et al.,* 1997). Removal of the neuroectoderm will increase the potential of mesoderm to become fated to cardiomyocytes suggesting that this tissue also has an inhibitory effect on cardiogenesis (Fishman and Chien, 1997). Indeed, it has recently been reported that members of the Wnt family of morphogens may be the source of this inhibitory signal (Marvin *et al.,* 2001; Schneider and Mercola, 2001; Tzahor and Lassar, 2001). A recent study in chick showed that ectopic expression of *Wnt-1* or *Wnt-3a* can mimic the block in cardiogenesis that is observed from cells in the neural tube (Tzahor and Lassar, 2001). In a study of *Wnt-1* signaling in *Xenopus,* it was shown that the Wnt antagonists *Dkk-1* and *Crescent,* but not the frizzled domain-containing antagonists *Frzb* and *Szl,* can induce heart induction from marginal zone explants (Schneider and Mercola, 2001). Moreover, expression of *glycogen synthase kinase 3β,* which inhibits Wnt signaling, also promotes cardiac cell programming (Schneider and Mercola, 2001). A third report using the chick model also shows that Wnt inhibition promotes cardiogenesis from posterior mesoderm (Marvin *et al.,* 2001). Based on these findings, a model was derived that involves a delicate interplay of morphogenic signals that, when present at the right time and the right concentration, convey this specific signal to the nucleus of a predestined cardiomyocyte. What implements this signal?

It is understood that BMP signaling is conveyed through a complex interaction of ligand with cellular receptors that activate a family of proteins termed Smads (via phosphorylation) in vertebrates (Raftery and Sutherland, 1999). There are three subtypes of Smad factors, which are DNA-binding proteins regulated by translocation into the nucleus, that either directly or through interactions with other transcription factors modulate gene expression. A more detailed discussion of Smads is provided later.

In *Xenopus,* the organizer also plays an essential role in specification of the cardiac lineage, suggesting a role for axial signals in heart development (Fishman and Chien, 1997). Currently, in higher organisms, the combinations of transcription factors that implement the program of cardiac cell specification are not understood.

B. Cardiac Morphogenesis

The molecular pathways controlling left–right asymmetry and thus the direction of cardiac looping are well understood (Yost, 1995, 1999). In *Xenopus* and zebra

fish, communication between the notochord and cardiac cells determines both the dorsal–ventral and left–right axes of the forming heart tube (Danos and Yost, 1996). The signaling molecules XWNT-8, sonic hedgehog, and nodal have been shown to mediate morphological patterning of the developing heart in *Xenopus* and chickens (Danos and Yost, 1996; Levin *et al.,* 1997). Both *sonic hedgehog* and *nodal* are expressed asymmetrically on the left side of the neural tube. This asymmetry results from activin-like molecules interacting with the activin receptor IIa which is expressed on the right side of the neural tube of developing embryos. This interaction inhibits *sonic hedgehog* expression on the right side, which in turn inhibits sonic hedgehog's activation of nodal. The complex expression pattern of these genes is coordinated with extracellular matrix and cell adhesion proteins to drive sidedness of the morphogenic process (Lyons, 1996; Trusk *et al.,* 1996). Interestingly, two mutations in the mouse, one naturally occurring and the other the result of a transgenic insertion, affect left–right axes formation. The first mutation, *situs inversus* (*iv*), shows a randomized left–right asymmetry (Layton, 1976). The hearts of *iv*−/− embryos loop normally to the right or exhibit leftward looping at a frequency of 50% (Layton, 1976). The insertional mutant, *inversion of embryonic turning* (*inv*), shows more than an 85% switch in left–right asymmetry in the homozygous state (Yokoyama *et al.,* 1993). These two mutations map to different chromosomes and exhibit altered expression of nodal, indicating that these genes are upstream of the known regulators of left–right patterning (Lowe *et al.,* 1996). Intercrosses of *iv* and *inv* result in inverted asymmetry ratios similar to that of *iv*, indicating that the *iv* mutation is dominant to that of *inv* in the establishment of left–right patterning (Overbeek, 1997). The results of these studies establish that a molecular gradient involving the signaling molecules sonic hedgehog, nodal, and perhaps other members of the transforming growth factor-β (TGF-β) superfamily controls the direction but not the process of morphogenic looping. The transcription factors within the cardiomyocytes that respond to these signals are largely unknown. Recently, the bicoid-related homeodomain transcription factor Pitx2 has been shown to be a downstream target of the sonic hedgehog/nodal signaling cascade controlling left–right asymmetry (Logan *et al.,* 1998; Piedra *et al.,* 1998; Ryan *et al.,* 1998; Yoshioka *et al.,* 1998). *Pitx2* expression is induced by sonic hedgehog and nodal, exhibits altered expression in the *iv* and *inv* mice, and etopic expression of *Pitx2* alters left–right asymmetry (Ryan *et al.,* 1998). Because *Pitx2* is clearly downstream of the TGF-β signaling cascade controlling left–right asymmetry, it is likely that this transcription factor is a critical downstream mediator of the left–right asymmetry program (Logan *et al.,* 1998; Piedra *et al.,* 1998; Ryan *et al.,* 1998; Yoshioka *et al.,* 1998).

The deleted gene within the *inv* mice has been identified and has been termed *inversin* (Morgan *et al.,* 1998). The *inv* mutation was rescued with a yeast artificial chromosome (YAC) clone encompassing the region of the transgene insertion. Deletion analysis shows that a single deleted gene spanning 47 kb was identified as a 1062-amino acid (aa) product with tandem ankyrin-like repeat sequences. Characterization of complementing and noncomplementing YAC transgenic families

revealed that correction of the *inv* mutant phenotype was concordant with integration and intact expression of the inversin gene, suggesting that this ankyrin repeat protein sits atop the molecular pathways controlling left–right asymmetry (Morgan *et al.,* 1998).

Clearly, external signals sent from overlying tissues control cell specification and morphogenic looping of the heart. These events set the stage for an alteration in the gene expression within the cells that become programmed to become heart tissues. In starting coverage of the various transcription factors, note that in regarding a comparison of cardiac and skeletal muscle myogenesis, no transcription factor has been determined to be cardiac specific. In the skeletal muscle paradigm, myogenic bHLH gene expression is restricted to cells that are fated to become muscle. The observation that no factor shown to be important in cardiogenesis is expressed exclusively within the developing heart indicates that the genes that convey MyoD/Myf5 specification within the cardiac lineage have yet to be identified. Another possibility is that specific combinatorial interactions from the pool of known genes form cardiac-specific complexes, which convey the extracellular signals to the nucleus to orchestrate cardiac cell programming. Next, a discussion of the known transcription factors that are implicated in cardiogenesis and their arrangement within a molecular road map of heart formation is presented.

III. Transcription Factors in the Developing Heart

A. Smads: The Convergence of Extracellular Signaling and Transcription

As mentioned previously, BMP signals are implemented within cells by ligand binding to extracellular receptors, which phosphorylate an evolutionarily conserved group of DNA-binding proteins termed Smads. There are three subtypes of Smads: R-Smads, which are regulated directly via phosphorylation from the extracellular receptors; co-Smads, which interact with R-Smads but are not modified directly by the receptors; and anti-Smads, which antagonize the actions of BMPs/activins and other TGF-β superfamily members (Raftery and Sutherland, 1999). The interrelationship of ligand to receptor to the individual Smads is complex and not without controversy and thus will not be discussed. What is relevant here is that Smad activation via translocation to the nucleus is essential for the specification of the cardiac lineage and several recent studies have provided data that have begun to link this signal to transcriptional programming of the cardiomyocyte.

A clear link to extracellular signaling implementing the cardiac program via Smad activation comes from the study of the *Drosophila* homeobox transcription factor *tinman* promoter. *Tinman* expression is essential for the formation of the dorsal vessel in flies (Azpiazu and Frasch, 1993; Bodmer, 1993; Xu *et al.,* 1998). Within the downstream *tin D* enhancer of the *tinman* gene are eight Smad

cis elements, which bind the co-Smad, *Medea,* and possibly the R-Smad, *MAD*. Also contained within the *tin D* enhancer are other *cis* elements that are required for proper expression when stimulated by the BMP factor *decapentaplegic* (*dpp*), suggesting that interactions with other transcription factors are necessary for implementation of *dpp* signal. It will be interesting to identify the exact *cis* elements and *trans* factors that coordinate function along with *Medea, MAD,* and possibly other Smad factors from this enhancer because these factors will greatly bridge the gap in understanding the earliest transcriptional events in the programming of a cardiomyocyte.

In vertebrates, recent reports show the anti-Smad, *Smad6,* playing an important role in cardiogenesis. In a study of chick embryogenesis, expression of *Smad6* is detectable in the mesoderm fated to become the heart at HH stage 5 and is expressed strongly through the developing heart in regions where BMP2 and -4 are observed (Yamada *et al.,* 1999). Early expression overlaps with the reported expression of the *tinman* homolog *Nkx2.5,* suggesting that like *tinman* regulation, *Nkx2.5* expression may be directly regulated by Smads. In mouse, a null mutation in *Smad6* was generated via homologous recombination. Expression analysis of murine *Smad6* assayed by β-galactosidase activity from the null allele shows similar expression characteristics as reported in the chick study (Galvin *et al.,* 2000). Phenotypically, $Smad6^{-/-}$ mice survive to adulthood and exhibit both vascular and cardiac defects, which include hyperplasia of the cardiac valves and outflow tract as well as septation defects (Galvin *et al.,* 2000). These phenotypes are suggestive of endocardial cushion defects and consequent vascular abnormalities. Mice null for the activin TGF-β responsive R-Smad, *Smad2,* completely lack mesoderm and 20% of $Smad2^{+/-}$ mice show severe gastrulation defects indicating that the level of expression is critical for normal function (Nomura and Li, 1998). The BMP-responsive R-Smad *Smad5* knockout mice die between Embryonic (E) Days 9.5 and 11.5 from various tissue abnormalities, including some heart defects similar to those observed in *BMP2* knockout mice (Chang *et al.,* 1999; Yang *et al.,* 1999).

Considering the size of the TGF-β superfamily, the variety of extracellular receptors and Smad factors, and their ability to combine in different configurations to elicit specific signal transduction cascades, complete understanding of cardiac specification control through these factors will take time. Nevertheless, the link between induction signal and transcriptional events within the unprogrammed mesodermal cell precursor is established; only the details of Smad regulation and interactions need be determined.

B. Homeobox Genes in Cardiogenesis

1. *Hox* Genes

Homeobox genes are transcription factors that contain a helix–turn–helix DNA-binding motif (the homeodomain) and are among the most diverse and important in

development. This class of transcription factors first gained attention from studies of the homeotic or *Hox* cluster genes (*HOM* genes in *Drosophila*). In humans there are 4 clusters of *Hox* genes (*a–d*) and each cluster contains 9–11 *Hox* genes which play an essential role in establishing anterior–posterior identity of body structure (Krumlauf, 1994; Manak and Scott, 1994). When expression of a *Hox* gene within a cluster is disrupted, the specific body structures that form within that expression domain fail to form. Instead, the tissues are transformed into body structures found normally within the expression zone of the *Hox* gene that lies anterior or posterior to the disrupted transcription factor (Krumlauf, 1994).

Evidence that *Hox* genes play role in cardiogenesis derives from studies of chick embryos in which retinoic acid (RA), a known inducer of *Hox* gene expression, causes a posteriorization of the heart when administered to primitive streak-staged embryos (Satin *et al.,* 1988; Yutzey *et al.,* 1994, 1995). Polymerase chain reaction screening analysis of early chick hearts shows that *Hoxd-3, a-4,* and *d-4* are expressed. *Hoxd-3* message can be detected prior to heart tube fusion. In RA treatment, upregulation of *Hoxa-4, d-3,* and *b-5* is observed, suggesting that expression of these genes alters normal cardiac patterning (Searcy and Yutzey, 1998). Moreover, *Msx-1* (*Hox-7*) and *Msx-2* (*Hox-8*) are both expressed in chick cardiogenesis from stages 15+ to 37 in the atrioventricular and the outflow tract regions and cells that coincide morphologically with the cardiac conduction system, implicating a role in heart development (Chan-Thomas *et al.,* 1993).

In addition to the *Hox* cluster genes, numerous other homeodomain proteins have been identified and appear unlinked scattered across the chromosomes. It is clear from examining cardiac morphogenesis that a segmental anterior–posterior organization exists—ventricles anterior and atria posterior—and experimental evidence suggests that *Hox* genes play a direct role in establishing this organization. There is also strong experimental evidence that heart development is driven by a set of cardiac–specific transcription factors that are expressed within the heart in a modular fashion (e.g., ventricular specific), thus creating an independent internal organization scheme for heart formation. Of course, several of the genes that are expressed in such a manner are homeobox factors, and these genes are known to play an essential role in cardiogenesis.

2. "If I Only Had a Heart": *Drosophila's Tinman*

In *Drosophila,* mesoderm induction is controlled by the bHLH transcription factor *twist,* which in turn activates the expression of other genes that later convey regional mesodermal identity. The homeobox transcription factor *tinman* (NK4, msh-2) is regulated by *twist* and plays an essential role in the formation of the dorsal vessel or heart of the fly. Cloned in 1989 by a degenerate screen for new homeodomain proteins, *tinman* represents a new class of homeoproteins. Members of the NK homeoprotein family have a highly conserved homeodomain that contains a distinct tyrosine at position 54, a 10-aa TN domain in the amino portion

of the protein, and a transactivation domain (Fig. 2, see color insert). The highly conserved homeodomain of *tinman* and its vertebrate homologs recognizes the *cis*-acting consensus sequence TNAAGTGG, which is present in the promoters of the downstream target genes (Evans, 1999). *Tinman* is expressed at an early stage in the subdivision of the developing mesoderm of *twist*-positive cells (Azpiazu and Frasch, 1993). *Tinman* expression first becomes restricted to the dorsal mesoderm via expression of the BMP homolog *dpp* and then is further restricted to transient expression in the visceral mesodermal cells of the splanchnopleura. Final restriction of *tinman* expression is mediated by the action of the secreted factor *wingless* to the cells of the dorsal tip mesoderm, which are the progenitor cells fated to become heart (Azpiazu and Frasch, 1993; Harvey, 1996). In *tinman* mutant flies, the dorsal precursor cells never become specified to the cardiac lineage; thus, the dorsal vessel never forms. *Tinman* expression is not only linked to the cell specification of the heart but also linked to the upregulation of cardiac genes, such as *β-tubulin* and *dMEF2,* which is necessary for all fly muscle differentiation (Gajewski *et al.,* 1997; Kremser *et al.,* 1999; Ranganayakulu *et al.,* 1995). Moreover, regulation of *tinman* expression is dependent on Smad factors *Medea* and possibly *MAD,* which convey the extracellular signals of *dpp* to the nucleus (Xu *et al.,* 1998). From these observations, it is clear that *tinman* expression occurs at the earliest time points of *Drosophila* cardiac cell specification and is required for the expression of critical genes involved in proper implementation of the fly cardiac gene program. Because *Hox* gene are evolutionarily conserved between flies and vertebrates, discovery of *tinman* and its functions initiated a search of vertebrate NK homeoproteins with the hope of finding a specifying transcription factor for mammalian cardiogenesis.

3. NK Genes in Vertebrates

Using *tinman* as a starting point, several groups quickly discovered vertebrate homologs. Several of these NK family genes show cardiac expression within fish, frog, chick, mouse, and man and have 63–67% amino-acid identity with *tinman* (Evans, 1999). The most highly studied of these NK factors is *Nkx2.5/Csx. Nkx2.5* and *tinman* show high amino acid identity within both the homeodomain and the TN domain (Fig. 2; Izumo, 1993; Lints *et al.,* 1993). In addition to these domains, Nkx2.5 contains a NK2-specific domain, which is conserved within the vertebrate genes. In adult mice, *Nkx2.5* expression is primarily cardiac specific, with lower levels of expression found in the spleen, stomach, thyroid, and tongue (Kasahara *et al.,* 1998; Lints *et al.,* 1993). Extraembryonic expression has also been implicated, which is consistent with visceral mesoderm expression of *tinman* in the fly (Tanaka *et al.,* 1999b). Expression of human *Csx* has been shown to be induced by BMPs, which are known inducers of mesoderm that become programmed to the cardiac lineage and mirror *dpp* induction of *tinman* in fly cardiogenesis (Shiojima *et al.,* 1997).

In mouse detailed dissection of the *Nkx2.5* promoter revealed a highly modular array of *cis*-acting elements that independently coordinate ubiquitous heart expression (Lien *et al.,* 1999; Reecy *et al.,* 1999; Schwartz and Olson, 1999; Searcy *et al.,* 1998; Tanaka *et al.,* 1999a). Modular transcriptional control has been seen in an increasing number of genes (Firulli and Olson, 1997) and it is interesting to speculate on the advantage of having numerous cardiac-restricted *cis*-acting elements compared to a single cardiac enhancer. Multiple transcriptional elements likely allow for fine control of *Nkx2.5* expression within the heart because each enhancer responds to unique set of upstream genes (Firulli and Olson, 1997; Schwartz and Olson, 1999). Prominent players in *Nkx2.5* regulation include GATA factors and Smad factors via binding of BMPs (Schwartz and Olson, 1999). To date, analysis of Nkx2.5 regulatory proteins has revealed factors that are more broadly expressed than *Nkx2.5* and play important roles in the development of other tissues. In human *Csx,* a 965-bp element that contains several E-boxes, homeobox binding, and CarG-like elements confers cardiomyocyte-predominant expression (Shiojima *et al.,* 2000). It is also reported that Nkx2.5 and the MADS box transcription factor MEF2C can cross-regulate/autoregulate their own expression in P19 cells induced to the cardiac lineage (Skerjanc *et al.,* 1998). Based on regulatory data and the observed temporal expression of *Nkx2.5/Csx* in the early heart, it appears that this *tinman* homolog is one of the earliest expressed transcription factors that marks the developing heart in mammals.

In functional studies using gene targeting, null alleles of *Nkx2.5* were generated by two groups and the resulting phenotypes of these mice show an embryonic lethality between E9 and 10.5 showing arrest of cardiogenesis just prior to cardiac looping (Lyons *et al.,* 1995; Tanaka *et al.,* 1999a). Cardiac marker analysis in both studies shows that expression of *atrial natrioretic peptide (ANP), β-type natrioretic peptide (BNP), MLC-2v, MEF2C, HAND1 (eHAND),* and *Msx2* are disrupted in the Nkx2.5 mutant hearts (Lyons *et al.,* 1995; Tanaka *et al.,* 1999a). Chimeric analysis using double-null *Nkx2.5/Csx* ES cells showed that very few cells lacking a functional *Nkx2.5* gene are observed to contribute to the heart (Tanaka *et al.,* 1999a). Although clearly essential for normal murine heart development, *Nkx/Csx* is not essential for cardiac cell specification because morphogenic and whole mount *in situ* analysis shows that cardiomyocytes have developed (Lyons *et al.,* 1995; Tanaka *et al.,* 1999a). This is in contrast to the lack of functional *tinman* flies where the cardiomyocytes of the dorsal vessel do not develop (Azpiazu and Frasch, 1993; Bodmer, 1993). This finding could be explained by considering that the genetic program of cardiogenesis in the fly is much simpler than that in vertebrates, although it is molecularly conserved. Indeed, comparing transcription factors in flies and mice, the mouse genome generally contains a larger number of genes within a transcription factor family. For example, the single *dMEF2* gene, which is directly regulated by *tinman* in flies and is necessary for proper development of the dorsal vessel, has four mammalian homologs, MEF2A–D, some of which are essential for normal heart development (Bodmer and Venkatesh, 1998).

A comparison of the function of *tinman* and *Nkx2.5* raises the following question: Is the difference in the observed phenotypes of a *tinman* null fly and *Nkx2.5* null mouse the result of a different functional role for the two proteins or is this an evolutionary refinement of function of Nkx2.5 allowed for by gene amplification of cardiac-expressed NK2 family? It is clear from *Drosophila* rescue experiments that *Nkx2.5/Csx* cannot completely rescue the *tinman* null phenotype, suggesting that factors are not functionally identical. *Nkx2.5* expression in the *tinman* mutant background resuces the visceral muscle defects but not the cardiac defects in the mutant flies (Harvey *et al.,* 1999; Ranganayakulu *et al.,* 1998). This result is observed when other cardiac expressed NK genes are expressed (Harvey *et al.,* 1999; Ranganayakulu *et al.,* 1998). Through domain swapping experiments, a 42-aa region of tinman located at the amino-terminal end of the protein that has no homology to any region of Nkx2.5 has been determined to hold the cardiogenic-inducing activity of *tinman* (Fig. 2; Ranganayakulu *et al.,* 1998). When this region of tinman is placed within the amino terminus of Nkx2.5, subsequent expression of this chimeric protein shows rescue of the cardiac phenotype in *tinman* mutant flies (Ranganayakulu *et al.,* 1998). The results of these experiments suggest that although *tinman* and *Nkx2.5/Csx* are related and have cardiogenic function, their exact roles in cardiogenesis are divergent. This suggests that *tinman* function is duplicated in vertebrates by the multiple NK-2 genes or that the molecular mechanism controlling cardiogenesis has diverged such that NK genes do not play the same role that *tinman* plays in flies. Because multiple Nkx genes are expressed within cardiogenesis in a species-specific manner, addressing this question is a challenging undertaking.

4. Other NK-2 Genes

Many other NK-2 genes have been shown to play a role in cardiac development; however, the roles these genes differ between species (Evans, 1999; Harvey *et al.,* 1999; Tanaka *et al.,* 1998). *Nkx2.3, -2.6, -2.7, -2.8, -2.9,* and *-2.10* are expressed in the hearts of some species but not others. These differences support the idea that the molecular pathways controlling cardiogenesis can diverge in evolution where the temporal–spatial expression of highly related genes can change from species to species because family members can replace the functions of each other. This type of evolutionary drift can be seen with myogenic bHLH factors, where myogenin is the first bHLH factor expressed in *Xenopus* skeletal myogenesis and Myf5 is first expressed in mouse (Molkentin and Olson, 1996; Scales *et al.,* 1990). When examining this model, one would predict that genes whose role and expression are less variant between species such as *Nkx2.5* contain unique properties that cannot be replaced easily.

Nkx2.3 is expressed in the developing hearts of frogs, fish (early in cardiogenesis), and chicken (late in cardiogenesis) but not in the developing heart of the mouse (Buchberger *et al.,* 1996; Fu *et al.,* 1998; Pabst *et al.,* 1997). Functional data

on the role of *Nkx2.3* derive from the frog, employing injection of a dominant repressor *NK2.3* construct into embryos (Fu *et al.,* 1998). Results indicate that frogs injected with either a dominant repressor *NK2.3* or *Nkx2.5* construct show an increase in cardiac mutations over control, whereas coinjection of 2.3 and 2.5 yields a synergistic increase in mutation frequency suggesting an overlapping function for 2.3 and 2.5 (Fu *et al.,* 1998). In chick, *Nkx2.3* expression can be detected at Hamburger–Hamilton (HH) stage 18 compared to detection of *Nkx2.5* at HH 5 and Nkx2.7 at HH 7 (Evans, 1999). Because expression of *Nkx2.3* begins much later in chick cardiogenesis, it is likely that the role of *Nkx2.3* has become less prominent in the cardiac molecular program. Furthermore, because the targeted disruption of *Nkx2.3* in the mouse shows no cardiac defect, it is clear that *Nkx2.3* has evolved out of the heart-building business somewhere between frog and man (Pabst *et al.,* 1999).

Nkx2.6/Csx is expressed in mice within the developing outflow tract of the heart overlapping with Nkx2.5 expression (Biben *et al.,* 1998). Mice homozygous null for *Nkx2.6* show no observable phenotype as well as an expansion of *Nkx2.5* expression to the lateral side of the pharynx (Tanaka *et al.,* 2000). Because it is possible that a redundancy of function exists between *Nkx2.5* and *Nkx2.6,* the intercross of these mice will need to be done to determine the role of *Nkx2.6* in murine cardiogenesis, specifically its ability to compensate for *Nkx2.5.*

Nkx2.7 appears to play an important role in the cardiac program of zebra fish. Expression of *Nkx2.7* in the fish embryo appears soon after the initiation of gastrulation within the cardiac mesoderm and precedes the expression of *Nkx2.5* and -2.3, suggesting a potential *tinman*-like role in fish (Lee *et al.,* 1996). There are no reported *Nkx2.7* homologs from other species. If the apparent uniqueness of *Nkx2.7* holds true, then further study through mutagenesis in zebra fish may prove valuable in gaining new information on the role of this unique NK family member.

In chicken cardiogenesis, *Nkx2.8* can be detected in the developing heart at HH 7, slightly later than *Nkx2.5* but prior to *Nkx2.3* (Brand *et al.,* 1997; Evans, 1999; Reecy *et al.,* 1997). As is the case with *Nkx2.7,* no homologs for *Nkx2.8* have been reported in other species, again suggesting that *Nkx2.8* may be a unique player in the chicken heart program.

In *Xenopus, Nkx2.9* has been isolated and shown to be expressed within the developing cardiac mesoderm just prior to differentiation (Newman and Krieg, 1998). As differentiation proceeds, *Nkx2.9* expression is rapidly downregulated, suggesting a role as a possible block for implementation of the cardiac program until the proper time. The mouse homolog of *Nkx2.9* has been identified but, like many of the other Nkx genes, its expression has drifted such that it is not detectable in murine cardiogenesis. This supports the idea that the high amino acid and functional identity that the NK homeodomain family exhibits allows for overlapping functions permitting genetic drift of expression between species (Pabst *et al.,* 1998).

Recently, a new member of the NK-2 family, *XNkx2.10,* was cloned in *Xenopus* and is expressed at early stages of the developing frog heart (Newman *et al.,* 2000). Transactivation studies show that *XNxk2.10* is capable of activating the

transcription of a minimal cardiac actin promoter shown to be regulated by *Nkx2.5* and that it shows synergy when cotransfected with serum response factor (SRF) or GATA-4 (Newman *et al.,* 2000). Although it is not yet known if the mammalian homolog of *XNkx2.10* is expressed similarly, further study of this NK gene should prove interesting.

5. The *Iroquois* (*Irx*) Homeobox Genes

Recently, six homeodomain transcription factors were isolated in mice which show identity to the genes of the *Drosophila Iroquois* complex and are expressed within the central nervous system, otic vesicle, branchial epithelium, limbs, and heart. The genes are conserved throughout evolution and, like their distant cousins in the *Hox* clusters, are implicated in playing a role in tissue patterning (Bosse *et al.,* 1997, 2000; Bruneau *et al.,* 2000; Christoffels *et al.,* 2000; Peters *et al.,* 2000). Unlike the single fly complex, the mammalian genes form two clustrs: *Irx1, -2,* and *-4* map to a cluster on chromosome 13, and *Irx3, -5,* and *-6,* map to mouse chromosome 8 (Peters *et al.,* 2000). *Irx1–5* are reported to exhibit unique segmented cardiac expression and the recently discovered *Irx6* gene has no reported expression (Christoffels *et al.,* 2000; Peters *et al.,* 2000). *Irx1* and *-2* were the first transcription factors shown to be expressed within the ventricular septum, which later become restricted to the myocardium of the atrioventricular bundle and bundle branches of the ventricular conductive system, suggesting a role for these factors in establishing cardiac structural identity (Christoffels *et al.,* 2000). *Irx3* expression is upregulated in the trabeculated region of the ventricles, where *Irx4*'s expression is similar to the ventricular pattern of *MLC-2v* (Christoffels *et al.,* 2000). *Irx5* is expressed within the endocardium lining and atrial and ventricular myocardium, which suggests a role in the pathway defining the formation and/or maintenance of the chamber myocardium that differentiates from the primary myocadium after cardiac looping (Christoffels *et al.,* 2000).

Based on the phenotype of the *Irx* mutation in *Drosophila,* speculation on the role of this gene family in murine cardiogenesis focuses on positional identity within the heart is attractive. Because *Irx* cardiac gene expression appears later than that of Nkx and other cardiac transcription factors, this putative role would fit. Evidence that at least one *Irx* gene is directly downstream of *Nkx* genes was reported by Bruneau *et al.* (2000), where *Irx4* ventricle expression is shown to be directly modulated by Nkx2.5 and HAND2 (dHAND). There are no published studies analyzing *Irx* null alleles or direct downstream targets for *Irx* transcriptional regulation, so we must wait for these types of experiments to be completed to add more details to this new and exciting family of players in cardiogenesis.

6. *PAX3*

In the *splotch* mutation in mice, homozygous animals die at E14 exhibit congenital heart defects involving the outflow tract, similar to DiGeorge syndrome and

ablation of neural crest in the developing chick (Conway *et al.,* 1997; Epstein *et al.,* 2000; Kirby *et al.,* 1983). The *splotch* mutation is a result of mutations within the *PAX3* gene, a paired homeodomain factor that is implicated in many genetic disorders (Epstein *et al.,* 1991, 1993; Li *et al.,* 1999; Vogan *et al.,* 1993). *PAX3*'s paired domain and paired type homeodomain are both capable of binding DNA. These domains interact synergistically with a sequence harboring an ATTA motif and a GTTCC sequence, the cores of the *cis* elements for the paired domain and the homeodomain, respectively (Chalepakis *et al.,* 1994). Initially, disruption of *PAX3* was thought to affect neural crest cell migration. However, recently it has been shown through fate mapping studies that *PAX3*-deficient cells indeed migrate into the outflow tract but at reduced numbers, and thus may affect the rate of migration (Epstein *et al.,* 2000). Because *PAX3* also has a putative role in inhibiting differentiation of migrating skeletal muscle myoblasts from the somites to the limb bud, it is possible that *PAX3* plays a similar function in neural crest (Epstein *et al.,* 1995). Clearly, further investigation of the mechanism of function will prove an interesting area of research.

C. MADS Box Transcription Factors

The MADS box is a highly evolutionarily conserved protein domain found in organisms ranging from yeast and plants to man. The name derives from the founding members of this family: *MCM1, a*gamous, *d*eficiens, and *s*erum response factor (Shore and Sharrocks, 1995). Members of this family exhibit diverse roles in development, including flower development, activation of immediate early gene expression, and activation of tissue-specific gene expression. In animals, both skeletal and heart development depend on the function of MADS box transcription factors and understanding of the mechanisms by which these factors convey tissue-specific gene activation and/or upregulation of immediate early genes has been a challenging scientific undertaking.

1. SRF

SRF is a 67-kDA ubiquitous nuclear factor that, like all MADS proteins, binds as a dimer. SRF was originally identified as a DNA-binding protein of the serum response element (SRE) that conveys transcriptional activation of the *c-fos* gene (Norman *et al.,* 1988; Treisman, 1987, 1992). During vertebrate embryogensis, *SRF* expression is highly enriched in all developing muscle (Belaguli *et al.,* 1997; Croissant *et al.,* 1996). Consistent with this observation, the consensus DNA binding site for SRF, 5′-CCWTATAWGG-3′ (also termed a CarG box), was also identified in muscle-specific structural genes, such as *calponin, SM22, myosin light-chain 1a (MLC-1a),* and *smooth, skeletal,* and *cardiac* α*-actins* (Catala *et al.,* 1995; Li *et al.,* 1996; Mack *et al.,* 2000; MacLellan *et al.,* 1994; Miano *et al.,* 2000;

Sartorelli *et al.,* 1990; Solway *et al.,* 1995). These findings indicated a paradox regarding how a ubiquitous transcription factor that is enriched in muscle during embryogensis can control both immediate early gene expression (growth) and expression of genes associated with terminal differentiation (inhibition of growth). The answer to this paradox has several parts. First, SRF is alternately spliced. Recently, four unique SRF isoforms have been identified and these isoforms have varied transcriptional effects on the regulation of the *SM22* gene (Kemp and Metcalfe, 2000). It is likely that these SRF isoforms are capable of differentially regulating immediate early, skeletal, and cardiac-specific genes. Regulation of SRF DNA binding via competition of other transcription factors for CarG/SRE elements has also been observed. This type of regulation can be demonstrated with the zinc finger-containing GLI–Kruppel-like factor, YY1. YY1 is known to have overlapping DNA binding specificity for CarG elements and is a negative regulator of transcription, which is antagonistic to SRF activity (Lee *et al.,* 1992). During myogenesis, DNA-binding activity of SRF accumulates, whereas DNA binding of YY1 is downregulated (Lee *et al.,* 1992). When myoblasts are inhibited from differentiation by treatment with BrDUrd, YY1 activity is upregulated at the expense of SRF binding. Thus, by competing for the same *cis*-acting element, YY1 and SRF can alter transcriptional response between growth and differentiation target genes.

Although this mechanism helps explain the paradox, it does not fully account for the specific actions of SRF on tissue-specific gene expression. The remainder of the explanation is addressed in the form of combinatorial interactions of SRF with a variety of other transcription factors, which is a common mechanism recognized to control biological function. As stated previously, SRF binds DNA as a dimer and dimerization is essential to function. In experiments in which the DNA-binding domain of SRF is mutated but dimerization is unaffected, transcription of α-actin is downregulated (Croissant *et al.,* 1996). The mutated SRF functions in a dominant-negative fashion, titrating out endogenous SRF into inactive dimer complexes.

Among the numerous proteins that interact with SRF and facilitate transcriptional modulations are SAP1 and -2, Elk1, pHOX (MHOX), the myogenic bHLH factors, and the high-mobility group protein SSRP1 (Cserjesi *et al.,* 1992; Dalton *et al.,* 1993; Dalton and Treisman, 1992; Groisman *et al.,* 1996; Grueneberg *et al.,* 1992; Shipley *et al.,* 1994; Simon *et al.,* 1997; Spencer *et al.,* 1999). Interestingly, it has been shown that SRF directly interacts with the initiator binding protein TFII-I, which facilitates the formation of stable higher order complexes of SRF and Phox1 (Grueneberg *et al.,* 1997). Most relevant to cardiac-specific functions of SRF are combinatorial interactions with Nkx2.5, XNkx2.10, GATA-4, and the TEA domain factor TEF-1 (Belaguli *et al.,* 2000; Chen and Schwartz, 1996, 1997; Gupta *et al.,* 2001; Newman *et al.,* 2000). In all the previously mentioned combinatorial interactions the result of the protein–protein interactions is synergistic activation of transcription. Taken together, these data suggest that DNA binding of SRF and its interactions with other factors are requirements for driving tissue-specific gene expression in tissues such as the heart. SRF seems to function as an

interaction platform that facilitates communication of tissue-specific transcription factors and general transcription factors coordinating specific regulatory events. It is likely that many more transcriptional regulators will be found to interact with SRF and that the specific isoforms of SRF as well as modifications to the SRF proteins such as phosphorylation will modulate these interactions. Indeed, recently it was shown that SRF-mediated transcription is regulated by the Rho family of GTPases and that these signaling mechanisms also mediate histone H4 hyperacetylation (Alberts *et al.,* 1998; Hill *et al.,* 1995; Treisman *et al.,* 1998). Because destabilization of nucleosomes is a required event in initiation of transcription of many genes, the convergence of a signaling pathway on both histone acetylation and SRF activation supports its role as a base platform for building combinatorial transcription complexes. It has also been shown that caMKII can phosphorylate SRF at multiple residues, some within the MADS domain which are likely to affect DNA binding (Fluck *et al.,* 2000). The mouse knockout of SRF has recently been reported. $SRF^{-/-}$ mice show an embryonic lethal phenotype exhibiting a complete lack of mesoderm, misfolded ectodermal and endodermal cell layers, and no primitive streak (Arsenian *et al.,* 1998). This phenotype is suggestive of a central role for SRF in the formation of mesoderm and clearly indicates that understanding the specific function of SRF in cardiac development will require a tissue-specific knockout approach to specifically remove this critical factor from the developing heart.

2. The MEF2 Factors

The MEF2 (also called RSRFs) family of MADS box transcription factors also shows a high level of evolutionary conservation between species in both protein identity and biological function. In mammals and birds there are four MEF2 factors termed MEF2A, -B, -C, and -D. In zebra fish there are three MEF2s, and in *Xenopus* there are two MEF2 factors (SL1 and SL2). *Drosophila* contains a single MEF2 gene, *dMEF2* (Breitbart *et al.,* 1993; Chambers *et al.,* 1992; Leifer *et al.,* 1993; Lilly *et al.,* 1994; Martin *et al.,* 1993, 1994; McDermott *et al.,* 1993; Nguyen *et al.,* 1994; Pollock and Treisman, 1991; Ticho *et al.,* 1996; Yu *et al.,* 1992). In addition to the MADS box, MEF2 proteins share a high amino acid identity within the MEF2 domain, which is located carboxy to the MADS box and is required for DNA binding and dimerization. The consensus MEF2 binding site is YATWWWWTAR, and it is found in the promoters of many muscle-specific genes, such as *desmin, α-MHC,* and *MLC-2v,* and in some nonmuscle genes such as c-*jun* (Fickett, 1996; Han and Prywes, 1995; Molkentin and Markham, 1994; Navankasattusas *et al.,* 1992). Thus, like SRF, MEF2 factors are associated with the expression of genes are associated with cell growth such as expression of c-*jun* and the proliferation of smooth muscle cells after balloon injury and terminal differentiation of skeletal and cardiac myoblasts (Black and Olson, 1998; Firulli *et al.,* 1996; Han and Prywes, 1995). Like all MADS domain proteins, MEF2s

bind DNA as homo- or heterodimers and are alternately spliced; thus, there is a wide range of MEF2 complexes in species with multiple MEF2 genes (Fig. 3, see color insert).

In murine embryogenesis, MEF2B and -C expression is first detected in the cardiac crescent at E7.75, and at E8.0 expression of both MEF2A and -D is observed (Edmondson *et al.,* 1994; Molkentin *et al.,* 1996; Subramanian and Nadal-Ginard, 1996). Throughout the later stages of embryogensis, the four MEF2 factors are continually expressed. Other sites of *MEF2* expression are the developing skeletal and smooth muscle and brain (Edmondson *et al.,* 1994; Leifer *et al.,* 1993; Lyons *et al.,* 1995; Molkentin *et al.,* 1996; Subramanian and Nadal-Ginard, 1996). In the adult *MEF2* mRNA expression is near ubiquitous; however, DNA binding activity is detectable only from muscle and brain, indicating posttranscriptional regulation (Gossett *et al.,* 1989; Suzuki *et al.,* 1995; Yu *et al.,* 1992). Evidence to support this derives from experiments in which a 428-bp sequence from the 3′ UTR of *MEF2A* was fused to the CAT reporter gene (Black *et al.,* 1997). The results of these transfection experiments show that the *MEF2A* UTR confers translational repression in myoblasts and that the repression is relaxed when myoblast differentiation is induced.

In the fly, there is only one MEF2 factor; however, it is expressed in the muscle and neuronal tissues, suggesting conservation of function. Indeed, *dMEF2* expression is first observed within mesodermal precursor cells prior to specification of the somatic and visceral muscle lineages (Lilly *et al.,* 1994; Nguyen *et al.,* 1994). Expression of *dMEF2* requires the mesodermal determinants *twist* and *snail* and the homeobox-containing gene *tinman,* which is also required for visceral muscle and heart formation (Cripps *et al.,* 1998, 1999; Gajewski *et al.,* 1997; Lilly *et al.,* 1994; Nguyen *et al.,* 1994). In flies lacking functional *dMEF2* expression there is a complete lack of muscle differentiation, suggesting that *dMEF2* is essential for smooth, skeletal, and cardiac myogenesis (Bour *et al.,* 1995; Lilly *et al.,* 1995; Ranganayakulu *et al.,* 1995). Myoblasts are present in these flies as assayed by expression of *tinman even, skipped, nautilus,* and *β3-tubulin,* showing that cell specification is not affected. Taken together, the results of these experiments show that *dMEF2* plays an essential role in all muscle cell differentiation and thus is a common component of all myogenesis.

In the mouse the MEF2 picture is more complex. There are four MEF2 genes and their overlapping expression and ability to readily form homo- and heterodimers suggest overlapping function. Indeed, in the case of *MEF2B* null mice there is no observable phenotype (Black and Olson, 1999). The *MEF2C* gene has also been knocked out, showing an embryonic lethal phenotype due to inhibited cardiac function (Lin *et* al., 1997). Detailed analysis of $MEF2C^{-/-}$ mice shows that development precedes normally to the linear heart tube stage E8.0–8.5. At E9.0 a clear reduction in size in comparison to wild-type and heterozygous littermates is evident (Lin *et al.,* 1997) (Fig. 4, see color insert). Cardiac looping appears abnormal, with the phenotypic lack of a right ventricle similar to that observed

in *dHAND* null mice (Lin *et al.,* 1997; Srivastava *et al.,* 1997). On the cellular level, endocardial and cardiomyocytes are present but appear disorganized. On the molecular level many cardiac-specific genes are downregulated, including *ANP, α-myosin heavy chain (α-MHC),* and *MLC-1a.* Other factors such as *MLC2a* and *-2v* are expressed normally, showing that some level of cardiac specification and differentiation occurs. Expression of *dHAND* initially appears normal; however, at the time of cardiac looping *dHAND* expression is downregulated within the ventricular region of the heart (Lin *et al.,* 1997; McFadden *et al.,* 2000). By *in situ* hybridization, the expression of *eHAND* is reported to be unaffected in the *MEF2C*$^{-/-}$ mice (Lin *et al.,* 1997). In our studies designed to examine the intercross of the *MEF2C* and *eHAND* knockouts, we observed a specific loss of eHAND expression at E9.0–9.5 within the left ventricular region of the developing heart as assayed by lac-z staining (Fig. 4). In contrast, *eHAND* expression within the outflow tract and lateral mesoderm is unaffected (Fig. 4). This discrepancy is difficult to assess, but it suggests that *eHAND's* expression in the ventricle is dependent on *MEF2C* or, like *dMEF2* in the fly, a lack of complete myocyte differentiation has occurred. Not surprisingly, *MEF2C*$^{-/-}$ *eHAND*$^{-/-}$ mice appear phenotypically similar to *eHAND* null mice (A. Firulli, unpublished results) and our molecular analysis of these animals is currently under way.

Due to the large size of introns and complex arrangements of exons in the 5′ UTRs, the mammalian *MEF2,* genes analysis of the promoter regions of these factors has not been reported (B. Black, A. Firulli, J. Molkentin, and E. Olson, unpublished results). Fortunately, this is not the case with *fly dMEF2,* for which promoter analysis has revealed a transcriptional module that controls expression within the heart, muscle, and brain (Cripps *et al.,* 1999; Gajewski *et al.,* 1997; Schulz *et al.,* 1996). Expression of *dMEF2* in cardiac cells is dependent on two *tinman cis* elements which are located 180 bp apart within a 237-bp region located 5.5 kb upstream from the transcriptional start site (Gajewski *et al.,* 1997). Transgenic flies show *dMEF2* expression in four of six cardial cells within each hemisegment from this enhancer, consistent with *in situ* data showing *tinman* expression within these same four cells (Azpiazu and Frasch, 1993; Bodmer, 1993). More interesting is that *dMEF2 in situs* show expression in all six of the cells within the hemisegment, indicating that in these pairs of cells expression is *tinman* independent. Mutation of either *tinman* site abolishes *dMEF2* expression and ectopic *tinman* expression can expand expression of *dMEF2* to most regions of the embryo (Gajewski *et al.,* 1997). These *tinman* binding sites are also essential for *dMEF2* expression in somatic and visceral muscle lineages (Cripps *et al.,* 1999). Interestingly, ectopic *tinman* expression fails to activate *dMEF2* expression within a subset of cells in the ventral region of the embryo, indicating that a cofactor is lacking or that an inhibitor of *dMEF2* expression is expressed with these cells. The bHLH protein twist also plays a direct role in *dMEF* transcriptional control through an E-box located within a 175-bp enhancer located 2245 bp upstream of the transcriptional start site (Cripps *et al.,* 1998). Because twist is required for

specification of the mesoderm, this finding is not surprising. Also identified within the *dMEF2* cardiac enhancer is a GATA element that when mutated causes a switch of *dMEF2* expression from the cardial to pericardial cells, suggesting that specific combinatorial interactions contribute to cell-specific expression (Gajewski *et al.,* 1998). These promoter studies show that *dMEF2* lies directly downstream of the mesodermal specification genes as well as *tinman* and the GATA factor *pannier,* which are essential for cardiac development. Because there is still more to be learned from the *dMEF2* promoter (such as the factors controlling expression in the two cells within the hemisegment), further study will likely be fruitful and may prompt the diligent to study the mammalian genes.

Recently, many interesting studies have shown the importance of protein–protein interactions between MEF2 and other transcription factors; thus, like MEF2's cousin, SRF, can potentially act as a platform to build unique transcription factor complexes. In skeletal myogenesis combinatorial interactions between MEF2 and the myogenic bHLH factors have been shown to drive muscle-specific gene expression (Black and Olson, 1998; Kaushal *et al.,* 1994; Molkentin *et al.,* 1995). It is also reported that MEF2 can interact with the neuronal bHLH factor MASH1; however, no cardiac-specific transcription factor has been reported to interact with MEF2 (Black *et al.,* 1996; Mao and Nadal-Ginard, 1996).

Recently, interactions of MEF2 proteins associated with modification of DNA structure have further implicated MEF2 as a transcription factor platform from which to build transcriptionally unique complexes. Many groups have reported specific interaction of MEF2 with the histone deacetylases (HDACs)-4 and -5 and MITR (Lu *et al.,* 2000; Miska *et al.,* 1999; Sparrow *et al.,* 1999; Wang *et al.,* 1999). Association of MEF2 via its DNA binding domain with these HDACs is modulated by the calcium calmodulin-dependent protein kinase (CaMK) (Lu *et al.,* 2000). Activation by CaMK involves destabilization of the MEF2–HDAC interaction, freeing the HDAC from the DNA. The DNA binding domain is then free for protein modifications such as phosphorylation as well as interactions with other transcription factors. Because HDACs are involved in alterations in chromatin structure, via deacetylation of histones and the promotion of nucleosome formation, an elegant model can be deduced. MEF2-binding DNA under conditions in which it is transcriptionally inactive recruits HDACs to the DNA and promotes DNA structure inhibitory to transcription. When transcriptional activation signals are initiated, destabilization of the MEF2–HDAC interaction causes HDAC to leave the DNA, where it can conceivably interact with other transcription factors and repress transcription of genes antagonistic to the new program. Once free of HDAC, MEF2 is able to be modified and through protein–protein interactions acts as a platform for activating tissue-specific gene expression. Moreover, interactions with histone acetyltransferases would allow destabilization of the local nucleosomes, allowing other factors into the complex. Indeed, it is reported that MEF2C can specifically interact with p300/CBP via interaction within the MADS domain (Sartorelli *et al.,* 1997).

It is clear that both SRF and MEF2 are essential components of many developmental systems including cardiogenesis. Through their study, understanding the building of tissue-specific transcription factor complexes and insight as to how these factors interact with the factors controlling chromatin structure have been gained. Further study of the regulatory control and the interacting proteins within this superfamily of proteins will be an exciting and informative undertaking.

D. The Zinc Finger Transcription Factors

1. GATA Transcription Factors 4, 5, and, 6

The GATA family of transcription factors may be the most relevant to cardiac development because there is strong evidence for their role in cardiac morphogenesis and cardiac cell specification. The six members of the GATA family of transcription factors are expressed in a tissue-restricted manner and play important roles in tissue commitment and differentiation (Molkentin, 2000; Orkin, 1998). All GATA proteins contain a conserved Cys-X2-Cys-X17-Cys-X2-Cys type IV zinc finger DNA-binding domain that recognizes and binds the consensus motif (A/T)GATA(A/T) (Ko and Engel, 1993; Merika and Orkin, 1993; Molkentin, 2000; Omichinski *et al.,* 1993). Moreover, there is evidence that each GATA factor may have preferences for different related sequences (Ko and Engel, 1993; Merika and Orkin, 1993; Molkentin, 2000; Omichinski *et al.,* 1993). *GATA-1–3* are important for hematopoietic development (Orkin, 1998). The remaining three members of the family, *GATA-4–6,* demonstrate expression within mesodermal and endodermal cells which make up the gut, gonad, liver, lung, and heart (Molkentin, 2000). GATA *cis* elements are important in the direct regulation of a variety of cardiac expressed genes and have roles in both embryonic development and adult-onset cardiac disease (Molkentin *et al.,* 1997; Molkentin and Olson, 1997).

GATA-4–6 show an 85% level of amino acid identity within the zinc finger DNA-binding domains and 70% amino acid identity with GATA-1 and the *Drosophila* GATA factor *pannier* (Fig. 5, see color insert). GATA-4–6 show lower identity across regions located within their N termini (45%) and C termini (35%) (Charron and Nemer, 1999; Molkentin, 2000; Morrisey *et al.,* 1997). *GATA-4–6* are expressed in partially overlapping patterns within the precardiac mesoderm, heart, and gut epithelium (Arceci *et al.,* 1993; Kelley *et al.,* 1993; Laverriere *et al.,* 1994; Morrisey *et al.,* 1996).

Temporal spatially during embryogenesis, *GATA-6* is the first expressed, detectable in the primitive streak at E6.5 (Morrisey *et al.,* 1996, 1997; Parmacek and Leiden, 1999; Suzuki *et al.,* 1996) At E7.5 *GATA-4* and *GATA-6* genes are expressed in the primitive streak mesoderm, the mesoderm near the head fold and the extraembryonic visceral and parietal endoderm, simultaneously, the *GATA-5* gene is expressed in a restricted manner within the precardiac mesoderm that is

limited to the cardiogenic (Heikinheimo *et al.,* 1994; Morrisey *et al.,* 1996, 1997; Parmacek and Leiden, 1999). At E9.5 *GATA-4–6* are expressed in the endocardium and myocardium of the presumptive atria, ventricle, and cardiac outflow tract. *GATA-4–6* are also expressed in underlying septum transversum, which gives rise to cardiac myocytes and cells of embryonic liver (Arceci *et al.,* 1993; Heikinheimo *et al.,* 1994; Morrisey *et al.,* 1996, 1997). By E12.5 *GATA-4* and *-6* are expressed throughout the embryonic heart, whereas *GATA-5* becomes restricted primarily to endocardial cells lining the atria and the endocardial cushions until E16.5, when *GATA-5* expression is undetectable (Arceci *et al.,* 1993; Heikinheimo *et al.,* 1994; Morrisey *et al.,* 1996, 1997).

Clearly, members of the GATA family are expressed early in the mesodermal cells fated to become heart, however, their wide range of expression is in contrast to a role in cell specification or differentiation events. Confirmation of their role in cardiogenesis is provided by analysis of the GATA knockout mice. Null alleles for *GATA-4–6* have been generated via homologous recombination and phenotypes of these animals show a role for GATA in heart formation. Like the phenotype observed with the bHLH factor MesP1, *GATA-4* null mice exhibit cardia bifida due to failure of the anterior intestinal portal to close (Kuo *et al.,* 1997; Molkentin *et al.,* 1997). Due to the large endodermal component of the GATA-4 phenotype, it is possible that the heart defect is secondary to these defects. Supporting evidence derives from experiments using *GATA-4*$^{-/-}$ ES cells in differentiation assays. Results from these studies show that specification to the cardiac lineage can occur in these cells (Kuo *et al.,* 1997; Narita *et al.,* 1997; Soudais *et al.,* 1995). Recently, a human chromosomal deletion on chromosome 8p23.1 has been associated with congenital heat defects, and this deletion contains the *GATA-4* gene, further supporting its role in cardiac morphogenesis (Bhatia *et al.,* 1999; Pehlivan *et al.,* 1999). Experiments using an adenovirus-mediated antisense strategy show a role for GATA-4 in cardia-specific gene expression. Inhibition of GATA-4 and -6 in postnatal cardiomyocytes shows downregulation of many cardiac-expressed genes, including *ANP, BNP, cardiac tropinin I (cTnI), α-MHC,* and *β myosin heavy chain (β-MHC)* (Charron *et al.,* 1999). Taken together, these data suggest that cardiac morphology is affected in the *GATA-4* null animals and expression of cardiac-specific genes is inhibited, suggesting an essential role for GATA-4 in cardiogenesis.

GATA-5 null mice survive until adulthood, with the only reported phenotype being an abnormal urogenital tract in female mice (Molkentin *et al.,* 2000). *GATA-6* null mice are embryonic lethal and die between E5.5 and 7.5 as a result of extraembryonic defects involving visceral endoderm (Koutsourakis *et al.,* 1999; Morrisey *et al.,* 1998). Chimeric analysis using GATA-6 null ES cells shows a role in branching morphogenesis of the lung (Keijzer *et al.,* 2001). Interestingly, in zebra fish, a mutation in the *GATA-5* gene exhibits a cardiac phenotype that is similar to that observed in the *GATA-4* null mice (Reiter *et al.,* 1999). *GATA-5* mutant fish exhibit a reduced number of myocardial precursors and reduced expression of

several cardiac-specific genes including *Nkx2.5,* suggesting a role in implementation of the cardiac gene program (Reiter *et al.,* 1999). Moreover, overexpression of *GATA-5* induces the ectopic expression of *Nkx2.5* and can produce ectopic foci of beating myocardial tissue, strongly suggesting a cell specification function in fish (Reiter *et al.,* 1999). Based on these observations in mice and fish, *GATA-5* and *-6* have documented roles in cardiac-specific gene regulation and morphological patterning of the embryonic heart.

Due to the partially overlapping expression of GATAs in the heart, there is a possibility that functional redundancies between GATA factors affect phenotype. For example, it is reported that in the GATA-$4^{-/-}$ mice expression of *GATA-6* is expanded. Interestingly, in the *GATA-6* nulls, *GATA-4* expression is reduced (Kuo *et al.,* 1997; Molkentin *et al.,* 1997). In a recent review on GATAs (Molkentin, 2000), a model is suggested in which GATA-4 restricts expression of *GATA-6* and GATA-6 positively regulates *GATA-4.* This is an intriguing hypothesis that, if supported, could reveal an intricate balance of cross-regulation. It is also reported in this review that intercross of *GATA-4* and *-6* produces nonviable double heterozygote offspring, supporting the idea of overlapping functions and the requirement of not only the presence of GATA-4 and -6 but also proper regulation of the level of expression of each factor.

As eluded to previously, some of the most compelling data implicating GATAs in cardiomyocyte specification and differentiation are the large number of cardiac-specific genes shown to be directly regulated by GATA-4–6. In fact, of all the transcription factor families discussed in this review, GATA factor downstream target genes are among the best defined. The large number of cardiac structural genes regulated by GATAs as well as its regulation of *Nkx2.5* most clearly define this family of transcription factors as playing a direct role in cell specification and differentiation and not just in cardiac morphological changes. In the fly the GATA factor *pannier* regulates the MADs box transcription factor *dMEF2* in cardioblast cells of the forming dorsal vessel, reinforcing the evolutionary conservation of function in GATA factors so much so that the mouse *GATA-4* can substitute for *pannier* functions (Gajewski *et al.,* 1999).

It has been known for some time that situations in the adult that result in increases in blood pressure can result in cardiac cell hypertrophy and that this cell enlargement is associated with the reexpression of cardiac genes normally during embryonic development. Many of these marker genes, such as *ANP* and *β-MHC,* are regulated by GATA-4. Recently, GATA-4 has been demonstrated to interact with the transcription factor NF-AT3, whose transcriptional activity is regulated via nuclear localization by the calcium-dependent phosphatase calcineurin (Molkentin *et al.,* 1998). This finding not only supports the established data of GATA-4's ability to activate cardiac-specific gene expression but also establishes that GATA factors can and do interact with other transcription factors forming combinatorial complexes that affect transcription in a synergistic manner, and these combinatorial relationships are discussed later.

2. Seeing GATA through a FOG

The last zinc finger class of transcription factors to be discussed are the multitype Friend of GATA (FOG) transcriptional cofactors that were identified through protein–protein interaction experiments. The original member FOG-1 interacts via several of its zinc fingers with GATA-1 via its N-terminal finger and acts as a regulatory cofactor in erythoid development (Fox *et al.,* 1999; Tsang *et al.,* 1997). FOG and GATA-1 synergistically activate transcription from a hematopoietic-specific regulatory region and cooperate during both erythoid and megakaryocytic cell differentiation. *FOG-1* is not expressed within the heart; therefore, it is generally thought to function solely as a cofactor for GATA-1–3.

A second FOG member, *FOG-2,* has recently been identified and shown to interact with GATA-4–6 (Holmes *et al.,* 1999; Lu *et al.,* 1999; Tevosian *et al.,* 1999). Unlike *FOG-1, FOG-2* is clearly expressed within the developing heart as well as in brain and testis (Lu *et al.,* 1999; Tevosian *et al.,* 1999). Interestingly, interaction of GATA-4 and FOG-2 results in either synergistic activation or repression of GATA-dependent cardiac promoters, depending on the specific promoter and the cell type in which they are tested (Lu *et al.,* 1999). There is also evidence of functional redundancy of FOG-1 and -2. Overexpression of *FOG-2* rescues terminal erythroid maturation of $FOG^{-/-}$ hematopoietic cells, demonstrating functional conservation (Tevosian *et al.,* 1999).

Recently, two groups reported the generation of FOG-2 knockout mice and results of these studies show that the absence of FOG-2 results in embryonic lethality at approximately E13 due to defective cardiac morphology resembling the congenital heart defect tricuspid atresia (Svensson *et al.,* 2000; Tevosian *et al.,* 2000). Ventricular hypoplasia, a common atrioventricular canal, and numerous defects consistent with tetralogy of fallot were identified. There is also a block in the formation of the coronary vessels and a lack of a tricuspid valve. From these findings, it is clear that FOG-2 is important for normal cardiac morphogenesis and is required for formation of a tricuspid valve. However, FOG-2, like the other targeted knockout models that show a cardiac phenotype, cardiac cell specification is unaffected. There is also similarity to the observation from other knockout models (retinoic X receptor-α and NTEF) that suggests combinatorial coordination is essential for cardiogenesis in vertebrates.

In *Drosophila,* the *U-shaped* gene is structurally related to *FOG-2* and can interact with the GATA factor *pannier* as well as with mammalian *GATA-4* (Fossett *et al.,* 2000). Like *FOG-2, U-shaped* is expressed within the embryonic mesoderm in the cardiogenic region. Genetic analysis of *U-shaped* hypomorphic and null alleles reveals an increase in the number of cardiac precursor cells gauged by dMEF2 heart enhancer *lac-z* staining. A similar result is observed for pericardial cell numbers. Moreover, forced expression of *U-shaped* results in a decrease in cardial cell number. This phenotype stands in contrast to *pannier* loss of function embryos in which there is an absence of cardial cell precursors (Fossett *et al.,* 2000;

Gajewski *et al.,* 1999). This suggests that *pannier* and *U-shaped* have antagonistic roles. Indeed, this is demonstrated by the inhibition of transcriptional synergy that *U-shaped* confers to *pannier* and by *tinman* regulation of *dMEF2* heart enhancer. *U-shaped* mutants also exhibit a defect in cell migration that, together with the apparent antagonistic relationship with *pannier* in cells of cardiac lineage, suggests that a subtle balance of *U-shaped* and *pannier* expression is required for proper formation of the dorsal vessel.

It is perplexing that the fly and mouse phenotypes of *FOG-2/U-shaped* have an apparent difference in their effect on cardiac number. Even though the core molecular programs between flies and mice are conserved, there are differences, such as the conflicting results presented here, that beg greater understanding. Speculation on additional vertebrate *FOG* genes, additional cofactors, and so on should be considered and in time these questions will be resolved. It is clear that in both flies and mice *FOG-2* is essential for normal cardiogenesis and the differences may reflect the differences in organ complexity. Equally clear is that gaining understanding of the coordination of transcription factor actions through protein–protein interactions such as those observed between GATA and FOG is essential to fully understanding the initialization and implementation of the cardiac program.

3. Steroid Hormone Receptor Family

Steroid hormone type receptors such as the thyroid hormone receptor and retinoic acid receptor (RAR) also contribute to cardiac development. These receptors are regulated by hormone binding and exert their influence on transcription via DNA binding through their zinc finger motif. These receptors bind ligand in the cytoplasm and the functional complex translocates into the nucleus where they homo- and heterodimerize, binding the promoter regions of the target genes and thus modulating transcription (Doevendans and van Bilsen, 1996; Mangelsdorf *et al.,* 1995). This dimerization scheme is very elegant because thyroid receptor, vitamin D receptor, RAR, and RXR can all heterodimerize with each other and regulate transcription, forming a complex combinatorial array of possible biologically active complexes dependent on the various ligand concentrations. Much of the relevance to heart development within this family of factors lies with the RXRs, although thyroid hormone has been shown to regulate ventricular myocytes by determining the MHC isoforms after birth, mediating the switching of β-MHC isoforms to α-MHC isoforms (Lompre *et al.,* 1984; Morkin, 1993). As such, we focus on experiments focused on the role of RXR in cardiogenesis.

The three retinoic X receptor RXR-α, -β and -γ comprise a ligand-activated transcription factor family that functions as a heterodimer with other retinoid receptor (RARs or RXRs), thyroid, vitamin D, and peroxisomal proliferator-activated receptors (Mangelsdorf *et al.,* 1995). These receptors have all been knocked out in mice and individual knockouts exhibit divergent phenotypes. The *RXR-*γ null mutants are viable, fertile, and morphologically normal (Mark *et al.,* 1997). *RXR-*β

null mutants are viable but the males are sterile (Mark *et al.,* 1997). The interesting phenotype from the perspective of cardiogenesis is the *RXR-α* null mice, which die at approximately E13.5 from cardiac failure with evidence of ventricular wall thinning (Dyson *et al.,* 1995; Gruber *et al.,* 1996; Mark *et al.,* 1997; Sucov *et al.,* 1994). Molecular analysis of the *RXR-α* null mice shows an expanded expression region for *MLC-2a* into the ventricles. Because ventricle wall development is hypoplastic and shows an underdevelopment of trabeculations, this persistent expression of the atrial marker suggests alterations in ventricle cell development which could be due in part to altered gene programming and inhibited cell proliferation. In follow-up studies, the RXR-α receptor was conditionally knocked out using a Cre-Lox approach (Chen *et al.,* 1998). In this study, the Floxed *RXR-α* homozygous mice were crossed into a mouse line that expressed CRE recombinase via knockin to the *MLC-2v* locus. Results of this cross showed that RXR-α was specifically targeted in 80% of the ventricle cardiomyocytes; however, a normal phenotype resulting in viable mice was observed (Chen *et al.,* 1998). These are two possible explanations for this result. First, the effects of RXR-α are not cell autonomous for the cardiomyocyte lineage: that is, *RXR-α* expression in neighboring cardiomyocytes is sufficient to generate the required transcriptional events for ventricular cardiomyocyte development and chamber maturation. Second, although *MLC-2v* is expressed early in heart development, it is not expressed early enough and the critical regulatory events that require RXR-α occur prior to the onset of *MLC-2v* expression.

In the reported analysis of the *MLC-2a* promoter, there are four retinoid response elements in the regulatory region of the gene (Kubalak and Sucov, 1999), at least one of which functions to repress expression in ventricular myocytes in a dose-dependent manner and switches from transcriptional inhibition to transcriptional activation based on the ratio of homodimer to heterodimer (Kubalak and Sucov, 1999). Further studies of this promoter and its regulation by RXR and perhaps other nuclear receptors are required to gain a better understanding of the combinatorial control of these factors.

Considering these data, it is clear that retinoid-mediated transcription via RXR receptors is a key component of normal heart development and RXR-α appears to act as an inhibitor of ventricular cardiomyocyte differentiation and/or as a regulator of their proliferation. These functions are clearly controlled by the combinatorial arrangement of heterodimerization with RARs and other nuclear receptors that create an intricate balance in transcriptional control. It will be quite a challenge to elucidate these specific interactions and mechanisms. The use of knockout and conditional knockout mice has revealed a great deal of insight into the role of retinoic acid regulation in cardiogenesis. Continued analysis using mutated receptors, domain swapping, and even two-hybrid fishing will provide more clues. Recently, a cardiac-enriched RXR-binding protein has been reported to bind DNA and interact with the RXR-α AB region (Cresci *et al.,* 1999). Further investigations into the function of this factor and continued analysis of this complex

ligand-regulated transcription factor family will prove worth the effort in understanding the cardiogenic process.

4. HF-1b

The C_2H_2 zinc finger protein HF-1b binds to the HF-1b/MEF-2v site within the *MLC-2v* promoter and serves as a transcriptional activator (Zhu *et al.,* 1993). The HF-1b factor belongs to the group of factors that bind to MEF-2-like sites in cardiac muscle gene and regulate the process of cardiogenesis. This AT-rich site of the proximal MLC-2v promoter plays a critical role in inducing the *MLC-2v* gene during cardiac development (Navankasattusas *et al.,* 1994). The synergistic interaction between the transcription factors that bind the HF-1a site and HF-1b/MEF-2 confers an anterior–posterior gradient of gene expression, which could possibly play an important role in cardiac specification (Ross *et al.,* 1996).

The cardiac *MLC-2v* is expressed more specifically in the ventricular chambers and in negligible amounts in the atrial chambers (Lee *et al.,* 1992). This regional expression occurs early in cardiac development when the heart tube is still a linear structure (Chien *et al.,* 1993; O'Brien *et al.,* 1993). The HF-1b *cis* region is conserved between different vertebrate species suggesting an important regulatory role in this chamber-specific expression (Henderson *et al.,* 1989). Later studies have shown that EFI_A/YB-1, p30, HF-1b, and MEF2 control ventricular-specific expression of *MLC-2v* by binding the HF-1a and HF-1b/MEF2 *cis* elements (Zou and Chien, 1995). HF-1b activity has also been shown to regulate the electrophysiological transition between ventricular and conduction cell lineages. Recently, homozygous *HF-1b* null mice have been generated and exhibit severe conduction system defects undergoing sudden death in the adult (Nguyen-Tran *et al.,* 2000). From these data, it is clear that HF-1b plays potential roles in both chamber specification and *trans* differentiation of the cardiac conduction system, synergistically cooperating with the *trans* factors binding to HF-1a and possible antagonistic action via DNA-binding competition with MEF2.

E. Basic Helix–Loop–Helix Transcription Factors

The bHLH superfamily of proteins is a rapidly growing family of transcription factors whose members play a major role in the development programs of many tissues. The superfamily is characterized by the bHLH domain, a DNA-binding/dimerization motif that is a short stretch of basic amino acids followed by an amphipathic α helix and followed by a loop of varying length and then by another amphipathic helix (Klein, 1994; Massari and Murre, 2000). bHLH proteins bind DNA as either homo- or heterodimers, where protein–protein interaction is mediated by the α helices which juxtapose the basic regions of each factor to form a combined DNA binding domain that recognizes a canonical sequence known as

an E-box (CANNTG). This class of transcription factors leaped into the spotlight of developmental biologists with the discovery of *MyoD*. The finding that expression of this single gene in a fibroblast cell could cause it to become programmed to the skeletal myoblast lineage where the programmed cells would exit the cell cycle, fusing into beating myotubes, was a seminal observation (Tapscott *et al.,* 1988). Not long after this observation, a large number of bHLH factors were discovered. Many of these novel genes clearly play important developmental roles in the differentiation of tissues such as nerve, blood, dermis, and pancreas (Bain and Murre, 1998; Kageyama *et al.,* 1997; Lee, 1997a,b; Li *et al.,* 1995; Naya *et al.,* 1997).

The bHLH superfamily of transcription factors forms five individual classes (Massari and Murre, 2000) but can generally be characterized into two classes, the ubiquitously expressed class A genes and the tissue-specific expressed class B genes. Class A genes, such as the alternately spliced products of the *E2A* gene E12 and E47 and HEB, are expressed in all tissues and form hetero- and homodimers with bHLH genes from class B. Class B genes, such as those comprising the myogenic bHLH family, are restricted to expression in cells of the skeletal muscle lineage and form efficient heterodimers with class A factors. The myogenic bHLH proteins were experimentally shown to be inefficient at forming homodimers. This finding helped build the paradigm that class B factors required dimerization with a member of class A in order to form a bioactive complex. With the increasing understanding of the role that the myogenic bHLH genes play in controlling skeletal myogenesis, and the identification of E-box elements within the promoters of many cardiac-specific genes, numerous labs interested in heart development began searching for the elusive *"cardio D."* Initially, the search employed low-stringency cDNA library screens, which were soon determined to be ineffective because the bHLH domain is evolutionarily conserved as a secondary protein structure, not as a primary sequence. With the development of the yeast two-hybrid library screen, it was possible to search cDNA libraries using E proteins as bait, and shortly after this development many cardiac-expressed bHLH factors were identified.

1. The E Proteins, Id Proteins, and Class A Regulation

Because E proteins are ubiquitously expressed, by default they are expressed within the developing heart. With the exception of their roles in B and T cell development, E proteins are thought to convey specific transcriptional activation through the domains of their class B partners. That is, E12/Myod heterodimers will bind and activate skeletal muscle-specific genes, whereas E12/Beta-2 will bind to and activate pancreatic- and neuronal-specific genes. It can be seen from these examples that a combinatorial relationship exists in which different class A/class B heterodimers can create transcription factors with subtly different DNA-binding preferences and interaction properties within a single cell, establishing a very elegant system that allows for very fine control of gene expression. It is apparent when considering the

regulation of bHLH transcription that it is important to consider not only when and where a bHLH factor is expressed but also what other bHLH genes are coexpressed and what modifications allow the E proteins to choose their appropriate partner.

A well-established mechanism for E protein regulation is through competition for dimer partners of class B molecules with members of the Id family of HLH proteins. Id 1–4 lack a basic region for contact with DNA; thus, dimerization between Id and E proteins results in only one-half of a DNA-binding domain, thus locking the complex in an inactive form (Langlands *et al.,* 1997). Therefore, regulation of Id gene expression will dictate the participation of E proteins in transcriptional complexes. There are four Id genes in mammals, in which all have similar dimerization affinities with E proteins although they exhibit widespread partially overlapping expression patterns (Massari and Murre, 2000; Norton, 2000). In addition to their role in negatively regulating E proteins, Id factors have also been implicated in regulation of lineage commitment, timing of differentiation, angiogenesis, and cell cycle control (Norton, 2000). There are no reports in the literature showing a direct role of Id factors in cardiogenesis of heart morphogenesis; however, based on the Id family's known functions, it is likely that they play some regulatory role.

2. Giving Cardiogenesis a *HAND*

The first class B genes of the bHLH superfamily identified as playing a role in cardiogenesis are the HAND genes, *eHAND* (*HAND1, Hxt,* and *Thing1*) and *dHAND* (*HAND2, Hed,* and *Thing2*). Using E protein probes in the yeast two-hybrid system, several groups independently cloned the *HAND* genes. *HAND* genes show highly amino acid identity between species, suggesting conserved biological function (Angelo *et al.,* 2000; Cross *et al.,* 1995; Cserjesi *et al.,* 1995; Hollenberg *et al.,* 1995). Expression of the *HAND* genes is restricted to the developing heart, lateral mesoderm, and subpopulation of neural crest cells. *eHAND* is uniquely expressed in the extraembryonic mesoderm, whereas *dHAND* is uniquely expressed in the maternal deciduum (Cross *et al.,* 1995; Cserjesi *et al.,* 1995; Hollenberg *et al.,* 1995; Srivastava *et al.,* 1995). Cardiac expression of *eHAND* and *dHAND* initiates soon after cell specification (E7.0 in mice and HH stage 8 in chick). In the chick both *d-* and *eHAND* are expressed throughout the developing heart (Srivastava *et al.,* 1995). In mice, *HAND* heart expression is partially overlapping. Both genes are expressed throughout the cardiac crescent stage (E7.0). At the linear heart tube stage (E8.0) *dHAND* is expressed throughout the heart tube; however, *eHAND* expression becomes restricted to the outflow tract and the anterior region of the tube that is fated to form the left ventricle (Fig. 6, see color insert Firulli *et al.,* 1998; Srivastava *et al.,* 1995). As cardiac looping initiates, *dHAND* expression becomes restricted to the outflow tract and the anterior region of the heart tube fated to become the right ventricle (Fig. 6). We recently isolated the cardiac enhancer that controls the right-sided cardiac expression of *dHAND* (Fig. 6; McFadden *et al.,* 2000). Our data show that this enhancer organization is complex and that two

GATA *cis*-acting elements are necessary for normal transcriptional control of right ventricle expression (McFadden *et al.,* 2000). What is interesting about this finding is that GATA-4–6 are expressed throughout the developing heart, suggesting that an additional factor(s) interacts with GATA to initiate *dHAND* chamber-specific expression. Another possibility is a regional-specific block in GATA activation of the *dHAND* enhancer via a block in DNA binding, (binding of another protein or alteration in chromosome structure) or by a direct modification to GATA (phosphorylation/dephosphorylation or protein–protein interaction). It is unknown which GATAs can activate the enhancer. It is clear that GATA-4 can bind the enhancer *in vitro,* but *dHAND* expression is unaffected in *GATA-4* null mice (McFadden *et al.,* 2000). It is also reported that *GATA-4* is downregulated in the *dHAND* null mice, suggesting that *GATA-4* is downstream of *dHAND* in the cardiac program and that *GATA-5* or *-6* may lie upstream of *dHAND*. Currently, it is unknown if *dHAND* expression is altered in the absence of GATA-5 or -6; however, it is entirely possible that functional redundancy of the GATA factors ensures *dHAND* regulation.

The contrast of the sided expression of *dHAND* and *eHAND* in the mouse with the completely overlapping expression of *d-* and *eHAND* of the chick suggests that *HAND* genes are functionally redundant in the heart. This idea is supported by the finding that chick heart development is arrested at the cardiac looping stage (HH) when cultured embryos are treated with antisense oligonucleotides for both *d-* and *eHAND,* but development is unaffected when antisense oligos to either *d-* or *eHAND* are used separately (Srivastava *et al.,* 1995).

Both *d-* and *eHAND* have been successfully knocked out in the mouse and show morphological but not cardiomyocyte-specific abnormalities (Firulli *et al.,* 1998; Riley *et al.,* 1998; Srivastava *et al.,* 1997). *dHAND* null mice are embryonic lethal and die between E9.5 and 10.5 due to cardiac and vascular defects (Srivastava *et al.,* 1997; Yamagishi *et al.,* 2000). Molecular analysis clearly shows that cardiac cell specification has occurred and that all cardiac-specific cell marker tested genes, such as *α-MHC, MLC-2a,* and *MLC-2v,* are normally expressed (Srivastava *et al.,* 1997). The only gene reported to be downregulated in $dHAND^{-/-}$ mice is the transcription factor *GATA-4*. This is a surprising finding because one would predict that *GATA-4* lies upstream of *dHAND* based on temporal expression pattern during cardiogenesis, and its ability to bind the *dHAND* promoter; thus, it is a potential regulator of *dHAND* transcription. Further study of *GATA-4*/*dHAND* is needed to answer these "chicken or the egg" questions.

Morphological findings in *dHAND* null mice show that the region of the looping heart tube that will become the right ventricle is missing. Although the morphological phenotype could be the result of a looping defect, it is unlikely based on the finding that *dHAND* expression tracks with alterations in sidedness, placing it downstream of right–left polarity signals (McFadden *et al.,* 2000; Thomas *et al.,* 1998b). When *dHAND* cardiac expression is observed in the *dHAND* null mice using our *dHAND–lacZ* mice, there is a large reduction in the cell number

of *dHAND* expressing cells (McFadden *et al.,* 2000). This suggests that *dHAND* plays a role in cell proliferation or in inhibition of apoptosis. Increased apoptosis via TUNNEL analysis has been reported in *dHAND* null mice within the first and second branchial arches, supporting the latter possibility; however, more specific apoptosis screens have not been reported (Thomas *et al.,* 1998a). Further evidence comes from zebra fish mutant *hands off,* which maps to the fish *dHAND* locus (Yelon *et al.,* 2000). *Hands off* fish exhibit a reduced number of cardiomyocytes which exhibit a morphologically altered phenotype (Yelon *et al.,* 2000). Taken together, these observations suggest that *dHAND* is not playing a role in cardiac cell specification or differentiation and that it plays a role in morphological patterning that involves cell growth and/or programmed cell death regulation.

The phenotype of the *eHAND* null mice is more difficult to analyze in relation to cardiogenesis due to pronounced defects in extraembryonic mesoderm that result in embryonic lethality between E8.0 and 9.0 (Firulli *et al.,* 1998; Riley *et al.,* 1998). It is clear that in the *eHAND* null mice the cardiac cell lineage is specified and cell differentiation, as measure by the expression of cardiac-specific structural genes such as *MLC-2a* and *MLC-2v* is uncompromised (Firulli *et al.,* 1998). Heart morphology is altered; however, it is difficult to assess if this is a direct effect of *eHAND* or a secondary effect to the extraembryonic defects. Tetraploid rescue of *eHAND* null mice suggests a looping defect similar to that observed in the *Nkx2.5* mice, which are reported to show a downregulation of *eHAND* expression (Lyons *et al.,* 1995; Riley *et al.,* 1998). These data suggest that like *dHAND, eHAND*'s function in the cardiac program lies in the control of morphology, not in cardiac cell commitment or differentiation. Future efforts to engineer an *eHAND* conditional knockout will be necessary to fully explore *eHAND*'s role in cardiogenesis without contamination from other phenotypes or embryo fusion artifacts.

In our efforts to better identify the functional role of the *HAND* genes, we have recently determined that unlike many class B bHLH factors, *dHAND* and *eHAND* exhibit a promiscuous ability to dimerize with themselves and other class B bHLH factors (Firulli *et al.,* 2000). Considering the sided expression of *dHAND* and *eHAND* within the mammalian heart, it is clear that dHAND homodimers can form in the right ventricle and *eHAND* homodimers can form in the left ventricle. Thus, dHAND–eHAND heterodimers can form at the ventricle boundaries as well as in the outflow tract (Firulli *et al.,* 2000). Moreover, heterodimerization with E proteins and other coexpressed bHLH factors such as the HES-related transcription factors (HRTs) establishes a complex combinatorial relationship in which different HAND complexes create unique bioactive dimers that recognize different *cis*-acting sequences allowing for regulation of a diverse sets of genes. Our data also suggest that broad HAND dimerization characteristics allow for negative regulation of gene expression by titration of other bHLH factors into inactive complexes similar to the way in which Id factors sequester E proteins from binding DNA (Firulli *et al.,* 2000). This type of activity was recently reported for *eHAND* and a subset of bHLH factors in tissue culture (Bounpheng *et al.,* 2000).

Our current efforts are focused on investigating protein modifications to d- and eHAND such as phosphorylation and their effects on HAND protein dimerization and DNA binding affinities.

3. *HES*-Related Transcription Factors

The hairy and enhancer of split (HES) family of bHLH transcription factors responds to the Notch signaling pathway and functions as negative regulators of transcription. HES factors are unique bHLH factors in that they bind a variant of an E-box termed an N-box (CACNAG). Their transcriptional repression can be twofold; (i) they can repress by binding an N-box within the promoter of a regulated gene, and (ii) they can inhibit other bHLH factors from binding E-box sequences by the formation of inactive heterodimers (Kageyama *et al.,* 1997). HES factors are regulated by the Notch signaling pathway, promote cell growth by inhibition of differentiation, and help establish boundaries of gene expression (Greenwald, 1998; Kageyama *et al.,* 1997; Takke *et al.,* 1999; Wettstein *et al.,* 1997).

By both database analysis and yeast two-hybrid approaches, multiple groups have identified cardiac-expressed bHLH proteins in the hairy families HRT1 (CHF2, HESR1, and HEY-1), HRT2 (CHF1, HEY-2, and HERP1), and HRT3 (HEYL) (Chin *et al.,* 2000; Kokubo *et al.,* 1999; Leimeister *et al.,* 1999; Nakagawa *et al.,* 1999; Steidl *et al.,* 2000). HRT1–3 differ from hairy proteins in that they have a glycine in place of the conserved proline within the basic region and variation in WRPW *groucho* interaction domain (Nakagawa *et al.,* 1999). The factors also share a conserved C-terminal domain of unknown biological significance (Nakagawa *et al.,* 1999). *HRT1* and *-2* are expressed in both atrial and ventricular precursors at E8.0 in the mouse by whole mount *in situ* hybridization (Nakagawa *et al.,* 1999). Northern blot analysis shows low levels of *HRT2* expression as early as E7.5 (Chin *et al.,* 2000). *HRT1* and *-2* are downregulated in the adult heart, as are most fetally expressed transcription factors; however, it is interesting that *HRT3,* which is not expressed in the embryonic heart, is upregulated after birth (Chin *et al.,* 2000; Nakagawa *et al.,* 1999). There are no reported knockouts within this subclass of bHLH factors, so any role in cell specification or morphological patterning has yet to be determined. If the HRTs play a HES-like function in the heart one might expect to see growth arrest and premature differentiation such as that observed in *HES1* null mice (Ishibashi *et al.,* 1995; Tomita *et al.,* 1996). What is known about HRT factors is that the embryonic cardiac expression of *HRT1* and *-2* is not effected in either an *Nkx2.5* or *dHAND* mutant background, suggesting that they are not downstream targets of these factors (Nakagawa *et al.,* 1999). Recently, we have shown that the *HRT* genes are capable of forming heterodimers with both *d-* and *eHAND* in biochemical pulldown assays (Firulli *et al.,* 2000). Because *HRT* genes may play a role in specifying tissue boundaries from their identity to *HES* genes and *dHAND* and *eHAND* expression patterns suggest chamber-specific function, it is appealing to speculate that HRT/HAND dimers

could play a role in chamber specification or driving chamber-specific morphological events. In pharyngeal arch and limb bud development, *eHAND* (only in the pharyngeal arch), *dHAND,* and HRT genes are also coexpressed, further suggesting a common biological function via heterodimerization or an antagonistic relationship. As more data accumulate on HRT factors, these and other questions regarding their role in cardiogenesis will be addressed.

4. *MesP1* and *-2*

Two highly related bHLH genes, *MesP1* and *MesP2*, share a near identical bHLH domain and are both located on mouse chromosome 7 within a 16-kb region (Saga *et al.,* 1996). *MesP1* expression is reported to be the earliest molecular marker expressed in heart precursor cells as assayed by migration analysis of cells out of the primitive streak in the *MesP1* lac-Z knockin (Saga *et al.,* 1999). Lineage analysis shows that *MesP1*-expressing cells first move out of the streak and populate the amnion and then move into the region of the specifying myocardium but not the endocardium (Saga *et al.,* 1999). Phenotypic observations of $MesP1^{-/-}$ embryos show a nonfused heart tube at E8.25–8.5 compared to wild-type controls (Saga *et al.,* 1999). Molecular analysis of *eHAND* gene expression shows a switch from normal left-sided *eHAND* expression to expression on the right side in $MesP1^{-/-}$ mice (Saga *et al.,* 1999). *eHAND* expression within the presumptive left ventricle is clearly established at E8.0 (Firulli *et al.,* 1998). This finding suggests improper cell migration and possibly alterations in cardiac looping. *dHAND* expression is downregulated in the atrial and left ventricular regions of the forming heart as looping begins (Fig. 6; McFadden *et al.,* 2000). Because no alteration in *dHAND* expression can be observed in *MesP1* null mice, it is likely that looping has not initiated. Based on the observations of the delay in cell migration out of the streak, the lack of cardiac morphogenesis suggests that *MesP1* may play a role in the cellular organization program controlling looping.

This initiation of delayed migration in the $MesP1^{-/-}$ mice is likely due to expression of *MesP2,* which is expressed at low levels overlapping with *MesP1* (Kitajima *et al.,* 2000). Indeed, MesP1 can rescue segmentation defects in $MesP2^{-/-}$ mice, showing that these highly related factors share some redundant functions (Saga, 1998). Moreover, the *MesP1*/*MesP2* double null mice die at approximately E9.5 completely lacking a heart (Kitajima *et al.,* 2000). Examination of these double null mice shows a complete lack of a mesodermal layer between the endoderm and ectoderm. There is also an observed buildup of cells within the primitive streak showing a lack of cell migration. Specific marker analysis shows that double $MesP1/MesP2^{-/-}$ mice have a complete lack of craniocardiac mesoderm. Axial mesoderm can be detected; however, migration of these cells arrests after E8.5 (Kitajima *et al.,* 2000). Taken together, these studies show that *MesP1* and *-2* are essential for the cell migration of mesodermal cell precursors out of the primitive streak during gastrulation. Clearly, if the presumptive heart mesoderm is not in

place for the inducers of cardiac cell specification (BMPs, etc.) lineage commitment cannot take place. In the case of the *MesP1* and *-2* genes, cell specification and morphology are affected; however, experimental evidence suggests that the role of these genes may lie upstream of BMPs, Wnts, and as yet unknown endodermal factors.

5. bHLH/ZIP Factors

These two examples of bHLH factors are actually somewhat different from other members in the family in that they also contain a leucine zipper domain, which is employed by transcription factors such as *Fos* and *Jun* to form dimers and bind DNA. In the case of the bHLHZip factors, the basic region of the bHLH is used for binding DNA and the recognition sequence is still some form of an E box. The ZIP domain can affect the recruitment of other accessory factors and can also affect the kinetics of protein–protein interactions between the dimer pairs. It is also interesting that the following factors are widely expressed and have far-reaching biological functions in numerous tissues and systems, including controlling gene expression in cardiac development.

a. USF Analysis of the *MLC-2v* and *α-MHC* promoters identified critical E box *cis*-acting elements (Navankasattusas *et al.,* 1994; Ojamaa *et al.,* 1995). Both promoters contained an E-box CACGTG, which corresponds to the DNA binding site of the bHLH/ZIP protein USF (Navankasattusas *et al.,* 1994; Ojamaa *et al.,* 1995; Xiao and Ojamaa, 1998). USF-1 and -2 are ubiquitously expressed, highly conserved throughout evolution, and can form homo- and heterodimers with themselves. In these complexes USF dimerizes and creates its DNA-binding domain via the bHLH and the ZIP region adds stability to the protein–protein interaction (Bresnick and Felsenfeld, 1994; Lu and Sawadogo, 1994). USF can also interact with other bHLH factors or bZIP factors and has been shown to behave as transcriptional activators and repressors employing mechanisms that are similar to other bHLH proteins such as the *HAND* genes and *Id* genes (Kurschner and Morgan, 1997; Pognonec *et al.,* 1997; Sirito *et al.,* 1994). Many genes are known to be regulated by USF. Downstream targets include viral genes, metabolic genes, growth-related genes, tumor-suppressor genes, tissue-specific genes, and even ribosomal genes transcribed by RNA polymerase I (Ghosh *et al.,* 1997; Gregor *et al.,* 1990; Hale and Braithwaite, 1995; Navankasattusas *et al.,* 1994; Read *et al.,* 1993; Xiao and Ojamaa, 1998).

The effect of USF can be extremely specific despite its wide range of expression and function. In studies of the smooth muscle α-actin promoter, differential regulation of expression between smooth and skeletal muscle relied on two E-boxes and the differential binding of myogenin and USF (Johnson and Owens, 1999). USF function is also contrary. For example, binding of USF can inhibit the autoactivation of *XMyoD,* promoting continued cell proliferation, whereas USF inhibits

cell proliferation in rat embryo fibroblast (Lun *et al.,* 1997; Luo and Sawadogo, 1996). Taking this very complex set of data into account, the role of USF in the expression of any gene in any tissue needs to be considered carefully.

In the 250-bp *MLC-2v* promoter the *cis* element MLE-1 was identified by *in vivo* footprinting and shown to a CACGTG E-box known to bind USF elements (Navankasattusas *et al.,* 1994). Further investigation showed that USF can also bind to the HF-1a elements in the *MLC-2v* promoter. In the *α-MHC* promoter, mutational analysis of an E-box at position -47 showed that it was necessary for the contractile response in cultured cardiomyocytes and in the intact heart. Gel shift analysis using antibodies showed the *trans*-acting factor to be USF (Ojamaa *et al.,* 1995; Xiao and Ojamaa, 1998). Based on these studies it is clear that the actions of USF on cardiac genes involve communication/interaction with other transcription factors which, through combinatorial interaction, reflect the specific transcriptional effect. Gene targeting focused on gaining more information on the role of USF in heart development must employ a conditional system for heart-specific knockout because its broad-reaching and divergent functions will likely compromise data interpretation.

b. N-myc The bHLH/ZIP factor N-myc has been implicated to play a role in the delay of cardiomyocyte differentiation and/or cardiac cell proliferation. *N-myc* knockout mice die at E10.5–12.5 and exhibit underdeveloped hearts (Charron *et al.,* 1992; Sawai *et al.,* 1993). In compound heterozygotes that expressed 15% of normal *N-myc,* the cause of death appeared to result from cardiac failure stemming from hypoplasia of the compact subepicardial layer of the myocardium (Moens *et al.,* 1993). *N-myc* expression has been shown to be downregulated in the *Nkx2.5/Csx* null mice, suggesting function downstream of cardiac specification and that morphological defects in these animals may contain an N-myc component (Tanaka *et al.,* 1999a). Moreover, in the *β*-adrenergic receptor kinase knockout, hypoplasia of the ventricle myocardium is observed coincident with a reduction in *N-myc* cardiac expression, again showing a correlation with reduced proliferation and *N-myc* (Jaber *et al.,* 1996). An interesting question that has not been addressed in the literature is whether differentiation of the cardiomyocyte population in *N-myc*$^{-/-}$ mice can be accelerated. In summary, these observations show that *N-myc* is a key component in regulating the population of cardiac cells required to form the developing heart, employing control that involves cell proliferation and inhibition of differentiation.

6. bHLH PAS Domain Factors

The aryl hydrocarbon receptor (ahR) is a member of a growing family of evolutionarily conserved ligand-activated transcription factors which include the ahR nuclear translocator (ARNT)-1 and -2, hypoxia-inducible factor (HIF)-1*α* and -2*α* (EPAS1), and the *Drosophila* gene *single-minded*. These bHLH family members

also contain a Per–ARNT–Sim (PAS) domain, which facilitates protein–protein interactions and is required for heterodimerization specificity (Pongratz *et al.,* 1998). ahR and ARNT mediate functions that relate to toxicity of halogenated aromatic hydrocarbons such as dioxin (Gonzalez and Fernandez-Salguero, 1998). ahR is the only member of the family which is ligand stimulated. Upon ligand binding *ahR* translocates to the nucleus, where it can form a heterodimer with ARNT. The complex then binds DNA to modulate gene expression. In mice deficient in *ahR* and exhibiting hepatic and immune defects, nearly half died shortly after birth; however, survivors reached maturity and were fertile (Fernandez-Salguero *et al.,* 1995). In the adults, 46% died or were ill by 13 months of age and $ahR^{-/-}$ mice developed age-related lesions in several organs, some of which were apparent after only 9 months of age (Fernandez-Salguero *et al.,* 1997). Cardiovascular alterations included cardiomyopathy (100%) with hypertrophy and focal fibrosis (Fernandez-Salguero *et al.,* 1997). Gene targeting of *ARNT* and *HIF-1α* results in defects in the developing vascular system (Iyer *et al.,* 1998; Kozak *et al.,* 1997; Maltepe *et al.,* 1997). Interestingly, *EPAS1 (HIF-2α)* knockout mice are embryonic lethal late in gestation due to physiological and not developmental heart defects (Tian *et al.,* 1998). *EPAS1* is expressed in the organ of Zuckerlandl, which is the major source of catecholamines prior to maturity of the sympathetic nervous system and adrenal medulla (Tian *et al.,* 1998). Catecholamines are essential for normal function of the embryonic and neonatal cardiovascular system, regulating cardiac output; thus, $EPAS1^{-/-}$ mice die from bradycardia (Landsberg and Young, 1994; Tian *et al.,* 1998). It is clear that *EPAS1* is not directly involved in development but in maintaining function of the embryonic heart.

Members of these xenobiotic response genes are required embryonically for cardiac function and to date studies have not ruled out possible developmental function for this interesting subclass of bHLH factors.

F. Other Transcription Factors in Cardiogenesis

1. T-Box Transcription Factors

The T-box transcription factors are rapidly expanding superfamily proteins that are evolutionarily conserved from worms to man. T-box or T-domain is a highly conserved DNA binding motif that ranges from 160 to 180 aa in length and this family is exemplified by the mouse gene *brachyury*. Crystal structure analysis of the *Xenopus* T protein brachyury shows DNA binding to a 24-nt palíndromic sequence as a dimer and this is also reported to bind as a monomer (Kispert and Herrmann, 1993; Muller and Hermann, 1997). The consensus binding site for brachyury is centered on an invariant core AGGTG flanked by 8 nt 5′ and 7 nt 3′ that exhibit some variation (Kispert and Herrmann, 1993). This large DNA-binding

consensus suggests a high variability of DNA-binding sequences encountered in this class of transcription factors.

As mentioned previously, in *Drosophila dpp* is a BMP signaling molecule that is required for heart development in the fly. Many laboratories have searched for downstream targets of *dpp* signaling, and through these efforts a T-box-containing gene *optomotor* was identified and shown to share high sequence identity to the mouse *brachyury*. Subsequent screening for vertebrate homologs resulted in the identification of the Tbx superfamily of T-box factors. A large and growing number of the Tbx genes, *Tbx-2*, *-3*, *-5*, *-12*, and *-20*, have been shown to be expressed during cardiogenesis of fish, chick, mouse, and man, and *Tbx-1* is expressed within the cardiac neural crest cells (Ahn *et al.,* 2000; Begemann and Ingham, 2000; Carson *et al.,* 2000; Chapman *et al.,* 1996; Merscher *et al.,* 2001; Yamada *et al.,* 2000). *Tbx-1* maps to the DiGeorge locus on human chromosome 22 suggesting a potential role in this congenital disease (Chieffo *et al.,* 1997). In a recent study using a Cre-Lox approach, mice harboring a 1.5-Mb deletion that corresponds to the DiGeorge locus were produced (Merscher *et al.,* 2001). Using a transgenic mouse containing a human bacterial artificial chromosome containing the *Tbx-1* gene can partially rescue the phenotype observed in the 1.5-Mb deletion (Merscher *et al.,* 2001). Moreover, mice heterozygous for a null allele of *Tbx-1* exhibit conotruncal defects (Merscher *et al.,* 2001). These data clearly demonstrate a causative role of *Tbx-1* in DiGeorge syndrome; thus, it has an important function within cardiac neural crest cells that populate and migrate out of the pharyngeal arches. Whether the function of *Tbx-1* is controlling neural crest migration or some other cellular function, the study of *Tbx-1*'s biological role will be interesting and informative.

Tbx-2, *-3*, and *-5* exhibit overlapping expression in the cardiac crescent of the chick and expression closely overlaps with the expression of BMP2 and BMP4 throughout heart development (Yamada *et al.,* 2000). Moreover, BMP2 treatment shows induction of *Tbx-2* and *-3* in noncardiac tissues in which the BMP antagonist noggin downregulates expression linking these factors as direct BMP targets (Yamada *et al.,* 2000). *Tbx-2* has been shown to repress expression tyrosinase-related protein-I via DNA binding to a TCACAC motif, which share high identity with the core binding site of *brachyury,* but no definitive role in heart development has been determined (Carreira *et al.,* 1998).

The role of *Tbx-5* in cardiogenesis has implications in controlling cell growth and mutations in this gene are associated with the autosomal-dominant congenital heart and limb defects observed in Holt–Oram syndrome (Basson *et al.,* 1999; Hatcher *et al.,* 2001; Terrett *et al.,* 1994). Studies in which *Tbx-5* was overexpressed in the hearts of chicks showed reduced cardiomyocyte proliferation and these *in vivo* proliferative effects are abolished with a gly80Arg missense mutation (Hatcher *et al.,* 2001). Study of documented *Tbx-5* mutations identified in Holt–Oram syndrome suggested alterations in biophysical interactions with various *cis*-acting target sequences resulting in a wide range of observed abnormalities (Basson *et al.,* 1999). For example, mutations in the amino-terminal portion of the

T-box which makes contact in the major groove exhibit pronounced cardiac defects, whereas carboxy-terminal mutations that affect DNA binding via the minor groove produce pronounced limb defects (Basson *et al.,* 1999). It is interesting that *dHAND* mutations in zebra fish development show both cardiac and limb abnormalities (Yelon *et al.,* 2000). It is appealing to speculate that *Tbx5* and *dHAND* interact in a common program (cell proliferation?) which if proven would add insight into the function of both factors in cardiac development. Taken together, the rapidly growing Tbx family of transcription factors are expressed early in the cardiogenic program, show evidence of direct regulation by BMPs, and are causative of congenital heart defects in the human population. Gaining a better understanding of the total number of Tbx genes through the human genome sequence, further identification of those exhibiting cardiac expression, and analysis of the knockout and mutant phenotypes are likely to be an exciting and informative area of research.

2. EFI_A/YB-1

In analysis of the *MLC-2v* promoter, the HF-1a *cis*-acting element was identified as an important component of gene expression. Through an expression library screen using the HF-1a as a probe the transcription factor EFI_A the homolog of human YB-1, was found to bind and active transcription (Zou and Chien, 1995). YB-1 is a cold-shock domain-containing factor that was expressed in all human tissues examined; however, it is reported that in human 24-week-old embryos, high levels of *YB-1* mRNA were present in heart, muscle, liver, lung, adrenal gland, and brain. In contrast, low amounts of *YB-1* mRNA were found in thymus, kidney, bone marrow, and spleen (Spitkovsky *et al.,* 1992). YB-1 binds to an inverted CCAAT box motif both as single- and double-stranded complexes (Didier *et al.,* 1988; Spitkovsky *et al.,* 1992). YB-1 has been shown to interact with many factors, such as proliferating nuclear antigen, large T-antigen of human polyomavirus, the transcription factor AP2, and cardiac ankyrin repeat protein (CARP), and has functions in transcriptional activation/repression, replication, translation, and protection from cross-linking of DNA (Duh *et al.,* 1995; Mertens *et al.,* 1998; Safak *et al.,* 1999; Zou *et al.,* 1997).

YB-1's binding to the $HF1_A$ element allows for transactivation *in vitro,* and antiserum raised against the related factor YB-3 can inhibit *YB-1* DNA binding to the promoter (Zou and Chien, 1995). In an effort to understand how a ubiquitous transcription factor could regulate a cardiac-specific gene, a yeast two-hybrid screen was performed using YB-1 as bait (Zou *et al.,* 1997). The result of this effort was the cloning of CARP. By Northern analysis CARP is expressed in cardiac and skeletal muscle (Zou *et al.,* 1997). Immunostaining shows that endogenous CARP is localized in the cardiac myocyte nucleus. Cotransfection assays indicate that CARP can negatively regulate an HF-1–TK minimal promoter in an HF-1 sequence-dependent manner in cardiac myocytes (Zou *et al.,* 1997). CARP also

displays a transcriptional inhibitory activity when fused to a *GAL4* DNA-binding domain in both cardiac and noncardiac cell contexts. In *Nkx2.5* null mice, *CARP* is reduced suggesting a downstream position for *CARP* from *Nkx2.5* in the cardiac regulatory network (Zou *et al.,* 1997). Because CARP does not directly bind DNA, its association with YB-1 is necessary for its negative regulation of transcription and illustrates how tissue-restricted cofactors can combine with other factors to generate gene-specific mediated events. As with the other ubiquitously expressed factors, the ability to coordinate transcriptional specificity via interaction with other factors is essential for YB-1 cardiac-specific function.

3. TEA Domain Proteins: TEFs

Members of the TEA domain class of transcription factors have also been shown to play a role in cardiogenesis, and it is likely that many of the transcription factor families described here are highly evolutionarily conserved from plants to man (Blatt and DePamphilis, 1993). The TEA domain is a 66- to 68-aa domain predicted to form three α-helical structures that are not characteristic of a helix–turn–helix motif (Burglin, 1991). In vertebrates, TEF-1 is the parental member of TEA domain factors and was identified as binding to the following related *cis*-acting elements: SV40 GT-IIC (GGAATG), SphI (AGTATG), SphII (AGCATG), and muscle-specific M-CAT (GGTATG) enhancer. The M-CAT site is of interest because it is found in many muscle-specific genes such as α- and β-MHC, *cTNT,* and *sACT* (Larkin and Ordahl, 1999). A consensus DNA binding site that binds TEF-1 has recently been reported (Jiang *et al.,* 2000). Via binding site selection, 31 independent clones were obtained; 15 contained single binding sites and 16 contained direct repeats separated by a 3-bp spacer (Jiang *et al.,* 2000). The predominate consensus half-site was GGAATG (67%), and the other elements were of the form 5′-G(A)GA(T/C)ATG-3′ (Jiang *et al.,* 2000). *TEF* genes are implicated in transcriptional regulation as early as the two-cell stage suggesting multiple roles for these factors in a variety of genetic programs (Kaneko *et al.,* 1997; Kaneko and DePamphilis, 1998). Moreover, there is evidence that, like MEF2 factors, *TEF* genes are alternately spliced (Zuzarte *et al.,* 2000). Taken together, variations in the DNA-binding specificity and exon usage of TEF factors allow for these widely expressed factors to orchestrate tissue-specific gene control.

Four TEF factors (NTEF-1, RTEF-1, DTEF-1, and ETEF-1) have been described in vertebrates, and there are likely more. Expression patterns are overlapping and issues of functional redundancies clearly exist. The prototype member of the family, *NTEF-1,* is expressed in skeletal muscle with lower levels of expression observed in heart, pancreas, placenta, and brain (Blatt and DePamphilis, 1993; Shimizu *et al.,* 1993; Stewart *et al.,* 1996). During embryogenesis *NTEF-1* is ubiquitously expressed up to E10.5, at which time some tissue-restricted expression is observed (Jacquemin *et al.,* 1996). It is reported that *NTEF-1* null mice were generated using an insertional mutagenesis approach (Larkin and Ordahl, 1999), NTEF-$1^{-/-}$

mice are embryonic lethal at approximately E11.5 due to cardiac wall thinning and reduced trabeculations similar to the *RXR-α* null mice. This phenotype is not surprising due to the large number of cardiac structural genes that rely on MCAT *cis* elements for normal expression. What is surprising is that molecular analysis of these MCAT-dependent genes shows normal levels of expression. These results are quite puzzling and suggest that *NTEF-1* expression is not critical for early cardiogenesis, perhaps due to functional redundancy of other *TEF* genes and/or because the phenotype is a result of cardiomyocyte proliferation.

RTEF-1 is the second member of the TEF family of transcription factors and also shows high levels of amino acid identity between species. RTEF-1 mRNA, like NTEF-1, is alternately spliced, altering the coding sequence around the TEA domain, suggestive of altered transcriptional activity and variation in combinatorial interactions (Hsu *et al.,* 1996; Stewart *et al.,* 1994; Yockey *et al.,* 1996). Interestingly, rodent RTEF-1 shows 5-aa substitutions within the TEA domain compared to NTEF-1 but the human and chick RTEF-1 maintain higher conservation within the TEA domain (Hsu *et al.,* 1996; Jacquemin *et al.,* 1996; Stewart *et al.,* 1994, 1996; Yockey *et al.,* 1996). It is easy to assume that the amino acid changes in RTEF-1 reflect alterations in DNA binding specificity or preferences for different partners in combinatorial interactions; however, it is more difficult to apply this logic when considering that only the rodent RTEF-1 exhibits this diversion. Expression of *RTEF-1* is observed in both skeletal and cardiac muscle and placenta in humans and gizzard in chick. Interestingly, in chick, liver and brain expression is evident, whereas in humans no such expression is detected. Moreover, in mouse *RTEF-1* is detectable in the lungs and skin in addition to striated muscle. In our opinion this apparent variation in expression is highly suggestive of functional redundancy of the TEF family members. Because overlapping expression allows for reciprocal compensation there is no selection for expression patterns to maintain themselves in evolution. If one examines *HAND* expression in chick, *d-* and *eHAND* are expressed throughout the developing heart, whereas in mice the expression patterns are right- and left-sided, respectively. Moreover, both *HAND* genes must be knocked out (via antisense) to arrest chick cardiac development, whereas the single *HAND* gene knockouts show cardiac developmental abnormalities (Firulli *et al.,* 1998; Riley *et al.,* 1998; Srivastava *et al.,* 1995, 1997). It is possible that *TEF* genes have a similar relationship and therefore differ in the expression profiles between species.

Embryonic expression in the mouse follows closely that reported for *NTEF-1* showing uniform expression in early stages of development and later becoming restricted to skeletal muscle precursors (Jacquemin *et al.,* 1996; Yockey *et al.,* 1996). To date, there have been no reports on gene disruption for *RTEF-1,* and further speculation regarding its role in cardiogenesis must a wait completion of these studies.

DTEF-1 is highly expressed in the heart; however, only low levels are detected in skeletal muscle of the chick (Azakie *et al.,* 1996). *DTEF-1* transcripts are also

detected in the chick gizzard and lung and at low levels in kidney. There are two reported isoforms of DTEF-1 (DTEF-1A and DTEF-1B) whose differences are attributable to alternative splicing at the C terminus of the TEA DNA-binding domain (Azakie *et al.,* 1996). Murine embryonic expression is reported as broad, similar to that for *N*- and *RTEF-1* (Yasunami *et al.,* 1996).

Another TEF-1 family member, *ETF* (embryonic TEA domain-containing factor), has also been identified; however, there are no reports of cardiac expression in the adult or during embryogenesis (Jacquemin *et al.,* 1996; Yasunami *et al.,* 1995). Taken together, the importance of the MCAT *cis* element to the expression of cardiac-specific genes and the ability of TEF-1 family members to transcriptionally activate genes by binding MCAT elements along with the cardiac phenotype associated with *NTEF-1* strongly indicate that these factors are an integral component of heart development. It is also clear that the overlapping expression, extremely high amino acid conservation, alternate spliced isoforms, and large diversity in biological function will make dissecting the specific role of the TEA box transcription factors in cardiogenesis a challenging undertaking.

4. Sry-Box Transcription Factors

The *Sry* gene is required for mammalian male sexual determination. It binds DNA through an 80-aa high-mobility group (HMG) domain that allows factors to bind DNA with a varying degree of specificity. Interestingly, unlike most DNA-binding factors HMG domains contact DNA via the minor groove, resulting in dramatic DNA bending (Grosschedl *et al.,* 1994; Kamachi *et al.,* 2000; Wegner, 1999). In homology screens, factors found to be related within the DNA-binding domain to *Sry* have been termed *Sox* genes (*Sry* box genes). A large number of *Sox* genes have been identified and many have roles in development (Kamachi *et al.,* 2000; Prior and Walter, 1996; Schilham and Clevers, 1998; Wegner, 1999). The consensus DNA binding site for *Sox* genes has been deduced to be WWCAAWG, and all factors appear to bind this consensus (Wegner, 1999). Thus, because no *Sox* factor is expressed tissue specifically, how do *Sox* genes convey tissue- or developmental-specific transcription? The answer is through interaction with other transcription factors; a large body of work supports this conclusion (Kamachi *et al.,* 2000; Wegner, 1999).

Regarding cardiac development, one gene (*Sox-4*) has been identified to be involved. In the mouse knockout of *Sox-4,* embryos die at E14 from circulatory failure resulting from abnormal development of the endocardial ridges into the semilunar valves and outlet portion of the muscular ventricular septum (Schilham *et al.,* 1996). Mice appear normal at E13, after which *Sox-4*$^{-/-}$ mice develop edema and die within 24 h. Increased heart rate is observed which indicates valve malfunction (Schilham *et al.,* 1996). Null mice also exhibit septation defects affecting the outlet portions of the ventricles and great arteries, resembling the phenotype of the human congenital defect common arterial trunk type I and II. It will be

interesting to determine what other transcription factors interact with *Sox-4* to maintain endocardial ridge development, and further study into this and the determination of the potential role of other *Sox* factors in cardiogenesis may yield greater understanding of congenital heat diseases.

5. CMF-1 E-Box Binding without the bHLH Motif

CMF-1 was originally isolated and characterized from a chick embryo expression library and was found to bind E-box and dimerize with ubiquitous HLH proteins (Buckingham, 1994b; Wei *et al.,* 1996). Further analysis of CMF-1 revealed that it was not related to bHLH factors, having a higher amino acid identity to the centromeric protein F, mitosin, and LEK1, and is hypothesized to play a role in cell division and differentiation (Pabon-Pena *et al.,* 2000). It was found that disruption of *CMF-1* expression using antisense retrovirus inhibited the expression of *MHC* during differentiation of cardiac mesoderm; however, antisense virus had no effect on *MHC* expression in fully differentiated cardiomyocytes (Wei *et al.,* 1996). These results support the theory that CMF-1 is involved in the control of cell division and growth. Recently, cellular localization of CMF-1 protein was found to precede expression of cardiac structural genes (Pabon-Pena *et al.,* 2000). Interestingly, *CMF-1* expression is independent of BMP signaling control; however, *CMF-1* expression is not sufficient to induce cardiac-specific gene expression (Pabon-Pena *et al.,* 2000). In light of these findings, it is clear that *CMF-1* plays a role in cardiac cell growth and also in maintenance of expression of sarcomeric proteins. Further study of this interesting non-bHLH E-box binding factor will likely reveal a greater understanding of the link between differentiation and cell cycle control within the cardiomyocyte.

6. The Forkhead Transcription Factor Myocyte Nuclear Factor

Myocyte nuclear factor (MNF) is a member of forkhead domain transcription factors that play a role in cell proliferation and differentiation. *MNF* is expressed in the skeletal and cardiac muscle of the mouse embryo as well as in some regions of the brain (Garry *et al.,* 1997). In the adult expression is restricted to the satellite cells committed to the skeletal muscle lineage. The *MNF* message is alternately spliced, producing two proteins (MNF-α and MNF-β) which are reciprocally expressed in satellite cells from injured muscle (Lai *et al.,* 1993; Yang *et al.,* 1997). In the *MNF* knockout, animals survive to adulthood and exhibit defects in muscle regeneration due to injury (Garry *et al.,* 2000). There is also a binary phenotype observed in $MNF^{-/-}$ at E9.5 showing evidence of growth retardation, pericardial edema, and ventricular dilation (D. Garry, personal communication). Although it is not completely clear what role *MNF* may play in cardiogenesis, its role in maintaining skeletal muscle satellite cell populations suggests that it may also play a role in keeping cardiomyocytes from exiting the cell cycle prematurely.

IV. Combinatorial Interactions of Cardiac-Expressed Transcription Factors

When considering the transcription of a given gene and the isolation and characterization of its promoter, without exception multiple *cis*-acting elements, whether TATA boxes or E-boxes, are arrayed in unique arrangement. This arrangement creates an on/off switch, but in reality it creates a complex regulatory circuit that controls not only on/off but also where, when, and how much. The *trans*-acting factors, the transcription factors that bind these various *cis* elements, can exhibit activity on their own; as long as a *cis* element is in the general location of a promoter, adding the *trans* factor will affect transcription. In a simple system, adding *cis* elements and *trans* factors would simply provide an additive relationship in activity; however, we all know that this is not the case. In many cases, adding two factors creates synergy, and this synergy implies communication (cross talk) that addresses the details underlying on/off, such as when, where, and how much. As alluded to previously, protein–protein interactions likely play a role in this cross talk between individual transcription factor complexes that are arrayed across a gene's promoter. This type of communication clearly occurs in cardiogenesis and understanding the combinatorial interactions between the transcription factors that regulate when, where, and how much will be a major scientific challenge in the near future.

In considering the transcription factors implicated in cardiac development the GATA and Nkx classes of factors by both temporal expression, analysis of knockout phenotype, and known downstream target genes are at the highest position in the known cardiogenic program. Not surprisingly, for both of these classes there are documented cases of interactions with other transcription factors that modulate their transcriptional effect. In the case of GATA known partners are FOG-2, NF-AT3, Pit-1, Pu-1, and Nkx2.5. GATA also interacts with the P300/CBP protein, which via its HAT activity can alter chromatin confirmation by destabilization of nucleosomes allowing for ready access to other *trans* factors and an open DNA confirmation conducive to transcription. Not quite a transcription factor, P300/CBP interacts with a large number of transcription factors such as MEF2 and makes contacts with the general transcription factor machinery (TAFs and TFIID). This interaction calls attention to the often ignored role of DNA structure and the prerequisite communication between the enhancer binding factors, the general factors, and RNA polymerase II in the regulation of gene expression. In the *P300* knockout cardiac defects are observed. Is this phenotype due to direct effects of P300 or an indirect effect of braking down the combinatorial network required by GATAs, MEFs, and other P300 interacting factors in cardiac structural gene expression? Why is there a heart defect from such a central player in transcriptional regulation? The reason is likely that the heart is the first organ to form during emrbyogenesis and thus is the most likely tissue to be affected. In analysis of the *ANP* promoter

both GATA and Nkx *cis* elements are present. Studies of Nkx2.5 regulation of this promoter show synergistic interaction of Nkx2.5 with GATA-4 (Shiojima *et al.,* 1999). The synergy was found to be dependent on Nkx2.5 DNA binding but was independent of GATA-4 DNA binding, strongly suggesting that direct interaction between the factors facilitated the effect. Moreover, in analysis of artificial gene promoters positive synergy is observed with GATA-4 and Nkx2.5 on multimerized Nkx *cis* elements in contrast to negative regulation of GATA-4 transcriptional activity by Nkx2.5 on a promoter driven by GATA *cis* elements. These results suggest that additional factors are involved in the GATA–Nkx complex and/or that the specific arrangement and type of *cis* elements available for protein binding can play a function in dictating biological output. This implies that the position of the *cis* elements does matter. You can move a *cis* element and add a *trans* factor and you will get a transcriptional effect, but will this complex still talk to the other *cis* elements in the same way? This question has yet to be answered.

Using MEF2 as an example, protein–protein interactions show synergy in skeletal muscle with the myogenic bHLH factors and DNA binding is not a prerequisite (Molkentin and Olson, 1996). Recently, such as MEF2 has been shown to interact with P300/CBP as well as with members of the HDAC family. Because MEF2 is bound to DNA, HDAC binds the basic region and represses transcriptional activity. When the "on" signal is conveyed this interaction is destabilized, releasing the HDAC to perhaps form nucleosomes in another region of the genome and MEF2 is then able to activate transcription.

In the case of MEFs and bHLH proteins, these factors bind DNA as homo- or heterodimers. Not only is a combined DNA-binding domain created but also a combined target capable of interactions with specific sets of proteins. In examining the well-characterized promoters, such as *MLC-2a* and the milieu of factors MEF2, YB-1, CARP, Nkx, and GATA that are known to bind and affect transcription. When considering the combinatorial possibilities between these factors, we can see how general, specific, and nontranscription factors can clearly define on/off, where, when, and how much. There is documented functional redundancy of related transcription factors and this redundancy will be reflected in the combinatorial circuitry. Like a backup circuit, a missing factor shuts a circuit and a backup circuit takes its place. If there is no GATA, there is no P300 recruitment, if there is MEF2, P300 is targeted to the transcription complex. This idea may help explain why all the reported knockouts with heart defects show cardiac cell specification and expression of many of the downstream target genes that they regulate. It may also suggest that gene targeting of *cis*-acting elements that control tissue-specific expression will fail to show the expected phenotype due to these complex interaction compensations. In organisms such as *Drosophila,* the circuits are simpler and removing one transcription factor has a more pronounced effect. Consider Christmas lights: In the older type, one bulb would burn out and they would all not work. In the newer type, a bulb burns out but the rest work fine. Why? Because they are wired more efficiently and the circuitry compensates.

V. Concluding Remarks

In skeletal muscle the myogenic bHLH genes are exclusively expressed and their upregulation in a cell can initialize a programming of that cell to the skeletal muscle lineage. This unique situation has not been encountered in the cell programming of other tissues, certainly not the heart. Does this mean that there is no *cardioD*? It is possible that it exists, but based on the transcription factors that have been identified to date and the understanding of how these factors can communicate via limitless combinatorial relationships, lack of *cardioD* in the genome of mice and men would not be difficult to understand. With the entire human genome at our fingertips, if more transcription factors lie out there in the pool of approximately

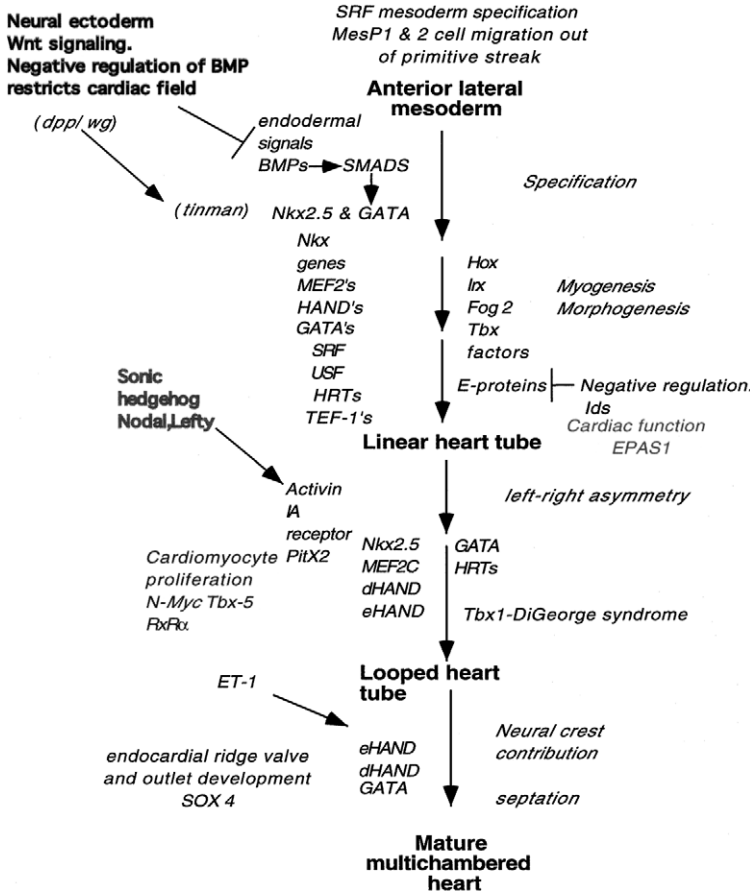

FIG. 7 Flowchart of transcription factors and inducers that modulate cardiogenesis.

30,000 genes they will quickly be fished out and analyzed. Figure 7 attempts to lay out the positions and functions of the various factors described in this review. The challenge is not the *in situs* or knockouts; the challenge is to examine the instruments within the orchestra of cardiac cell specification and differentiation and listen to how they play off each other, understanding the melodies and the harmonies that make up this great symphony.

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Neuroactive Steroid Mechanisms and GABA Type A Receptor Subunit Assembly in Hypothalamic and Extrahypothalamic Regions

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Gonadal- and neuronal-derived steroids are capable of altering brain functions through two basic mechanisms: slow (genomic) and rapid (novel nongenomic membrane) types of activities. The genomic activities that are circumscribed to the numerous neuronal and glial expressed receptor actions involve transcriptional processes regulated largely by classical steroids. On the other hand, rapid nongenomic activities are linked to the stereoselective interactions of potent neuroactive steroids. It appears that both of these steroid mechanisms can be successfully evoked at the ligand-gated heteroligomeric GABA type A receptor. However, the precise structural prerequisites and type of molecular steroid interactions implicated in this neuronal target have not been fully investigated. This article reviews the most common subunits (α, β, and γ) of the native GABA type A receptor involved in signaling pathways of slow and rapid steroidal mechanisms. Different β-containing compositions ($\alpha_1\beta_{1-3}\gamma_2$) are necessary for the slow type of mechanism, whereas different α-containing constructs ($\alpha_{2-6}\beta_{1/2}\,\gamma_{2/2L}$) are linked to the rapid type. Because of the major role played by neuroactive steroids in GABA-dependent neuroendocrine and sociosexual events, distinction of the specific subunit combination is essential not only for elucidating neuronal communicative expressions during such events but also for elucidating their potential neuroprotective role in neurodegenerative disorders.

KEY WORDS: GABA type A receptor, Bimodal modulation, Estrogen, Neurosteroids, Genomic regulation, Fast-acting modulators, Hypothalamus.

I. Introduction

Until the past few decades, neurobiologists had largely argued in favor of the sex steroids estrogen and progesterone controlling the numerous brain functions by mechanisms that are linked to gene transcription and protein synthesis. In this classical genomic model for steroid action, it is necessary that the hormones freely cross the plasma membrane and induce conformational changes of their respective intracellular receptors upon binding. Afterwards, the activated intracellular steroid homo- and/or heterodimer receptor complexes are translocated to the nucleus, where sex steroids by binding to specific regulatory regions of DNA promoter are capable of alterating transcriptional functions (Pettersson *et al.,* 1997). At the same time, these ligand-activated receptors have also been associated with the modification of transactivational responses through protein–protein interactions of other gene transcriptional factors (McEwen, 1991; O'Keefe *et al.,* 1995). Recently, significant neurotrophic activities exerted by estrogens on neuronal developmental and neurochemical differentiation properties of specific brain sites, which are connected to the highly dimorphic physiological and behavioral activities, have accounted for a resurgence of interest in the morphofunctional role of this class of hormones. Such interest seems to stem mainly from the steroid-dependent neurogenic plasticity of different brain regions (McEwen *et al.,* 1997) occurring as early as the first postnatal days.

In fact, it is in this biological phase that steroid receptor-enriched (androgen, progesterone, and α/β subunits of estrogens) brain areas are profoundly active (McEwen, 1992; Kuiper *et al.,* 1997) via genomic mechanisms (McEwen, 1991) toward the topographical and morphological arrangements of synaptic formation in both hypothalamic and extrahypothalamic regions (Lewis *et al.,* 1995; Toran-Allerand, 1996; Compagnone and Mellone, 1998). Although the classical genomic response is in perfect synchrony with protein-regulated functions, this model cannot account for all steroid responses because some biological functions induced by steroids occur within seconds or a few minutes (Wong *et al.,* 1996) in the absence of gene transcriptional activities. As a consequence, a great amount of attention has been directed toward the involvement of steroids in neuronal excitability responses via the cell surface. In this particular interaction, steroids called neurosteroids for the first time (Baulieu, 1981) and currently recognized as "neuroactive steroids" (Baulieu, 1998; Rupprecht and Holsboer, 1999) rapidly regulate membrane ion conductance activities in the central nervous system (CNS) through specific interactions with some of the major neurotransmitter receptor systems, such as the heteroligomeric ion-gated GABA type A (Majewska *et al.,* 1986; Harrison *et al.,* 1987).

Modulatory activities of neurosteroids have also been described on glycine-activated chloride channels and on neural nicotinic acetylcholine and glutamate receptor complexes (Valera *et al.,* 1992; Yaghoubi *et al.,* 1998). In most cases,

the stereoselective structural and conformational requisites of the previously mentioned sex hormones tend to represent a necessary step for the facilitation of steroid-dependent transmission mechanisms in endocrine functions and in cognitive and spatially regulated tasks (Maren *et al.,* 1994; McEwen *et al.,* 1997). Included in the list of this class of steroids are the two most potent 3α-hydroxy-reduced pregnane neurosteroids (3α-hydroxysteroids), the allopregnanolone 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP) and 3α,5α-tetrahydrodeoxycorticosterone (THDOC), which are capable of producing anxiolytic, hypnotic, anesthetic, and anticonvulsant activities (Majewska, 1992; Lambert *et al.,* 1995). These effects are accomplished by neuroactive steroids either potentiating or inhibiting GABA-gated currents via their allosteric interaction with a distinct GABA type A neurosteroid site (Majewska *et al.,* 1986; Puia *et al.,* 1993; Zhu and Vicini, 1997). Another group of steroids that is also considered important for the neuroactive steroid class is that which is currently recognized as excitatory steroids: dehydroepiandrosterone and pregnenolone plus their conjugated sulfate ester.

Within the neuroendocrine circuits, particularly those of the hypothalamic medial preoptic area that comprise one of the largest populations of cells expressing gonadal steroid receptors (Simerly *et al.,* 1990), the GABAergic–steroid interaction reportedly induces alterations of synaptic morphology (Inoue *et al.,* 1993) with consequent key roles on reproductive events such as the preovulatory surge of gonadotropin release (Watson *et al.,* 1995). In the same manner, other reproductive activities, such as pregnant and maternal behaviors, seem to also depend on steroid interactions occurring within this major neurotransmitter receptor system of other hypothalamic sites (Amico *et al.,* 2000). On the basis of the diverse sex steroid mechanisms involved in neuronal propagating systems, this review discusses recent developments in the field of brain steroids, with attention devoted to the selective estrogenic (genomic) and neuroactive steroid (membrane type) modulatory responses induced by some of the major GABA type A receptor subunit compositions.

II. Influence of Sex Steroids on Adult Central Nervous System

The cerebral pool of estrogens and neuroactive steroids, in addition to peripheral steroidogenic derivation, provides brain cells with discrete regulatory functions. They behave like hormones secreted by gonads and placenta, like parahormones synthesized from circulating androgens and progestins (in close proximity to neuron targets), and like neurosteroids when synthesized by oligodendrocytes. Within the brain, neuroactive steroids and estrogens are able to exert a strong influence on neural functions beyond the neurendocrine system, including mood, fine motor skill, and cognitive behaviors (Luine and Rodriguez, 1994; O'Neal *et al.,*

1996; McEwen *et al.,* 1997). This is accomplished either through their interaction with intracellular receptors or via membrane-bound receptors with their end product being mostly linked with modulatory functions of the major neurotransmitter systems through enzymatic induction of the neuromediator and/or receptor subunit assembly, respectively (Sagrillo and Selmanoff, 1997; Maitra and Reynolds, 1999). Earlier studies established that it was the actions of intracellular receptors, occurring mostly in the traditionally known hypothalamic and preoptic regions but also in prominent extrahypothalamic regions such as the amygdala, cortex, and hippocampus, that dominated the multiple cell signaling pathways. In the classical genomic model, it was the highly active role of gonadal hormones (particularly estrogens) that was responsible for the triggering of gene transcriptional factors such as cAMP response element binding protein and the brain-derived neurotrophic factor (BDNF). Although estrogens have been recognized as a key regulatory element of molecular neuronal communicative responses of neurotransmitter receptor systems, in the case of GABA type A receptor subunit constituents, it is unknown which type of mechanism predominates.

A. Estrogens

Mapping studies of the 18-carbon-length aromatized steroid (estrogen) in different hypothalamic and extrahypothalamic regions showed a discrete density of estrophilic neurons containing receptors for this sex hormone (Simerly *et al.,* 1990). Recent studies have demonstrated that estrogens have a general enhancing effect on neuronal GABA type A receptor function via a dramatic increase in axosomatic and axodendritic spine synapses of the GABAergic neuronal population in both these estrogenic target areas (Párducz *et al.,* 1993; Weiland and Orchinik, 1995). In certain conditions, a downregulating effect of estrogens on glutamic acid decarboxylase and its major product (GABA), through low levels of BDNF, has pointed to a reduction in both size and frequency of miniature inhibitory postsynaptic currents. Consequently, changes of estrogen-dependent neuronal excitatory and inhibitory drives are directly involved in the altered biochemical neuronal functions, although the variations of intracellular calcium levels are likely the same conditions responsible for the formation of new dendritic spines (Murphy *et al.,* 1998a). Regarding the specific estrogenic activities at the receptor level, it has been shown that the neuroselectivity actions of steroids depend on precise structural parameters (Fig. 1), such as an allopregnanolone skeleton (i.e., the A ring is reduced in the 5α-conformation), which are lacking in estrogens (Gee *et al.,* 1988). These structural steroid requisites suggest that the greater structural and/or functional neural maintenance exerted by different levels of estrogens is preferentially of the genomic type. As a consequence, the receptor-regulated transcription mechanisms of this sex steroid seem to be the dominant means of hormonal expression involved in the modification of the nuclear gene program of brain cells, especially

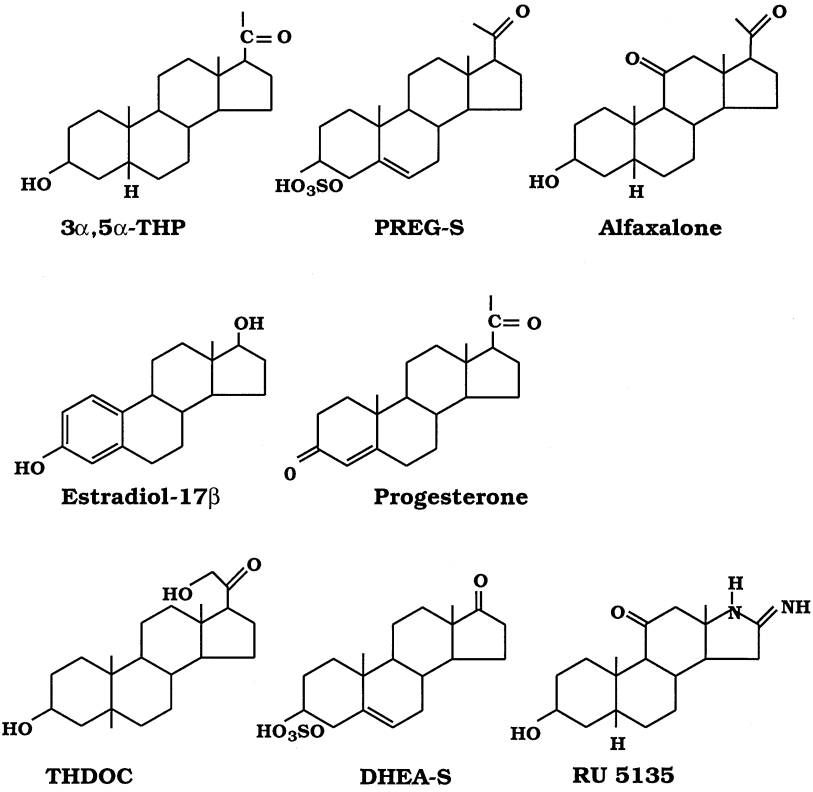

FIG. 1 Chemical structure of natural occurring neuroactive steroids and synthetic steroids. DHEA-S, dehydroepiandrosterone sulfate; PREG-S, pregnenolone sulfate; RU 5135, 3α hydroxy-16 imino-5β-17-azandrostan-11-one; THDOC, tetrahydrodeoxycorticosterone; 3α-5α-THP, 3α hydroxy-5α-pregnan-20-one.

during the various phases of the estrous cycle (Woolley and McEwen, 1993) such as the local intrinsic GABAergic biphasic modulation of luteinizing hormone secretion (Herbison *et al.,* 1991; Wagner *et al.,* 2001).

Dendrites, which are considered to be the main receptive site of the neuron, can be exposed to altered patterns of spacing and shaping, thus affecting excitatory and inhibitory postsynaptic potentials (Van Pelt *et al.,* 1997). Moreover, morphological studies have revealed a net change in the number of axodendritic spines in the ventromedial hypothalamus (VMN), in the pyramidal and dentate regions of the hippocampus during the estrous cycle (Inoue *et al.,* 1993), as well as in the different developmental stages (Rasia-Filho *et al.,* 1999). Hence, the morphological and functional changes of neurons in dentate and pyramidal hippocampal areas following elevated levels of estradiol indicate that there is an estrogenic component associated with an amelioration of cognitive functions such as learning and

memory processes (O'Neal *et al.,* 1996; Warren and Juraska, 1997). Since these changes occur in brain regions that are enriched by intracellular steroid receptors (Simerly *et al.,* 1990) or that display robust responses to estrogens, an intense synaptogenesis role of this sex steroid during certain stages of the reproductive cycle is suggested (Gazzaley *et al.,* 1996; Woolley and McEwen, 1996). Indeed, estrogen replacement in both sexually active rats and those exhibiting an altered estrous cycle improved spatial reference memory (Markowska, 1999), indicating that most reproductive and nonreproductive neuroprotective estrogenic roles, promoted by estrogen α receptors (Ogawa *et al.,* 2000), are predominantly of the genomic type. The receptor that is involved in cognitive functions has recently been noted to represent a critical link in the estradiol-mediated protection against brain dysfunction (Dubal *et al.,* 2001) and the prevention of apoptotic neuronal cell death (Harms *et al.,* 2001), whereas the β receptor seems to be vital for astrocyte and neuronal survival in morphological aberrations of some degenerative diseases (Wang *et al.,* 2001).

Interestingly, this class of steroids is not only capable of producing physiological effects through the mediation of estrogen receptors but also has displayed neuroprotective functions via local hormonal effects on neuronal cell lines lacking functional estrogen receptors (Morley *et al.,* 1992; Green *et al.,* 1997). In this case, neuroprotection independent of estrogen receptors has been demonstrated to necessitate elevated nonphysiological estrogen doses (Sawada *et al.,* 1998), whereas only low doses are required for the optimal facilitation of estrogen receptor-mediated gene transcription. In addition, neuroprotection independent of female sex steroid receptors has been shown to occur in neuronal cultures in which the estrogen receptor antagonist tamoxifen, which has been demonstrated to increase synaptic density (Silva *et al.,* 2000), did not interfere with *N*-methyl-D-aspartate (NMDA)-induced neuronal death (Weaver *et al.,* 1997). An activity that appears to be closely related to the endogenous antioxidant capacity of the estrogen molecule (Behl *et al.,* 1997) might in part be the consequence of its modulatory neurotransmission activity mainly in neuronal pathogenesis that is linked to status epilepticus (Reibel *et al.,* 2000), depressions (Halbreich, 1997), Alzheimer's and Parkinson's disease, and overall cognitive impairments of not only the telencephalic regions (Twist *et al.,* 2000) but also the nigrostriatal regions (Leranth *et al.,* 2000). Of the most important neuronal receptor systems involved in this kind of activity (NMDA and dopamine), monoamine oxidase A is a key target even though, as shown by its significant estradiol-dependent reductions in both *in vivo* and *in vitro* experimental models (Ma *et al.,* 1995; Agrati *et al.,* 1997), the estrogenic effect indicates an antidepressant activity, whereas the underlying neuroprotective validity of this hormone remains to be resolved. Other neurotransmitter systems, such as the cholinergic and serotoninergic systems, are also implicated in the previously mentioned diseases, and estrogens may thus decrease the effective symptoms of Alzheimer's disease either through reduction of G proteins coupled to serotonin receptors (Mize and Alper, 2000) in postmenopausal women (Halbreich, 1997) or

through upregulation of choline acetyltransferase (Baskin *et al.,* 1999) and tyrosine hydroxylase (Leranth *et al.,* 2000). In addition, the influence of estrogens has been extended to other neuronal pathogenesis, such as the short-term restoration of middle cerebral artery occlusion (Hawk *et al.,* 1998) and fimbria–fornix lesions (Shi *et al.,* 1997), which precludes the involvement of nongenomic mechanisms primarily in brain regions known to be devoid of nuclear receptors for this class of steroids (Smith *et al.,* 1988; Wong *et al.,* 1996). Results from electrophysiological studies, in which estrogens accounted for enhanced modulatory neuronal functions within seconds, tend to link the partial antioxidant effect of this sex steroid to neuroprotective role in some brain regions and for particular dysfunctions (Green *et al.,* 1997). The importance of dimorphic components on the levels of steroid-specific subunit sequence interactions of the major membrane neurotransmitter receptor systems such as GABA type A has been considered (Flood *et al.,* 1992; Facciolo *et al.,* 2000). Indeed, estrogens have been shown to prevent and rescue tissue from focal ischemic damage to a greater degree in females than in males (Zhang *et al.,* 1998; Liao *et al.,* 2001) by activating neurotrophins (Toran-Allerand, 1996) and their receptors against neurotoxicity associated with glutamate (Singer *et al.,* 1999). On the other hand, estrogens may be exerting a similar activity by directing the catabolism of β-amyloid precursor protein toward the nonamyloidogenic pathway (Goodman *et al.,* 1996) or by controlling the synthesis of TAU, another important component of the neurofibrillary tangle (Ma *et al.,* 1993). Further biomolecular studies will be required before the effects and the estrogen-dependent neuroprotective mechanisms of cerebral vascular disorders can be fully addressed and exploited for potential therapeutic application.

B. Neuroactive Steroids

Since the discovery of the modulatory actions of GABA type A receptors by psychoactive drugs, researchers have obtained numerous data on the alteration of GABA-stimulated chloride conductance by steroids. This coincides with the proposal that not only steroids synthesized *de novo* in the CNS but also the potent tetrahydro metabolites 3α-hydroxysteroids (Fig. 1) be considered as neuroactive steroids (Majewska, 1986). In early works, neuroactive steroids efficaciously displaced *t*-butylbicyclophosphorothionate (TBPS; preferential ligand of the chloride ion channel receptor) from the chloride channel as well as enhanced the binding response of muscimol and benzodiazepines (BZDs). The expression of recombinant GABA type A receptors and subsequent electrophysiological recordings have enabled the molecular characterization of neurosteroid effects at the subunit level (Puia *et al.,* 1993). Their rapid action indicates a probable membrane-mediated effect because no intracellular receptor has been shown to exist even though the oxidation of pregnanes has been demonstrated to regulate gene expression via progesterone intracellular receptors (Rupprecht and Holsboer, 1999). In this class

of steroids it is necessary to recall the anesthetic alphaxalone and the anticonvulsant 3α-hydroxylated, 5α-, or 5β-reduced metabolites of progesterone and THDOC. Although their highly lipophilic nature enables them to easily cross the blood–brain barrier, the brain exhibits a highly active capacity to synthesize these hormones without the aid of peripheral sources. The endogenous steroid products termed neuroactive steroids, which can be synthesized *ex novo* from sterol precursors (Jung-Testas *et al.,* 1989) due to the elevated activity of the cytochrome P_{450} enzymes (Corpéchot *et al.,* 1997), appear to produce similar conductance activities through modifications of the GABA type A receptor properties (Gee *et al.,* 1988). Preparations of synaptoneurosomes and brain membranes have been used to demonstrate that the various steroid effects are caused by either a change in the binding affinity or a change in the number of binding sites (Lopez-Colome *et al.,* 1990). In conjunction with these studies, it was possible to link the positive allosteric neuromodulation of 3α,5α-THP and THDOC—characterized by a 5α-reduced pregnane derivative, an α-hydroxyl group at C3, and a ketone at C20—to enhance burst duration and channel opening frequency in a comparable manner to that observed for barbiturates (Zhu and Vicini, 1997). As a consequence, the predominance of this class of steroids in the different encephalic regions has extended its participation in other receptor-dependent neurochemical processes of the major inhibitory neurotransmitter system via nongenomic mechanisms. These include modifications in mRNA levels of the rate-limiting enzymes ($GAD_{65/67}$), which are involved in the synthesis of GABA with subsequent variations of GABA level, and/or density differences of GABA type A receptor binding and subunit mRNAs (O'Connor *et al.,* 1988; Herbison *et al.,* 1995; McCarthy *et al.,* 1995). From the previous considerations, and also from similar reproductive and antiaggressive effects induced by BZD agonists (Skolnick *et al.,* 1985; Saldivar-Gonzales *et al.,* 1993), it is possible to ascribe strong mimicking actions of GABA type A and BZD sites to 3α-hydroxysteroids (Harrison *et al.,* 1987; Majewska *et al.,* 1992), whereas an inhibiting effect seems to instead characterize the chloride ion channel receptor (Gee *et al.,* 1988; Canonaco *et al.,* 1990). Moreover, a potential pharmacological value of the different neuroactive steroids has supported their clinically beneficial role not only in a variety of normal behavioral activities but also in several neuropsychiatric disorders that are characterized by changes of emotional state and of the neuronal excitability level. In this context, recent physiologically and pharmacologically induced fluctuations in plasma and/or brain concentrations of allopregnanolone have made it possible to establish that this neuroactive steroid is capable of modulating GABA type A receptor plasticity and associated behaviors (Weiland and Orchinik, 1995; Serra *et al.,* 2000) as well as exerting sedative and anxiolytic properties (Bitran *et al.,* 1991) and maintening homeostasis during hormonal responses to stress (Patchev *et al.,* 1994). From these studies, it is clear that neuroactive steroids interact in a stereoselective manner with a site that is distinct from those of both BZD and barbiturates. Despite the considerable amount of attention given to the hypothesis that this putative-specific steroid

binding site is located on the GABA type A receptor complex, there is no biochemical evidence of a steroid recognition site due to the multiphasic concentration–electrophysiological responses (when applied extracellularly) and to the different receptor binding effects of the 3α-hydroxysteroids (Gee *et al.,* 1995). However, it appears that heteroligomeric combinations of the major subunits of this receptor system might account for the irregular neurosteroidal activities. Thus, although this aspect has opened up an ever-growing field regarding molecular GABA type A-dependent steroid mechanisms, there is no clear consensus on the type of recombinant GABA type A receptor subunit composition involved in the sequential and specific conformational state implicated in the expression of such mechanisms.

III. Relevance of the Recombinant GABA Type A Receptor as a Target of Steroidal Activity

A large body of evidence has revealed that GABA type A receptors, along with the nicotinic acetylcholine receptor, the glycine receptor, and the 5-HT3 receptor, belong to a superfamily of homologous transmitter gated-ion channels which are able to exert a fast type of synaptic transmission (Sigel and Buhr, 1997). The sites of the heterooligomeric receptor system are targets of a variety of pharmacologically and clinically important drugs which are responsible for anxiolytic, anticonvulsant, and sedative–hypnotic activities. Among these drugs are the allosteric modulators that do not interact directly with the GABA type A binding site but exert their action by binding to additional sites of this receptor complex that in turn induce conformational changes and hence influence the binding properties of other sites (Sieghart, 1992). Consequently, it is evident that the close interaction among the different receptor sites of this molecular complex—specific sites for GABA, BZDs, convulsants, barbiturates, and steroid hormones—is structurally related to the GABA type A receptor subunits (Olsen and Tobin, 1990; Rabow *et al.,* 1995). Knowledge of the previous relationships stemmed from early molecular studies of this receptor system in which the screening of brain cDNA libraries with oligonucleotide probes, constructed according to the available sequences, led to the identification of numerous related subunits (Sieghart, 1995).

A. Pharmacology and Structure of GABA Type A Receptor Subunits

The heterooligomeric GABA type A receptor protein has been shown to consist of at least seven classes of genes encoding a long list of subunits. Of the 19 different mammalian subunit genes, those forming the heteromeric assemblies of α, β, and γ subunits (Fig. 2) are fundamentally not only considered the most common, due

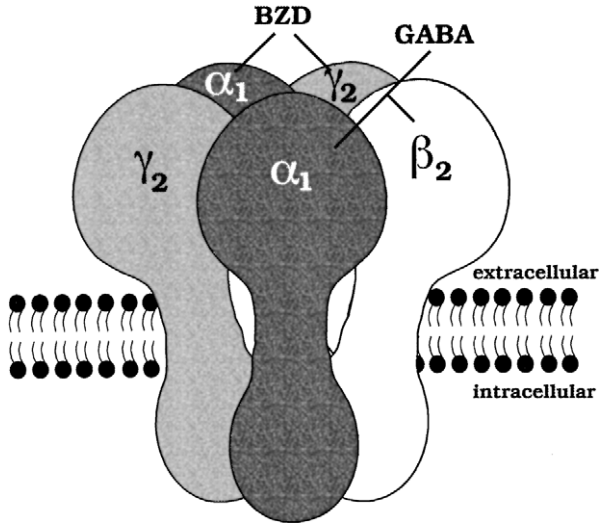

FIG. 2 A schematic and representative model of the major GABA type A receptor subunits along with the number of isoforms constituting each subunit. Even though numerous subunit combinations are possible, the $\alpha_1\beta_2\gamma_2$ combination appears to be the most widely distributed type. To date, seven classes of subunits have been described; however, only the three most common subunit classes are displayed since the positions of the other four are still in dispute.

to elevated numbers of isoforms (6α, 4β, and 3γ), but also constitute the composition that determines the overall biophysical and pharmacological properties of the GABA type A receptor (MacDonald and Olsen, 1994; McKernan and Whiting, 1996; Barnard *et al.,* 1998). The other subunits, which are numerically inferior (1δ, 1ε, 1π, and 3ρ) inclusive of splice variants, especially of γ_2 (γ_{2S} and γ_{2L}), have only recently been included (Whiting *et al.,* 1990; Korpi *et al.,* 1994; Barnard *et al.,* 1998; Neelands and MacDonald, 1999; Moragues *et al.,* 2000), even though, for example, the ρ subunit is generally related to GABA type C receptors (Wang *et al.,* 1995). The structural and functional predominance of these major subunits stems mainly from the specificity of subunit–GABA type A agonist and/or antagonist interaction.

In particular, studies on imunoprecipitation (Amin and Weiss, 1993), receptor (Ebert *et al.,* 1994), and behavior (DeLorey *et al.,* 1998) showed that the β subunits are highly specific for the binding of muscimol to its GABA type A receptor site (Wafford *et al.,* 1994; Taylor *et al.,* 1999). On the other hand, only α subunits, from the demonstration that residue 101 is the main recognition site for BZDs (Davies *et al.,* 1998), have been shown to be photoaffinity labeled by BZDs (Sigel and Buhr, 1997). γ Subunits are specifically required for conductance of chloride receptor channel and are essential for allosteric modulation of BZDs (Pritchett *et al.,* 1989; Sigel *et al.,* 1990; Sigel and Buhr, 1997). Interactions of BZDs at

the receptor level, together with their long history of therapeutic application as anxiolytic, anticonvulsant, sedative–hypnotic, and antiaggressive, have strongly influenced studies on the GABA type A receptor system. In this context, and due to the presence of high-affinity binding sites for BZDs in vertebrate brain, researchers originally recognized GABA type A and BZD sites as basic sites of this neurotransmitter receptor complex.

Each of the protein molecules contained within the GABA type A receptor complex consists of a large hydrophilic NH_2 terminal and a cystine loop formed by two conserved cysteines. This formation is followed by four putative transmembrane domains and a large intracellular loop which contains possible phosphorylation sites (Olsen and Tobin, 1990). Within each subunit class, the various members exhibit homologies in thier amino acid sequences of approximately 60–80% (Sieghart, 1995). The coexistence of GABA type A receptors, the nicotinic acetylcholine receptors, some glutamate receptors, and the strychinine-sensitive glycine receptors in a superfamily of transmitter-gated ion channels is likely due to the homology in their amino acid sequence and in the structure of their subunits as well as to the necessity for five subunits in the formation of the channels (Sigel and Buhr, 1997). The assembly of the heterooligomeric GABA type A receptor is a complex, multistep process involving the encoding of a single gene for each subunit (Klausberger *et al.,* 2000), and the various genes for the individual subunits cluster on different chromosomes.

For example, the genes encoding α_5 and β_3 subunits, together with genes for the α_4 and γ_3 subunits, have been localized on chromosome 7 of mice, which corresponds with human chromosome 15 (Danciger *et al.,* 1993). On the other hand, in humans chromosomes 4 and 5 contain the genes encoding $\alpha_{1/2}$, α_1, $\beta_{1/2}$, and $\gamma_{1/2}$ subunits (Hicks *et al.,* 1994). Following the subsequent localization of other subunit genes, it was possible to demonstrate that the genes actually occur in groups, with each cluster containing genes of the α, β, and γ/ε classes. In line with the further isolation of the genes involved in the transcription of the specific mRNAs encoding the different subunits, it has been proposed that these clusters are derived from a series of gene duplication events from a single ancestral $\alpha\beta\gamma$ gene cluster (McLean *et al.,* 1995). Furthermore, efforts have been made to adapt the different expression systems for the isolation and pharmacological characterization of the recombinant GABA type A receptor system. Early attempts to rapidly encode the desired receptor subunits used direct injection of mRNAs in single *Xenopus* oocytes (Levitan *et al.,* 1988), but most of the present investigations dealing with the expression of the various recombinant receptors rely on transfected and endogenously expressed cell lines (Moss *et al.,* 1991).

These approaches, which are performed in binding studies as well as by eletrophysiological techniques, have proven to be much more useful for encoding the different subunits. It is now possible to obtain, according to the type of subunit sequence, the activation of one of the multiple recognition sites of the GABA type A receptor and hence the allosteric modulation of GABA-gated currents in transfected

cells by the potent neuroactive steroids (Puia *et al.,* 1993; Lambert *et al.,* 1995). Such observations also suggest that, due to probable sensitivity differences of the GABA type A receptor deriving from the distinct sequence combinations, there may be more than one type of mechanism accounting for the modulatory actions of the neuroactive steroids, including increasing the channel opening frequency and open time and prolongation of a slow phase of channel deactivation (Twyman and MacDonald, 1992; Zhu and Vicini, 1997). Further knowledge of the configurational and structural properties of this receptor system could provide a substantial contribution toward the elucidation of the biomolecular role of the various subunit combinations on behavioral activities expressed during normal neurophysiological situations, particularly during pathophysiological and psychological disorders such as stress, Cushing's syndrome, and epilepsy.

B. Steroid-Dependent Action via Genomic Mechanisms

In the classical genomic model, it was primarily the hypothalamic estradiol-dependent behavioral effects that focused attention on the direct sex steroid activity of specific recombinant GABA type A receptor sites. Earlier work reported successful facilitation of sociosexual behaviors, such as lordosis (Donoso and Zàrate, 1981) and aggression (Skolonick *et al.,* 1985; Shibata *et al.,* 1989), and also neuroendocrine secretion (Racagni *et al.,* 1982) when GABA was added either to the medial preoptic area and anterior hypothalamic area or to the ventromedial hypothalamic nucleus. These results are obtained for the same hypothalamic regions in which estradiol, alone or in combination with progesterone, readily promoted the previously mentioned activities (Takahashi *et al.,* 1985; Meisel *et al.,* 1990). From similar studies and after the demonstration of estrogen receptors colocalized to GABAergic neurons (Flugge *et al.,* 1986), there has been continuing interest in steroidogenic–GABA type A subunit interactions responsible for the numerous neurobiological events, especially during the different stages of the estrous cycle (McCarthy *et al.,* 1995; Grattan *et al.,* 1996). In this field, autoradiographic methods have proven to be useful experimental tools due to their ability to supply, in a regional-specific fashion, quantitative evaluations that have supported both genomically dependent and nondependent postsynaptic GABA type A receptor activity.

For the genomic type of steroid mechanism, the first important result obtained indicated that the estrogen-dependent variations of the preferential β-containing GABA type A receptor subtype agonist (muscimol) in dense steroid receptor hypothalamic areas such as the medial preoptic and anterior hypothalamic area (Fig. 3). Interestingly, lack of an estrogen-induced increase in muscimol binding was reported for the ventromedial nucleus of the hypothalamus in both rodents and other insectivores due to a reduced expression level of α_1 (Facciolo *et al.,* 1999; Davies *et al.,* 2000). This subunit, along with β_2, has largely been demonstrated in hypothalamic sites to be essential for the binding of muscimol (Davies and

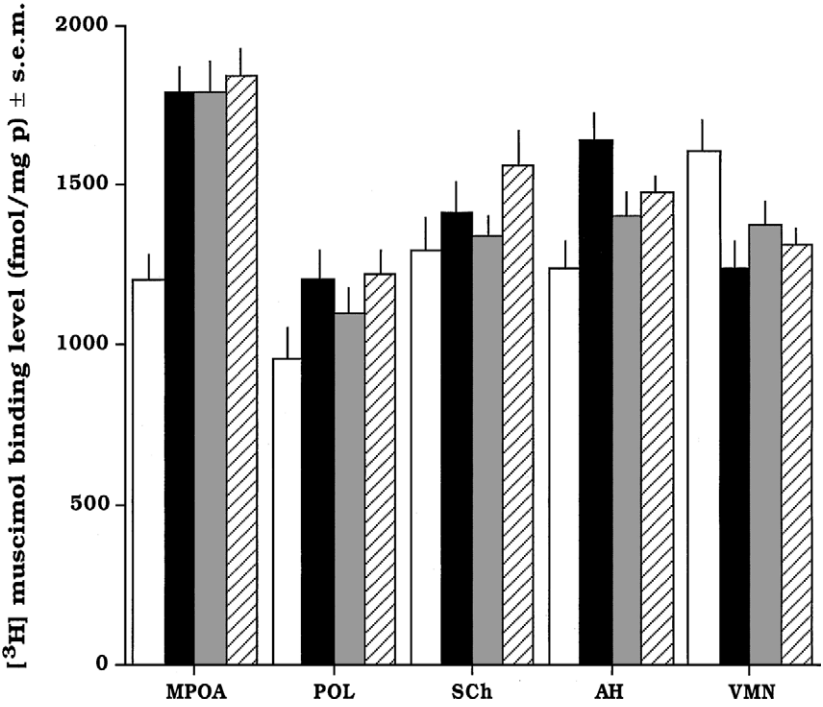

FIG. 3 Effects of estradiol (■), progesterone (▩), and estradiol + progesterone (▨) on β_2-containing GABA type A receptor and compared to controls (□) in some hypothalamic areas of the female rat. AH, anterior hypothalamus; MPOA, medial preoptic area; POL, lateral preoptic area; SCh, suprachiasmatic nucleus; VMN, ventromedial hypothalamus nucleus (data in part modified and integrated with those of Canonaco *et al.,* 1989a).

McCarthy, 2000) and that of the GABA type A receptor BZD site zolpidem (Crestani *et al.,* 2000), which binds at an affinity that depends on the subunit composition (Ruano *et al.,* 1992; Facciolo *et al.,* 2000). Note that no further estrogen-dependent binding changes were registered in both hypothalamic and extrahypothalamic areas, even after the administration of the naturally occurring and behaviorally potent derivative of 3α-hydroxysteroids (progesterone) (Fig. 4) (Canonaco *et al.,* 1989c, 1993a).

Other CNS regions in which the same combined treatment did not alter either the β-enriched or the γ-enriched GABA type A receptor subtype levels were the central canal and substantia gelatinosa of rat lumbar spinal cord (Schwartz-Giblin *et al.,* 1988). Note that the effects of both steroids have instead supplied contrasting activities in the same rodent brain regions, i.e., combined estradiol and progesterone treatment accounting for either enhanced (Perez *et al.,* 1986) or decreased (O'Connor *et al.,* 1988; McCarthy *et al.,* 1992) levels of β-enriched GABA type A receptors, especially in the hypothalamus of the female rat. These results are quite

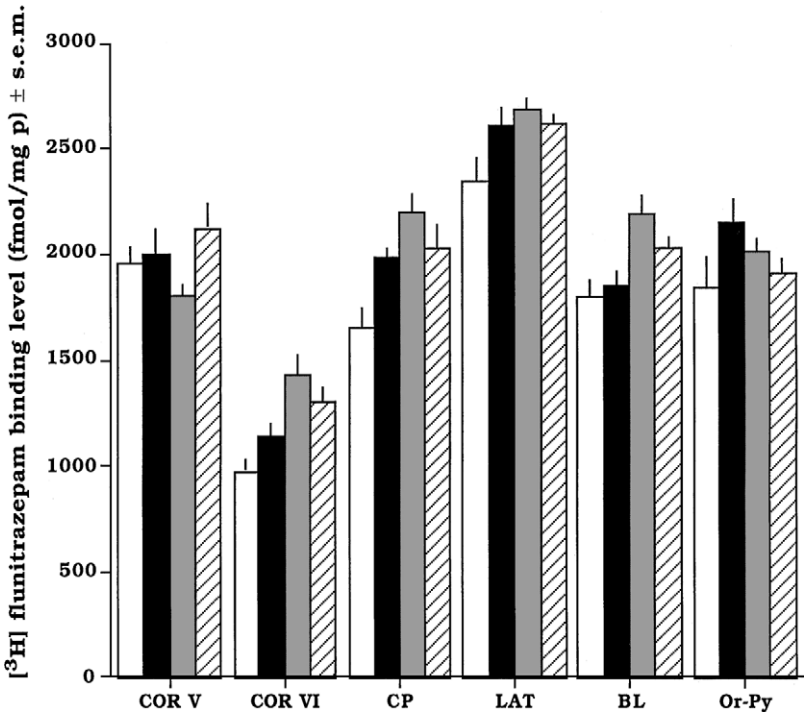

FIG. 4 Effects of estradiol (■), progesterone (▩), and estradiol + progesterone (▨) on α_1-containing GABA type A receptor and compared to controls (□) in some extrahypothalamic areas of the female rat. BL, basolateral amygdala nucleus; COR V–VI, cortex V–VI layer; CP, caudate putamen; LAT, lateral amygdala nucleus; Or–Py, oriens–pyramidalis CA1 layer (data in part modified and integrated with those of Canonaco *et al.,* 1989c, 1993a).

relevant if we consider that both steroids are responsible for reduced aggression and promotion of mating behavior in the female hamster (Takahashi and Lisk, 1985; Fraile *et al.,* 1986) but not in the female rat. It is possible that the differing and various estrogen- and/or progesterone-dependent modulatory changes of β and, to a lesser extent, γ subunit levels (Facciolo *et al.,* 2000), which are also correlated to the diverse behavioral expression patterns (Pratt *et al.,* 1993; Zhao *et al.,* 1996), might be operating in a species-specific manner. Interestingly, such a differentiated steroid-dependent relationship of the GABA type A receptor in rodents appears to be determined in the very early developmental stages since it is during this period that the type, levels, and pattern of mRNAs encoding for the various subunits are dramatically modified (Sieghart, 1995).

For example, even before synapse formation occurs, most neuronal cells contain mRNA for at least six subunits, of which the β_1 subunit mRNA seems to

be prevalent during the later part of the postnatal period, whereas α_2, α_3, $\beta_{2/3}$, and $\gamma_{1/2}$ subunit mRNAs, which are constant during the embryonic phase, are sixfold higher after the 10th day of development (Poulter *et al.,* 1992; Serafini *et al.,* 1998). Following this period, while α_2, α_3, and γ_1 transcript levels begin to decline, the quantities of α_1 and γ_2 mRNA begin to dramatically increase (Laurie *et al.,* 1992; Jones *et al.,* 1997). As a consequence, since steroid-induced organizational processes of some brain regions have not yet been completely terminated, a mismatch between levels of the different mRNA coding subunits and the pharmacological modulatory capability of the different GABA type A receptor components (Williamson and Pritchett, 1994) could very likely be accounted by a premature deficiency of gonadal-derived sex steroids. Thus, an early and continuous exposition to hormone influences does constitute a crucial and necessary step for the expression of mRNAs coding for the β-containing GABA type A receptors such as $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_{2/3}\gamma_2$, which represent the most frequent compositions of the fast type of synaptic inhibition (Benke *et al.,* 1996; Banks *et al.,* 1998; Serafini *et al.,* 1998). On the other hand, the combinations $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_3\gamma_2$, which are specific for type I (high-affinity) GABA type A receptor BZD site, appear at a later period (Poulter *et al.,* 1993; Ma and Barker, 1995; Sieghart, 1995; Davies *et al.,* 1998).

Recent studies, however, have considered the β subunit, and particularly β_3, a prime constituent of GABA type A receptor, and lack of such a subunit either leads to mortality or those that survive are accompanied by cleft palate and display frequent myoclonus and occasional epileptic seizures (Homanics *et al.,* 1997; Krasowski *et al.,* 1998). Moreover, these β-containing subunit combinations and that including β_1 appear to be the major subunit compositions that are preferentially regulated in a different fashion in extrahypothalamic and hypothalamic areas by stress levels of the steroids (Cullinan and Wolfe, 2000). In this context, the nongenomic inhibitory events of primarily β-containing GABA receptor activities in brain regions such as the cerebellum that lack detectable levels of intracellular steroid receptors (Smith *et al.,* 1987) may not be limited to just the fast-acting synaptic processes. Surprisingly, investigators have found that this steroid utilizes more than one type of signal transduction mechanism in which membrane potential changes induced by estrogens appear to include a cAMP-dependent kinase A system (Minami *et al.,* 1990). Other work evaluating the neuroprotective actions of estrogens on glutamate excitoxicity alterations of cortical neurons showed that these steroids rapidly induced an enhanced mitogen-activated protein kinase activity and in this manner defended neuronal functions (Singer *et al.,* 1999). In addition to the estrogenic influences arising from transcriptional factors, there is evidence indicating that estrogen receptors participate in mediating their effects via the interaction of intracellular signaling cascades—actions which strongly depend on excitatory amino acid-gated calcium channels (Mermelstein *et al.,* 1996). Whether these genomic mechanisms rely strictly on estrogen transduction signal is not known; however, phosphorylation or calcium-releasing processes are considered

to be key elements responsible for the estrogenic transcriptional regulatory events of neurotransmitter receptor systems (Watters and Dorsa, 1998). In the case of the GABA type A receptor system, prolonged exposure to steroids may trigger an increase in discrete dendritic subfields, which in turn alters the neuropharmacological properties of this receptor system in major brain regions of degenerative and mood disorders (Murphy *et al.,* 1998b; Howell *et al.,* 2000; Orchinik *et al.,* 2001).

C. Steroid-Dependent Action via a Membrane-Type Mechanisms

Although estrogenic influences on GABA type A receptor subunit interactions have provided important relationships, the contrasting effects of this class of steroids on neurotransmitter receptor activities have induced researchers to focus their attention on a local membrane type of mechanism in which the potent 3α-hydroxy neurosteroids have largely accounted for the facilitation of a sex steroid–GABA type A interaction. This mechanism is strongly supported not only from the elevated progesterone doses producing comparable GABAergic-dependent antiaggressive effects (Canonaco *et al.,* 1990; Bitran *et al.,* 1991) and playing a pivotal role during pregnancy and lactation (Thomas *et al.,* 1995), but also from the greater GABA type A and BZD receptor activities in hypothalamic and extrahypothalamic areas such as the hippocampus and cortex (Canonaco *et al.,* 1989b,c, 1993a; Schumacher and McEwen, 1989). Moreover, progesterone treatment alone and not concomitantly with estrogen proved to be sufficient for the increase in oxytocin mRNA levels in both paraventricular (Thomas *et al.,* 1995) and supraoptic nuclei (Fénelon and Herbison, 2000) via the alteration of the BDZ sites of the GABA type A receptor.

The well-described interactions of the 3α-hydroxysteroids at the intracellular level of the CNS areas, both enriched and lacking intracellular steroid receptors, result from the direct interaction with the neurosteroid site of the GABA type A receptor in a barbiturate-like manner (Majeswa *et al.,* 1986; Gee *et al.,* 1988; Canonaco *et al.,* 1993b). However, even though the specific neurosteroid site, which is important for progesterone-modulatory GABA type A activities, is an integral part of this receptor system, this does not necessarily imply a positive neurosteroidal action because coupling of GABA type A receptor components is not a sufficient condition to evoke any hormonal action. Rather, it seems that the specific subunit composition of the GABAergic receptor system determines the type of neurotransmitter receptor activity. Indeed, following the promotion and potentiation of GABA type A receptor-activated membrane current by the potent 3α-hydroxysteroid (3α,5α-THP) and the synthetic steroid alphaxalone, studies have revealed that the different α-containing subunit combinations account for the maximal efficacy of neuroactive steroids (Lan *et al.,* 1991; Lambert *et al.,* 1995; Maitra and Reynolds, 1999).

The enhanced binding potencies and maximal responses of 3α,5α-THP toward the major GABA type A agonist (flunitrazepam) and partial agonists of the recombinant GABA type A receptor are predominantly directed at the $\alpha_x\beta_3\gamma_2$ combinations ($x = 1, 3, 6$) in an allosteric manner (Maksay *et al.,* 2000). Specificity of these α subunits was further provided by the prevention of allopregnanolone synthesis by the 5α-reductase inhibitor finasteride, accounting for the reduction of progesterone-dependent increased levels of α_1, α_3, and α_5 subunit mRNAs in cerebellar granule cells (Follesa *et al.,* 2000). In an earlier study, we showed that the concomitant application of 3α,5α-THP and GABA accounted for the inhibition of TBPS binding (Canonaco *et al.,* 1993b) in hypothalamic and extrahypothalamic areas (Fig. 5), which were either enriched or devoid of steroid concentrating

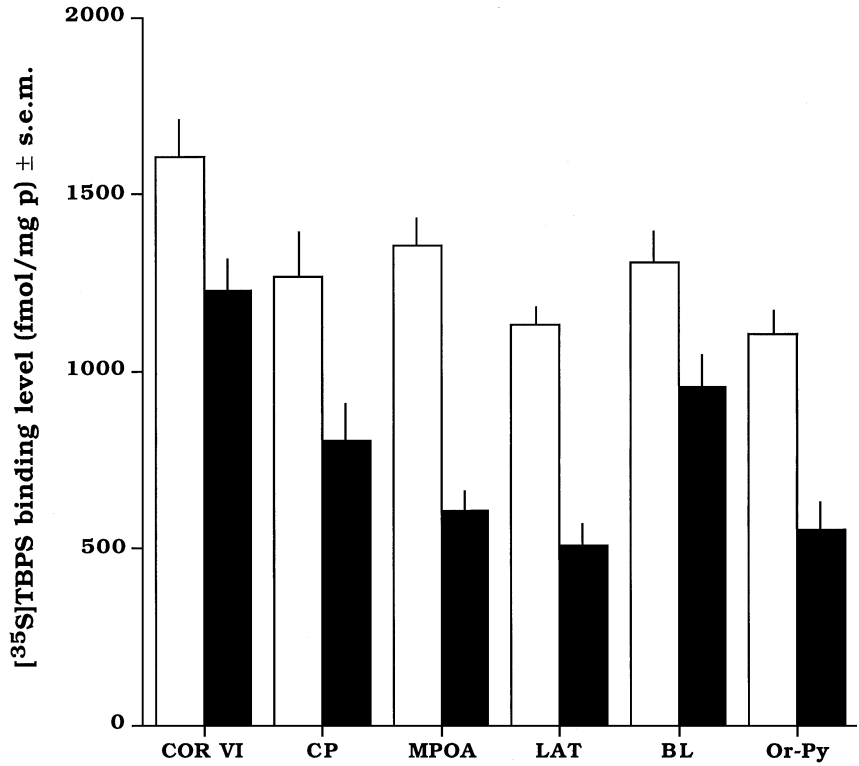

FIG. 5 Optimal *in vitro* binding effects of the neuroactive steroid 3α hydroxy-5α pregnan-20-one + GABA (■) on *t*-butylbicyclophosphorothionate (TBPS; preferential ligand of the chloride ion channel receptor) and compared to controls (□) in some forebrain areas of the *in vivo* treatment groups. For abbreviations see the legends to Figs. 3 and 4 (data in part modified and integrated with those of Canonaco *et al.,* 1993b).

cells, as in the case of the amygdala that are densely populated by noninducible progesterone receptors (MacLusky and McEwen, 1978). For this particular relationship, the GABA type A receptor was characterized by the following different α-containing subunit compositions: $\alpha_1\beta_1\gamma_2$, $\alpha_1\beta_1\gamma_{2L}$, $\alpha_3\beta_1\gamma_2$, $\alpha_5\beta_3\gamma_2$, and $\alpha_{4/6}\beta_2\gamma_2$ (Puia *et al.,* 1993; Connolly *et al.,* 1996; Buhr *et al.,* 1998). A similar binding capacity of 3α,5α-THP was detected for chloride channel receptor compositions $\alpha_5\beta_3\gamma_2$, $\alpha_1\beta_1\gamma_2$, and $\alpha_{1-3}\beta_1\gamma_{2L}$ (Zaman *et al.,* 1992; Sieghart, 1995), despite the fact that the potency of this last combination differs greatly between humans and other mammalian species (Belelli *et al.,* 1996). Of notable importance is the $\alpha_3\beta_1\gamma_2$ combination, considered functionally the most sensitive composition which binds at low affinities and displays the lowest potentiation capability (Canonaco *et al.,* 1997).

Consistent with the actions of α subunits is the progesterone withdrawal reaction in the pseudopregnancy model, causing a marked sensitivity reduction to another BDZ agonist (diazepam), a positive modulatory effect of classical BZD receptor antagonist (flumazenil), and an increased expression of the α_4 subunit mRNA (Smith *et al.,* 1998; Follesa *et al.,* 2000). In this case, the turnover of α subunits contributes to changes in the sensitivity of the GABA type A receptor to endogenous modulators as well as to receptor activity and behavior. As a consequence, the major GABAergic activities of the cerebellum granular layer are associated with the $\alpha_{4/6}\beta_2\gamma_2$ composition (Lüddens *et al.,* 1998). However, lack of a potent neurosteroid activity on chloride ion uptake in the granular layer, contrary to radioligand binding results, indicates that the previous combination is perhaps not the only one specific for this area. Recent studies have indeed shown that the success of the $\alpha_6\beta_2$ combination does not necessarily depend on the presence of γ_2 because it can also be combined with the δ subunit (Sieghart, 1995; Jones *et al.,* 1997). Even in the presence of this subunit, it is possible that the pharmacological properties of the $\alpha_6\beta_2$ combination do not modify since $\alpha_6\beta_2\delta$ was also devoid of any BZD potentiation effect (Zaman *et al.,* 1992; Lüddens *et al.,* 1998). In addition, work carried out on the GABA type A receptor δ subunit knockout mice revealed that the absence of this subunit did not inhibit 3α,5α-THP modulation of the GABA type A receptor (Zhu *et al.,* 1996). On the other hand, the presence of the δ subunit selectively attenuated behavioral responses to neuroactive steroids in the hippocampus (Mihalek *et al.,* 1999), which accounted for the social isolation-induced GABA-gated current and receptor functions (Serra *et al.,* 2000) in a regionally specific manner as shown in our laboratory (Canonaco *et al.,* 1993a,b).

Nonetheless, the previously discussed two subunits, on the basis of their specific binding activity toward the different GABA type A receptor agonists and antagonists, are considered to be the main targets responsible for neuroactive steroid actions. The γ subunit is required for normal channel conductance and for the formation and function of BZD sites. In particular, γ_2 is believed to contribute to at least 80% of receptor activity (chloride ion channel frequency) (Benke *et al.,*

1996; McKernan and Whiting, 1996), which plays an active role in neuroactive steroid–GABA type A interactions.

Recently, the splice variant, γ_{2L} has received attention because this variant, and not the γ_{2S} subunit of this receptor system, contains an additional phosphorylation site recognized by protein kinase C (Whiting *et al.,* 1990). As a consequence of this relationship and because of the specific γ_{2L}-dependent steroid-gated GABAergic functions, it has been possible to demonstrate that the success of pregnancy is tightly linked to high allopregnanolone levels, whereas these levels decline after delivery. This pattern was paralleled by low and high expression levels, respectively, of the γ_{2L} transcript (Concas *et al.,* 1998). The observation that the amounts of the different α isoforms did not change during pregnancy underlies the specificity and the necessity of the γ_2 subunit for neuroactive steroid–chloride ion channel functions. The low levels of gonadotropin-releasing hormone mRNA content in preoptic areas and medial septal neurons in knockout mice for the γ_2 subunit provide further support of the critically important role of this subunit on GABA type A–neuroactive steroid interactions.

On the basis of the previously mentioned differentiated maximal effects of neuroactive steroids, it appears that α isoforms, besides that of the γ_2 potentiating effect, can dramatically modify both GABA-gated current flow and binding activities through membrane-type mechanisms. The major role played by α subunits on these and other receptor functions is to a great extent supported by the fact that α_5 is a prime requisite for the development of a fully assembled GABA type A receptor complex (Fritschy *et al.,* 1997) and the absence of this subunit and $\alpha_{1,2/3}$ has instead been associated with the lack of any steroid-induced binding effects in the cortex, hippocampus, and striatum (Canonaco *et al.,* 1993b; Korpi *et al.,* 1999). In this context, how the predominance of the α isoforms is responsible for local membrane-type receptor mechanisms becomes a pertinent aspect. However, before such a relationship can be discussed, it is important to note that different states of the GABA type A receptor account for multiple active recetor conformations which are associated with chloride channel functions (Schumacher and McEwen, 1989). Evidence from electrophysiological and neuropharmacological studies indicates that these GABA type A-dependent receptor functions are modulated allosterically by neuroactive steroids (Harrison *et al.,* 1987; Puia *et al.,* 1993; Gee *et al.,* 1995) via a prolonged desensitized state as well as a slowdown recovery from desensitization, which is consistent with the increased probability of the chloride channel opening (Zhu and Vicini, 1997).

From recent studies, it seems that the channel properties, and hence the type of channel activity, rely heavily on specific subunits as demonstrated by the α_5- and α_4-containing GABA type A receptor being responsible for slow inhibitory GABAergic transients via short- and long-lasting openings, respectively (Maric *et al.* 1999), whereas the α_1- and $\alpha_{2/3}$-containing receptor parallels fast inhibitory transients. It is worth noting that allopregnanolone has a strong influence on the modification of the expression levels of the α_4 mRNA transcript (Grobin

and Morrow, 2000). Hence, it appears that the molecular success of neuroactive steroid action in the brain relies on intracellular cross talk between membrane and genomic mechanisms, with the former selectivity operating through channel kinetics that preferentially feature different α isoforms (α_{1-6}) and to a lesser extent β isoforms (β_{1-3}) and the latter seems to be controlled by the β isoforms (Fig. 6). Moreover, the different neuroactive steroid-dependent levels of mRNA encoding α subunits are a major element responsible for the efficacy of GABAergic actions (Smith *et al.,* 1998; Cullinan and Wolfe, 2000), which are in turn linked to the success of anxiolytic (Bitran *et al.,* 1999) and anticonflictual responses (Vanover *et al.,* 1999), particularly under different environmental conditions (Behringer *et al.,* 1996; Canonaco *et al.,* 1997; Facciolo *et al.,* 2000).

IV. Excitatory Neuroactive Steroids

As noted previously, the term neuroactive steroid was first introduced to name another important class of steroids that includes pregnenolone, dehydroepiandrosterone (DHEA), and their corresponding sulfate metabolites (Baulieu and Robel, 1996). This class of neuroactive steroid, which is found at high levels after gonadectomy and adrenalectomy (Baulieu, 1981), is synthesized in the CNS (Corpéchot *et al.,* 1997) and, like the 3α-hydroxysteroids, is also capable of rapidly interacting with the GABA type A receptor. The same steroidogenic enzymes that are located within oligodendroglia sites, where pregnenolone and DHEA are mainly formed, are involved in the synthesis from cholesterol of both the free and the sulfate ester forms of the neuroactive steroids through the participation of two cytochrome P450 enzymes having 17α hydroxylase and c17,20 lyase activities (Hu *et al.,* 1987; Jung-Testas *et al.,* 1989):

$$\text{Cholesterol} \xrightarrow{\text{P450}_{\text{scc}}} \text{pregnenolone} \xrightarrow{\text{P450}_{\text{c17}}} \text{DHEA}$$

Even though pregnenolone was found in the brain at concentrations higher than those of DHEA, they both displayed a bimodal effect toward the binding of [^{3}H]muscimol, i.e., increasing binding at nanomolar concentrations and decreasing it at micromolar concentrations (Majewska *et al.,* 1985), a relationship which has gained wide recognition for these neuroactive steroids in the treatment of some pathologies.

A. Pregnenolone

After the discovery of the previously mentioned bimodal binding relationship at the GABA type A receptor, researchers identified a proconvulsant and increased neuronal excitability when these neuroactive steroids were applied iontophoretically to neurons of the septopreoptic area (Carette and Poulain, 1984). Application of

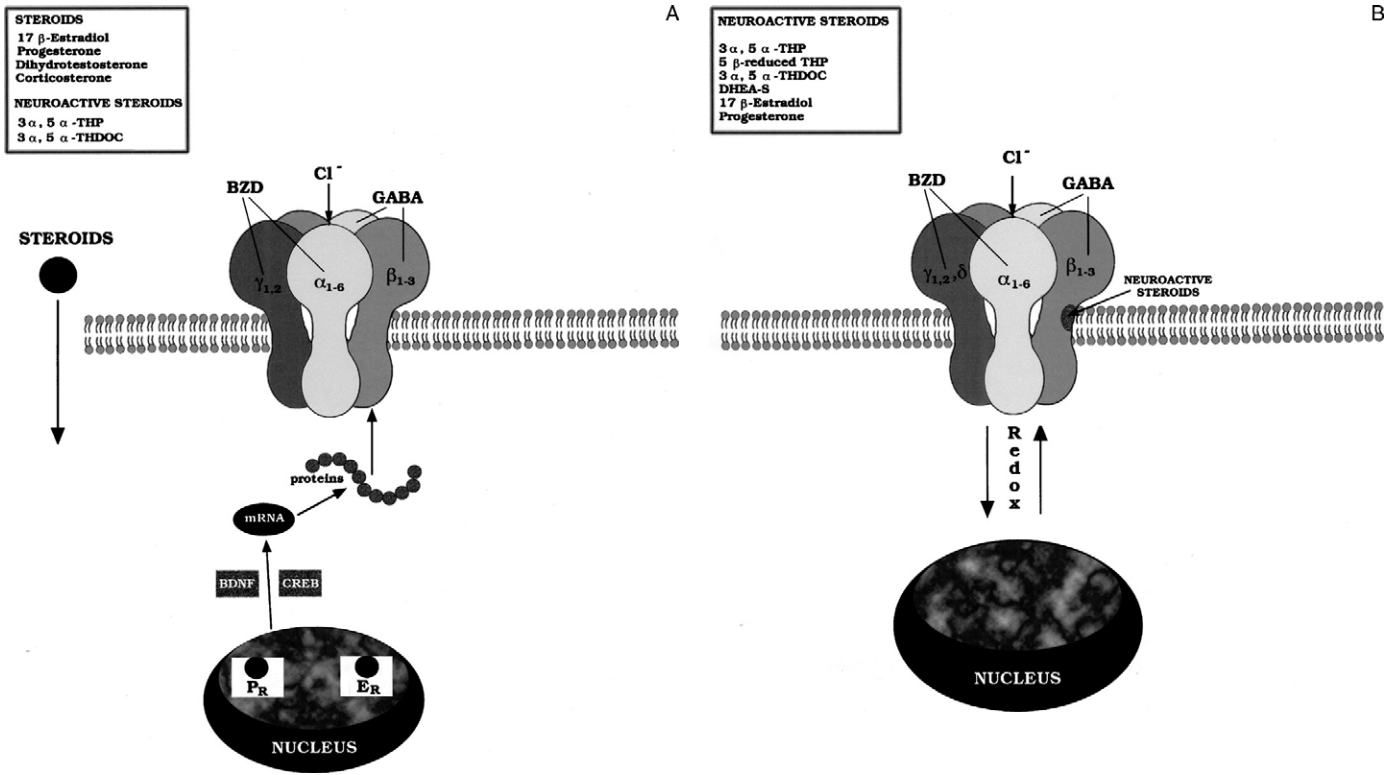

FIG. 6 Neuroactive-induced genomic and nongenomic types of steroid action. The recently defined neuroactive steroids include steroids that modulate the neurotransmitter receptor system through (A) slow-type or genomic action (hours to days) and (B) rapid-type or nongenomic action (milliseconds to minutes). In this type of mechanism, steroids (such as 17β-estradiol and progesterone, both defined as steroids and neuroactive steroids) are involved in neurotransmission of $GABA_A$ receptors through the assembly of certain subunit isoforms. BDNF, brain-derived neurotrophic factor; BZD, benzodiazepine; CREB, cAMP response element binding protein; DHEA-S, dehydroepiandrosterone sulfate; E_r, estrogen receptor; G, G protein; 3α,5α-THDOC, 3α,5α-tetrahydrodeoxycorticosterone; 3α,5α-THP, 3α,5α-tetrahydroprogesterone; 5β-reduced THP, 5β-tetrahydroprogesterone; PK, protein kinase; P_r, progesterone receptor.

pregnenolone at low micromolar concentrations competitively inhibited the binding of TPBS to the recombinant GABA type A receptor containing the $\alpha_1\beta_1\gamma_2$ subunit combination and antagonized GABA-induced chloride ion uptake in synaptoneurosomes and chloride ion conductance in cultured neurons (Majewska and Schwartz, 1987). The addition of similarly low concentrations enables pregnenolone to antagonize, in a analogous manner to BZD receptor inverse agonists, GABA type A receptor-mediated currents by reducing channel opening frequency. Despite the fact that the sulfate ester of this neuroactive steroid exerts light potentiation effects on BZD binding or inhibits barbiturate-induced enhancement of BZD binding, its excitatory action, via the negative modulatory GABA type A receptor effects (Majewska, 1992), has been considered of neurophysiological importance. Behavioral studies have attributed a potential cognitive role to pregnenolone and its ester product, as shown by the improvement of various memory tasks either by increasing the animal's natural performance or by antagonizing pharmacologically induced amnesia (Cheney *et al.,* 1995; Vallée *et al.,* 1997). The mechanisms underlying these cognitive functions of pregnenolone are unknown.

The first neurotransmitter receptor system that, due to the marked potentiation effect of pregnenolone sulfate on the alteration of calcium entry, is considered to be a preferential target of this neuroactive steroid is the NMDA receptor (Wu *et al.,* 1991). Indeed, the local perfusion of supraoptic vasopressin magnocellular neurons with pregnenolone sulfate provided a rapid nongenomic allosteric potentiation of glutamate-induced vasopressin secretion (Richardson and Wakerley, 1998) in a heavily active NMDA receptor-related hypothalamic site. Regarding the specific interaction of this neuroactive steroid on NMDA receptor-dependent memory processes, pregnenolone sulfate directly blocked retention deficits induced by competitive NMDA receptor antagonists in a Y-maze avoidance memory task (Mathis *et al.,* 1996), suggesting that this receptor system is a favorable target of pregnenolone mnemonic functions. Conversely, the cholinergic system, which is involved in the regulation of memory processes of the basal forebrain (Decker and McGaugh, 1991), is also considered an important target of pregnenolone sulfate. In fact, administration of this neuroactive steroid accounted for a marked release of acetylcholine in the frontoparietal cortex, which was in turn correlated to enhanced spatial memory performances (Pallarés *et al.,* 1998). On the basis of similar studies pregnenolone may be thought to promote mnemonic functions through either negative modulatory functions of the GABA type A receptor complex or via the positive modulation of the NMDA receptor resulting in an overall stimulatory effect of the cholinergic neurotransmission activities (Pallarés *et al.,* 1998).

B. Dehydroepiandrosterone

DHEA and its sulfate derivative, which are the most abundant steroids produced by the adrenal and thus are considered principal precursors of sex steroid synthesis,

are also capable of promoting similar antagonistic effects, such as the inhibition of GABA-induced chloride flow in a noncompetitive manner. In this case, the free neuroactive steroids do so in a less potent fashion (Mienville and Vicini, 1989). Contrary to the potent inhibiting effects of pregnenolone sulfate on TBPS binding, DHEA sulfate is devoid of such a capability and does not have any effect on BZD binding activity (Majewska, 1992). Moreover, even though recent studies have recognized pregnenolone sulfate as a weakly negative and positive modulator of both GABA and NMDA neurotransmitter systems, respectively, the simultaneous addition of DHEA in the synaptic regions of these two systems promotes a remarkable synergistic amplification of excitatory transmission at a notably lower concentration than would be required for either of the inhibitory or excitatory systems alone (Roberts, 1995).

This type of interaction, in addition to the fact that micro- and millimolar potencies are required for neuronal activity and that there is a lack of receptors for DHEA and its sulfate derivative, has made it difficult to address specific neurophysiological roles of these neuroactive steroids. However, DHEA and its sulfate product are able to maintain a hypothalamic–pituitary–adrenal homeostasis by acting as cortisol antagonists, particularly during prolonged glucocorticoid hyperactivity (Hechter *et al.,* 1997). Interestingly, in a condition such as social isolation, which is linked to a hyperactive hypothalamic–pituitary–adrenal axis, all other neuroactive steroids (with the exception of DHEA) were reduced (Serra *et al.,* 2000). Similar results indicate that this neuroactive steroid is an important factor in various neurobiological activities, ranging from regulation (the interaction of the NMDA receptor) of neocortical neuronal outgrowth and synaptic organization during embryogenesis (Compagnone and Mellone, 1998) to the modulation of neuropeptide secretion of the posterior and intermediate pituitary lobe through the differential actions of the GABA type A receptor (Hansen *et al.,* 1999).

In line with the differential effect of DHEA and its sulfate derivative on GABAergic activities, earlier studies support the mixed GABA agonistic/antagonistic properties on elevated rapid eye movement sleep and increased electroencephalogram activity in the sigma frequency following treatment with DHEA sulfate (Friess *et al.,* 1995). This activity also appears to fit well with the amelioration of memory processes (Flood *et al.,* 1988) and antidepressant effects in patients with major depression (Wolkowitz *et al.,* 1999). However, it is the neuroprotective role of this steroid that has gained particular attention. In this regard, investigators have begun to show that the formation of intracellular free radicals, induced by the accumulation of the β-amyloid peptide (a component of Alzheimer's disease plaques), is implicated in the resistance of glial cells to the toxic effects of such a peptide through the production of DHEA (Brown *et al.,* 2000). Moreover, this neuroactive steroid is capable of protecting some encephalic regions such as the hippocampus against oxidative stress-induced neuronal damage of Alzheimer patients (Bastianetto *et al.,* 1999) and neuronal injury following severe but transient cerebral ischemia (Li *et al.,* 2001) and oxygen–glucose deprivation-induced injury of

cerebellar granule cells (Kaasik *et al.,* 2001). Such studies provide a basis for potential clinical application of this class of neuroactive steroids after σ receptors, which have been shown to play a potent neuromodulatory role in cholinergic and NMDA receptor-mediated glutamatergic neurotransmission and have been reported to be important targets for both pregnenolone- and DHEA-induced mnemonic effects (Zou *et al.,* 2000).

V. Evolutionary Perspectives

Much remains to be answered about the structural and functional strategies adopted by the recombinant GABA type A receptor subunit combinations during their interaction with neuroactive steroids. For instance, how do neuroendocrine, behavioral, nutritional, and overall environmental cues influence this type of interaction in the different hypothalamic and extrahypothalamic regions. In this field, phylo- and ontogenetic research has and will continue to provide helpful solutions, especially if we consider that information regarding evolutionary neuroanatomy strategies of a particular neurotransmitter receptor system featuring specific subunits in a less laminated brain region might not only underlie roles of determinant brain regions but also indicate the importance of most of the specific subunits in distinct neurobiological activities. In particular cases, such as the projecting systems of the paleomammalian limbic area, thought to be the substratum of emotions (Squire and Kandel, 1999), and neomammalian cortical mantle, assumed to subserve cognitive functions (Armstrong, 1991), two telencephalic regions that couple to other telencephalic areas may provide significant information regarding the hierarchical molecular organization of the neuronal systems in the numerous behavioral processes. In this context, a systemic phylogenetic comparison of the labeling pattern of the BZD binding protein, which was referred to as an α subunit, supplied encouraging results regarding the subunit characterization of the GABA type A receptor complex. Indeed, the discovery of the BZD binding protein band varying between 49 and 53 kDa in mammals, whereas amphibians, reptiles, and birds present either a 53-kDa band or both a 53- and a 54-kDa band (Hebebrand *et al.,* 1987), suggested that perhaps more than one subunit characterize this GABA type A receptor site, not only in rodents but also in other vertebrates. Such differences are beginning to be recognized as the basis for the identification of the recombinant subunits involved in the assembly of the GABA type A receptor complex.

Regarding the neuroactive steroid interaction with this receptor complex, in an earlier study we demonstrated that the gonadal steroids, estrogen and testosterone, exerted strong effects on both β- and α-containing GABA type A receptor in hypothalamic and extrahypothalamic areas of the quail, with a greater effect on the former (Canonaco *et al.,* 1991a,b). Investigations dealing with conservative properties of steroid–GABA type A receptor interactions in cerebral areas of

submammals have increased. An important relationship that has emerged from these investigations is that the sensitivity of brain steroid target cells is very likely conserved in reproductive and nonreproductive behaviors of vertebrate phylogeny as shown not only by marked aromatase activity in frog (Guerriero *et al.,* 2000) and bird (Watson and Adkins-Regan, 1989) brain regions but also by neurosteroid activity in the brain of these animals (Do-Rego, *et al.,* 1998; Viapiano *et al.,* 1998).

Differential functional aspects of the steroidogenic component in relation to GABA type A receptor activity have been proposed. These differences vary from the presence of distinct subunits such as α_3 in frog neurons, involved in the regulation of neuroactive steroid biosynthesis (Do-Rego *et al.,* 2001), to the allosteric modulatory activity of neuroactive steroids in birds. However, for the latter class of vertebrates, it is perhaps the $3\beta,5\beta$ form rather than the $3\alpha,5\alpha$ neurosteroid that exerts a greater modulatory activity on the low-affinity GABA type A receptor (Viapiano and Fiszer de Plazas, 1998)—receptor affinity that is characterized by the prevalence of the different α subunits (Edgar and Schwartz, 1992). The variations reported in these two vertebrate classes indicate that differing and specific receptor mechanism have been adopted during the evolutionary development of GABA type A receptor–neuroactive steroid interaction. On the one hand, it might be the β position of the hydroxyl group that exerts internal regulatory loops by which neurosteroids may be regulating their own production during the early stages of brain development; on the other hand, it may be the type of subunit combinations of the receptor complex (Viapiano and Fiszer de Plazas, 1998; Do-Rego *et al.,* 2001). Additional studies are required before such a hypothesis be accepted, but it is tempting to speculate that in the early vertebrate phylogenesis the GABA type A receptor–neuroactive steroid interaction was a synthetic type of control mechanism and only later with the formation of laminated and more complex brain region did this interaction switch to an allosteric modulatory type—a correlation that may also be linked to the different and higher brain functions in mammals with respect to those of the other submammalian vertebrates.

VI. Concluding Remarks

The ubiquitousness of GABA in the CNS, particularly in areas enriched with steroid receptors, underlies its prevalent neuroactive steroid-dependent role in the expression of integrated brain functions. From the plethora of evidence regarding the dual steroid modulatory action, it has been largely accepted that the genomic estrogen-regulated variations are directed at the β-containing GABA type A receptors. Included in this type of activity are its synaptogenic-dependent roles, its influences on contextual learning and fear conditioning motor skills, as well as its neuroprotective role in Parkinson's disease, tardive dyskinesia, and cerebral ischemia. Nevertheless, in the past few years the rapid membrane type of

neurosteroid effects on the GABA type A receptor have received greater recognition. Due to the heteromeric assembly of different GABA type A receptor isoforms, the ability of neurosteroids to achieve allosteric modulatory actions in a bimodal nature on this receptor system containing differing α isoforms represents a key GABAergic structural feature in communicative brain processes. In fact, the high levels of α_{1-3} subunits and not necessarily $\gamma_{1/2}$ subunits along with either high pregnenolone sulfate or low 3α,5α-THP and THDOC levels have to some extend been accepted as a molecular basis of neuronal hyperactivity. These relationships could thus explain why chronic administration of 3α,5α-THP is able to evoke downregulation, heterologous uncoupling, and decreased efficacy of the GABA type A receptor in convulsive and stressful conditions (Yu *et al.,* 1996). They could also explain why increased mRNAs coding for $\alpha_{1/2}$ subunits and no variations of mRNAs coding for β_3, γ_1, or γ_2 subunits were observed in epileptogenic regions such as cortex and hippocampus (Pratt *et al.,* 1993). The *in vivo* administration of the phosphorothionate-modified antisense oligodeoxynucleotide (aODN) against the γ_2 subunit, which accounts for the inhibition of the specific gene expression without inducing any lethal conditions, has been shown to be responsible for convulsive threshold changes produced by BZD inverse agonists (Zhao *et al.,* 1996) and has also been shown to be involved in the pathogenesis of epileptical seizures (Karle *et al.,* 1998). In this context, the application of aODN to α and β subunits, hitherto considered preferential neuroactive steroid targets, could supply answers to why chronic administration of 3α,5α-THP is able to evoke down regulation, heterologous uncoupling, and altered kinetics of GABA type A-gated chloride ion conductances in GABA type A receptor–steroid interactions during neuroendocrine and stressful events (Richardson and Wakerley, 1998; Fénelon and Herbison, 2000; Serra *et al.,* 2000).

The developmental organization of adult synaptic physiology is becoming an important issue and recent studies have shown that this activity heavily depends on gonadal steroids inducing a neural remodeling through the calcium-dependent cell adhesion molecule N-cadherin, which participates in the plasticity of the adult nervous system (Monks *et al.,* 2001). It would be interesting to determine whether the plasticity of the GABA type A receptor is also altered genomically via the modification of this adhesion molecule and whether the estrogen receptors α and/or β or the neuroactive steroids that are implicated in such activities. Moreover, it is also important to identify the neuroactive steroid–GABA type A receptor subunit entity coupled to physiopsychological syndromes, such as depression, progesterone withdrawal, and protracted social isolation, i.e., whether they are due to a genomic action mediated by progesterone or its reduced product 5,α-dihydroprogesterone and/or to a concomitant local type of mechanism by allopregnanolone (Dong *et al.,* 2001). Clearly, there are many questions to be addressed. Novel neuroactive steroids specific for distinct subunit combinations (Hawkinson *et al.,* 1998; Vanover *et al.,* 2000) and the application of knockout models for certain

subunit genes might help us determine the direct roles promoted by the different GABA type A receptor subunits in the numerous behavioral activities as well as their protective role in neurodegenerative disorders and in progesterone-dependent coronary vasodilatory effects (Jacob and White, 2000). In future studies, it will be essential that the continuing experiments be conducted on the different vertebrate models with an eventual intention to beneficially apply them to humans.

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Tetraspan Vesicle Membrane Proteins: Synthesis, Subcellular Localization, and Functional Properties

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Tetraspan vesicle membrane proteins (TVPs) are characterized by four transmembrane regions and cytoplasmically located end domains. They are ubiquitous and abundant components of vesicles in most, if not all, cells of multicellular organisms. TVP-containing vesicles shuttle between various membranous compartments and are localized in biosynthetic and endocytotic pathways. Based on gene organization and amino acid sequence similarities TVPs can be grouped into three distinct families that are referred to as physins, gyrins, and secretory carrier-associated membrane proteins (SCAMPs). In mammals synaptophysin, synaptoporin, pantophysin, and mitsugumin29 constitute the physins, synaptogyrin 1–4 the gyrins, and SCAMP1–5 the SCAMPs. Members of each family are cell-type-specifically synthesized resulting in unique patterns of TVP coexpression and subcellular colocalization. TVP orthologs have been identified in most multicellular organisms, including diverse animal and plant species, but have not been detected in unicellular organisms. They are subject to protein modification, most notably to phosphorylation, and are part of multimeric complexes. Experimental evidence is reviewed showing that TVPs contribute to vesicle trafficking and membrane morphogenesis.

KEY WORDS: Membrane proteins, Vesicle trafficking, Endocytosis, Exocytosis, Vesicle biogenesis.

I. Introduction

Bidirectional transport between intracellular membrane-bounded compartments and the plasma membrane is mediated by cytoplasmic vesicles. The investigation of molecular principles that regulate the myriad of decisions necessary to ensure proper uptake and delivery of specific cargo within this transport system and the continued maintenance of these carriers remains a major challenge in modern cell biology, although interesting insights have been obtained into mechanisms of vesicle fusion and fission. Among vesicle proteins, integral membrane proteins with four transmembrane regions (TMRs) and cytoplasmically located ends are particularly abundant components of different vesicle types but whose function is still largely unknown. On the basis of their transmembrane topology and their vesicle localization, we propose to refer to these proteins as tetraspan vesicle membrane proteins (TVPs). They are the subject of this review. Based on similarities of the gene structure and amino acid composition of TVPs, three distinct families can be distinguished. In mammals the physins comprise synaptophysin, synaptoporin, pantophysin, and mitsugumin29, the gyrins encompass synaptogyrins 1–4, and the secretory carrier-associated membrane proteins (SCAMPs) include SCAMP isoforms 1–5. All polypeptides are encoeded individually by separate genes.

Available information on the cell-type-specific synthesis of mRNA and protein from TVP-encoding genes shows that some are universally expressed in many different cell types, whereas others are present only in a few specialized cell types, such as neuronal and neuroendocrine cells or skeletal muscle cells. Thus, individual TVPs are expressed in specific combinations with other TVPs that belong to the same family and/or to another family. Any given cell type is therefore characterized by a particular combination of TVPs. Conversely, each TVP exhibits a unique expression profile suggesting that gene expression is regulated individually and not in a coordinated fashion.

TVP expression is further complicated by the specific subcellular localization of each polypeptide in different cell types. Although family members colocalize in the same cellular organelles in most instances, they may not in others. It is therefore attractive to speculate that specific combinations of TVPs define structurally and functionally distinct vesicle populations. Despite the amazing abundance and ubiquity of TVPs in cytoplasmic vesicular carriers their function is still poorly understood. Accumulating evidence is reviewed that links TVPs to cellular membrane trafficking, especially by contributing to vesicle formation, endocytosis, and exocytosis. Furthermore, TVPs may be involved in certain specialized functions, such as neuronal plasticity and excitation–contraction coupling in skeletal muscle. The aim of the review is to combine available information on TVPs and to provide arguments to show that these polypeptides are structurally related despite their low amino acid sequence similarity and that they fulfill functions in cellular vesicle trafficking in a partially overlapping and redundant manner.

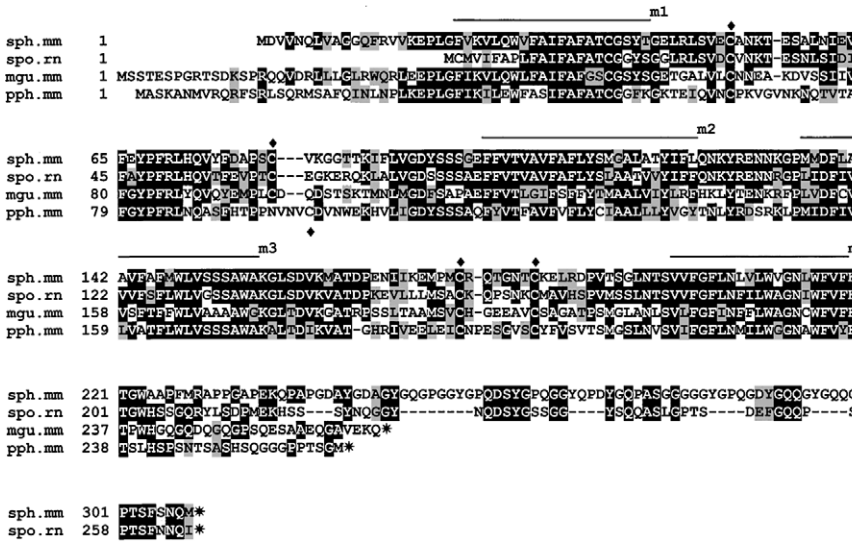
II. Diversity and Conservation of Polypeptide Features and Gene Organization

In this section, we summarize similarities and differences of TVPs and their encoding genes. We define features that are shared by all TVPs, those that are characteristic of each family, and those that are isoform specific.

A. Amino Acid Comparison

Figures 1–3 show amino acid sequence comparisons between TVP paralogs of the same or closely related species and between orthologs of different species. In this way, conserved versus divergent features are highlighted for each family. The most prominent common property of all TVPs is the presence of four hydrophobic domains that function as TMRs. These are also the parts of the molecules with the highest degree of sequence conservation. It is predicted that the amino and carboxy termini face the cytoplasm, whereas domains connecting TMR1 and -2 and TMR3 and -4 are directed toward the vesicle interior and will therefore be referred to as intravesicular loop 1 and intravesicular loop 2, respectively.

Figure 1 shows comparisons of physins. Percentages of identity and similarity of the amino acid sequences are given in Table I. To date, four different isoforms have been characterized in mammals (Fig. 1A): synaptophysin (also referred to as synaptophysin I), synaptoporin (also referred to as synaptophysin II), pantophysin, and mitsugumin29 (also referred to as TS28). Synaptophysin and synaptoporin are the most closely related family members, whereas pantophysin and mitsugumin29 are approximately equidistant from each other and from synaptophysin/synaptoporin. Most differences are noted in the amino and carboxy termini, which vary considerably in length and amino acid composition. In contrast, the four hydrophobic domains present the highest degree of amino acid sequence conservation. The connecting loop domains differ somewhat more but contain several conserved features, most notably in the presumptive intravesicular loop connecting TMR1 and TMR2. Furthermore, a pair of cysteine residues is present in both intravesicular loops of all mammalian physin isoforms. The same features are also found in physin orthologs of *Xenopus laevis,* chick, and *Torpedo californica* extending all the way to the nematode *Caenorhabditis elegans,* which is the only ortholog known to lack the cysteines in the second intravesicular loop (Fig. 1B). The *C. elegans* physin ortholog also has the longest cytoplasmic amino terminus and the shortest cytoplasmic carboxy terminus. Furthermore, its amino terminus includes an unusually hydrophobic stretch. Whether this serves as a TMR in the mature polypeptide or is only used as a transient leader sequence, even though it lacks features of a typical signal recognition sequence, is not known. In general, it can be concluded for physins that the high sequence conservation of the TMRs, and to a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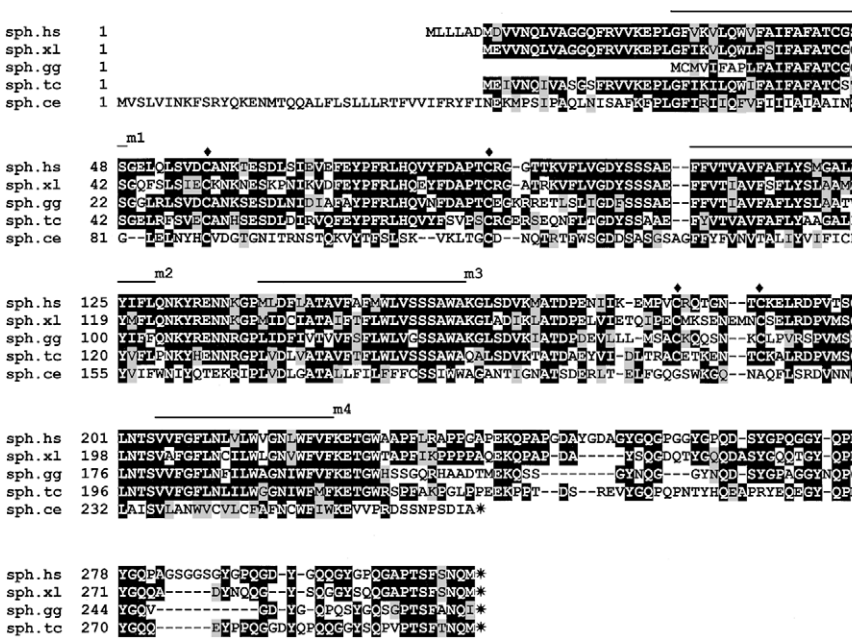
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sph.tc
sph.ce

B

TABLE I

Amino Acid Identity and Similarity (in Parentheses) of Paralogous and Orthologous Members of the Physin Family, the Gyrin Family, and the SCAMP Family[a]

Identity (%) and similarity [in parentheses (%)]						
Physin paralogs	sph.mm	spo.rn	mgu.mm	pph.mm		
pph.mm	47 (64)	49 (64)	42 (62)			sph.ce
mgu.mm	47 (65)	48 (63)			30 (47)	sph.tc
spo.rn	66 (78)			57 (69)	28 (44)	sph.gg
sph.mm			57 (67)	59 (72)	29 (43)	sph.xl
		73 (86)	66 (78)	70 (82)	27 (45)	sph.hs
	sph.hs	sph.xl	sph.gg	sph.tc	sph.ce	Physin orthologs
Gyrin paralogs	sgy1.hs	sgy2.hs	sgy3.hs	sgy4.hs		
sgy4.hs	40 (56)	41 (59)	42 (58)			sgy.ce
sgy3.hs	52 (64)	47 (59)			26 (43)	sgy.dm
sgy2.hs	49 (62)		37 (56)		23 (44)	sgy2.mm
sgy1.hs		88 (92)	38 (58)		31 (54)	sgy2.hs
	sgy2.hs	sgy2.mm	sgy.dm		sgy.ce	Gyrin orthologs
SCAMP paralogs	sca1.hs	sca2.hs	sca3.hs	sca4.mm	sca5.mm	
sca5.mm	46 (63)	51 (69)	45 (65)	56 (70)		sca.os
sca4.mm	48 (67)	47 (69)	47 (64)		25 (40)	sca.ce
sca3.hs	50 (61)	53 (70)		36 (51)	29 (39)	sca.dm
sca2.hs	53 (74)		41 (58)	36 (52)	28 (49)	sca1.hs
sca1.hs		sca1.hs	sca.dm	sca.ce	sca.os	SCAMP orthologs

[a]Corresponding amino acid sequence comparisons are shown in Figs. 1–3. For abbreviations, see the legends to Figs. 1–3.

FIG. 1 Amino acid comparison of physin paralogs (A) and physin orthologs (B). TMRs are denoted as predicted for murine synaptophysin in A and human synaptophysin in B. Multiple sequence alignment was done with the help of PIMA 1.4, and sequences were edited with BOXSHADE 3.21. Alignments were further optimized by hand. TMRs were only determined with the help of TMpred using standard settings. Black, identical amino acids; gray, similar amino acids; star, stop codon; m1–m4, transmembrane regions 1–4; diamonds, conserved cysteines. Gene Bank accession numbers: NM009305 for synaptophysin from *Mus musculus* (sph.mm), NM023974 for synaptoporin from *Rattus norvegicus* (spo.rn), NM008596 for mitsugumin29 from *M. musculus* (mgu.mm), AF081501 for pantophysin from *M. musculus* (pph.mm), AF196779 for synaptophysin from *Homo sapiens* (sph.hs), AF035014 for synaptophysin from *Xenopus laevis* (sph.xl), U27708 for synaptophysin from *Gallus gallus* (sph.gg), A60548 (protein) for synaptophysin from *Torpedo californica* (sph.tc), and AF038618 for synaptophysin-related *sph-1* gene from *Caenorhabditis elegans* (sph.ce).

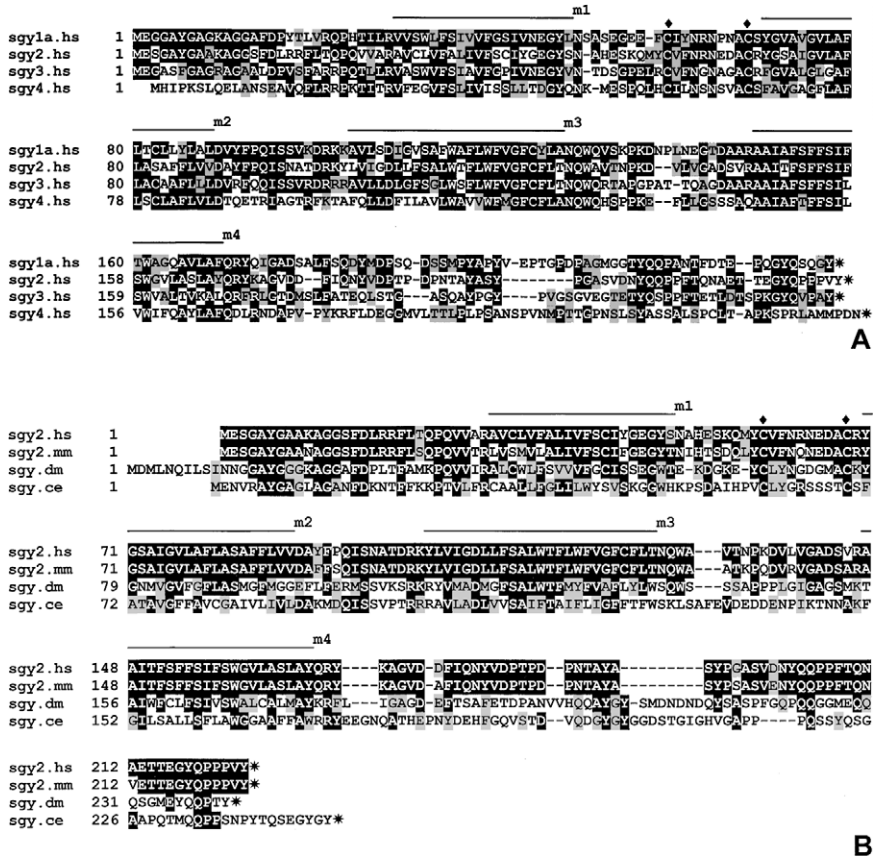

FIG. 2 Amino acid comparison of gyrin paralogs (A) and gyrin orthologs (B). Computer programs and denotations are the same as those described in the legend to Fig. 1. Gene Bank accession numbers: AL022326 for synaptogyrin 1a from *Homo sapiens* (sgy1a.hs), AJ002308 for synaptogyrin 2/cellugyrin from *H. sapiens* (sgy2.hs), AJ002309 for synaptogyrin 3 from *H. sapiens* (sgy3.hs), AJ011733 for synaptogyrin 4 from *H. sapiens* (sgy4.hs), AJ002307 for synaptogyrin 2/cellugyrin from *Mus musculus* (sgy2.mm), AAF58329 for synaptogyrin-related gene from *Drosophila melanogaster* (sgy.dm), and AF079373 for synaptogyrin-related gene *sng-1* from *Caenorhabditis elegans* (sgy.ce).

lesser degree that of the first intravesicular loop domain and the short cytoplasmic loop connecting TMR2 and -3, contrasts with the lower sequence conservation of intravesicular loop 2 and the considerable divergence in the cytoplasmic termini that also differ significantly in size.

A similar pattern of sequence conservation and diversity is observed for the gyrins (Fig. 2, Table I). Figure 2 shows four human paralogs termed synaptogyrin 1–4 (synaptogyrin 2 is indentical to cellugyrin) and orthologs from mouse, *Drosophila melanogaster,* and *C. elegans*. The TMRs are again, together with the

intravesicular loops, the parts with the highest degree of conservation. Furthermore, two conserved cysteine residues are present in intravesicular loop 1. The end domains show the most pronounced differences in length and amino acid composition. Human synaptogyrin isoforms are between 40 and 52% identical (Table I). Furthermore, sequence conservation between the human and murine synaptogyrin 2/cellugyrin isoforms and their *C. elegans* ortholog is in the same range as that observed for the physins (Table I).

Figure 3 and Table I show comparisons of SCAMP para- and orthologs. Two groups of SCAMPs can be distinguished: One group comprising mammalian SCAMP1–3 contains an extended amino terminus with three conserved NPF repeats, whereas the other group consisting of SCAMP4 and -5 lacks this domain entirely (Fig. 3A). Interestingly, within the rest of the molecule, similarities are spread evenly and are not restricted to specific subdomains. All family members are approximately equidistant. In constrast to physins and gyrins, SCAMP isoforms have been detected not only throughout the animal kingdom but also in plants, including *Arabidopsis thaliana, Pisum sativum* (pea), and *Oryza sativa* (rice) (Fernández-Chacón and Südhof, 2000; Hubbard *et al.,* 2000). Remarkably, even in these distantly related species amino-terminal NPF repeats are present, the basic transmembrane topology is conserved, and sequence similarities extend throughout the entire molecule (Fig. 3B).

Finally, to explore the similarities between the three TVP types, intraspecies comparisons between members of the different families are shown in Fig. 4. Although some similarities are noticeable, they are barely significant and the computerized fit did not properly align the four TMRs. Therefore, we conclude that TVPs can be grouped into three separate families with only marginal sequence similarities suggesting that possibly other polypeptides with the same basic structural features but lacking significant amino acid sequence similarities may qualify functionally as TVPs.

B. Gene Structure in Mammals

1. Physins

Based on cDNA cloning four distinct physin mRNAs have been identified coding for synaptophysin, synaptoporin, pantophysin, and mitsugumin29 in several mammals, including human (Südhof *et al.,* 1987b; Leube, 1994), rat (Leube *et al.,* 1987; Südhof *et al.,* 1987a; Knaus *et al.,* 1990; Leube, 1994), mouse (Eshkind and Leube, 1995; Haass *et al.,* 1996; Gaitanou *et al.,* 1997; Shimuta *et al.,* 1998), rabbit (Takeshima *et al.,* 1998), and cow (Johnston *et al.,* 1989b). These cDNAs are encolded individually by single genes which have been characterized in detail for synaptophysin in human (Özcelik *et al.,* 1990; Bargou and Leube, 1991), rat (Bargou and Leube, 1991) and mouse (Özcelik *et al.,* 1990; Eshkind and Leube, 1995; McMahon *et al.,* 1996), for synaptoporin in mouse (Leube,

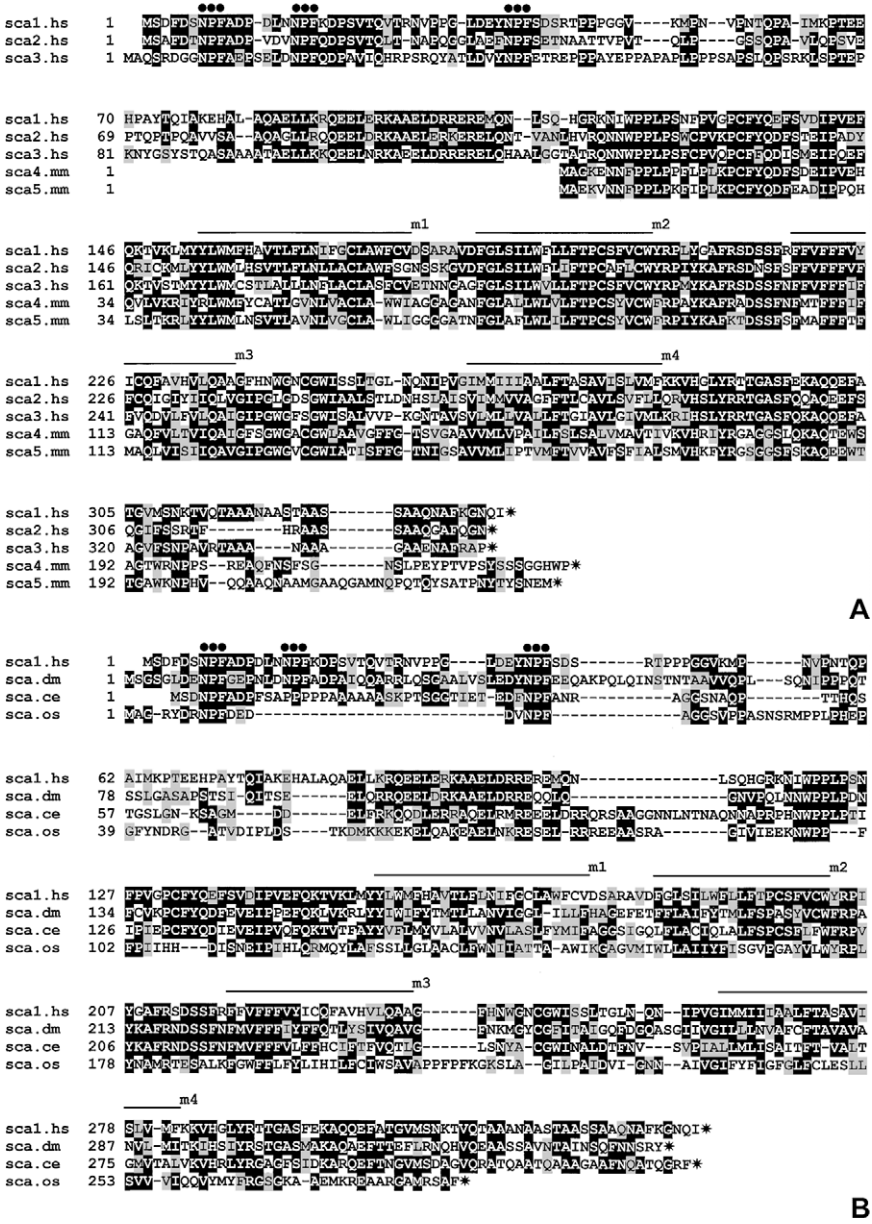

FIG. 3 Amino acid comparison of SCAMP paralogs (A) and SCAMP orthologs (B). Denotations are the same as those described in the legend to Fig. 1. Conserved NPF repeats are marked by dots. Gene Bank accession numbers: AF038966 for SCAMP1 from *Homo sapiens* (sca1.hs), AF005038 for SCAMP2 from *H. sapiens* (sca2.hs), BC005135 for SCAMP3 from *H. sapiens* (sca3.hs), AF224721 for SCAMP4 from *Mus musculus* (sca4.mm), AF241833 for SCAMP5 from *M. musculus* (sca5.mm), AF241834 for SCAMP-related gene from *Drosophila melanogaster* (sca.dm), AF225921 for SCAMP-related mRNA from *Caenorhabditis elegans* SCAMP (sca.ce; gene name *scm-1*), and AF225922 for SCAMP-related mRNA from *Oryza sativa* (sca.os).

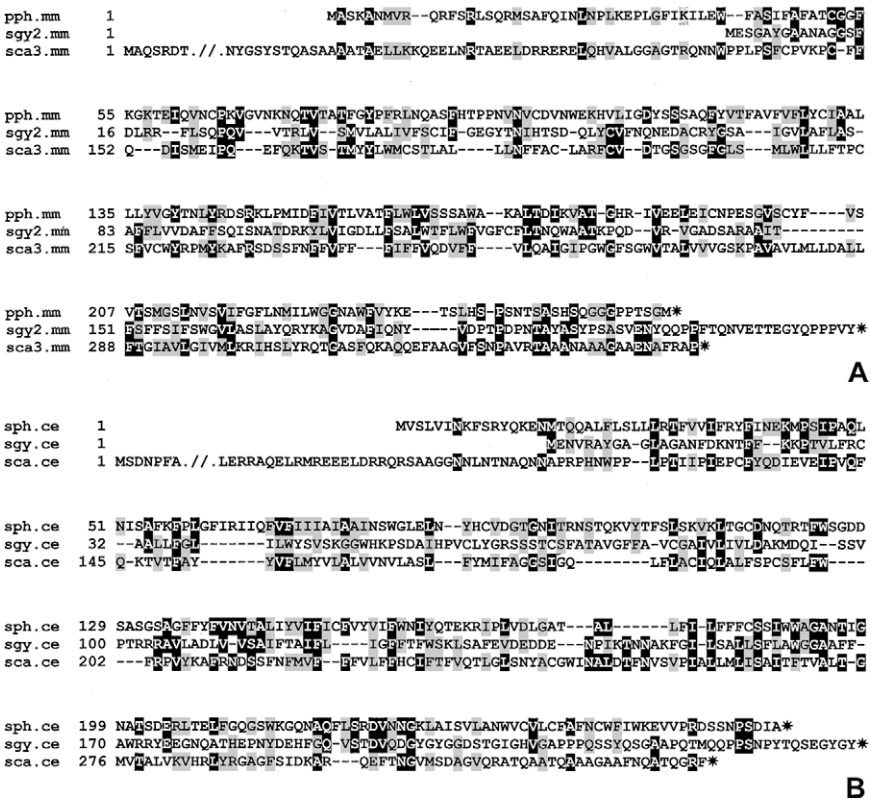

FIG. 4 Amino acid comparisons of members of TVP families in mouse (A) and *Caenorhabditis elegans* (B). Denotations are the same as those described in the legend to Fig. 1. Gene Bank accession numbers: AF081501 for pantophysin from *Mus musculus* (pph.mm), AJ002307 for synaptogyrin 2/cellugyrin from *M. musculus* (sgy2.mm), AF005036 for SCAMP3 from *M. musculus* (sca3.mm), AF038618 for the synaptophysin-related *sph-1* gene from *C. elegans* (sph.ce), AF079373 for synaptogyrin-related gene *sng-1* from *C. elegans* (sgy.ce), and AF225921 for SCAMP-related mRNA from *C. elegans* (sca.ce; gene name *scm-1*).

unpublished observations), for pantophysin in mouse (Haass *et al.,* 1996), and for mitsugumin29 in mouse (Shimuta *et al.,* 1998). The respective genes are not clustered: The synaptophysin gene is located on the X chromosome in human, mouse, and rat (Özcelik *et al.,* 1990; Szpirer *et al.,* 1998), whereas pantophysin and synaptoporin have been mapped to autosomes in mouse (Leube *et al.,* unpublished observations). The recently published human genome sequence also showed that each physin gene is located on a different chromosome. The protein-coding regions of all four genes are interrupted by five introns in all mammalian species examined with excatly conserved splice sites (Fig. 5). Introns, however, differ in length and nucleotide composition among species and between the different genes

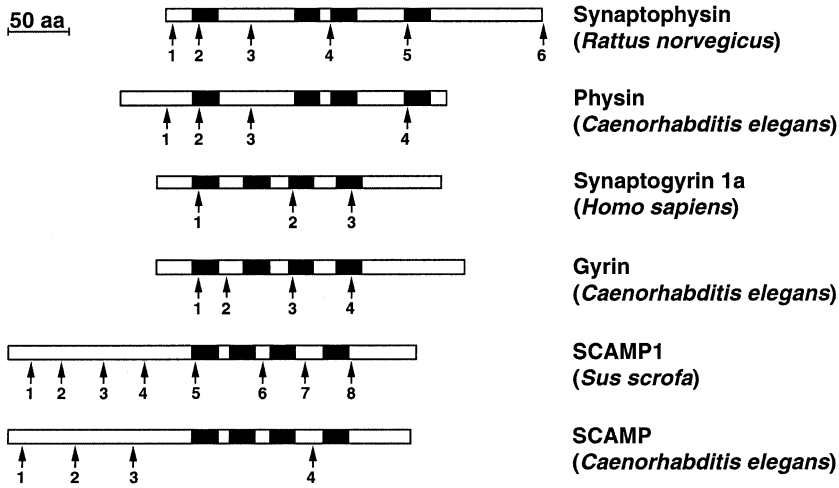

FIG. 5 Schematic representation showing comparison of domain organization of representative TVPs in mammals with that of their *Caenorhabditis elegans* orthologs. TMRs are drawn as black boxes. The relative intron positions determined from the respective genes are demarcated by arrows. Note the conserved features within each family. Gene Bank accession numbers: NM012664 for synaptophysin from *Rattus norvegicus,* AF038618 for synaptophysin-related gene *sph-1* from *C. elegans* (physin), AL022326 for synaptogyrin 1a from *Homo sapiens,* AF079373 for synaptogyrin-related gene *sng-1* from *C. elegans* (gyrin), Y15710 for SCAMP1 from *Sus scrofa,* and AF003739 for SCAMP-related gene *scm-1* from *C. elegans* (SCAMP).

within one species (Özcelik *et al.,* 1990; Bargou and Leube, 1991; Eshkind and Leube, 1995; Haass *et al.,* 1996; McMahon *et al.,* 1996; Shimuta *et al.,* 1998; Janz *et al.,* 1999). Interestingly, the synaptophysin gene contains an additional intron in the 3′ untranslated region (Özcelik *et al.,* 1990; Bargou and Leube, 1991). Whether this intron contributes to the regulation of synaptophysin expression is not known, but it has been proposed to work in concert with a conspicuous AT-rich domain in the 3′ noncoding region that could be important for regulation of mRNA stability (Johnston *et al.,* 1989b; Bargou and Leube, 1991). Alternative splicing of synaptophysin does not seem to be of importance since, with the exception of murine AtT20 cells (Buckley *et al.,* 1987), only single hybridizing synaptophysin mRNA bands have been detected in Northern blot hybridization experiments of mammalian cells (Leube *et al.,* 1987; Südhof *et al.,* 1987b; Bargou and Leube, 1991; Gaitanou *et al.,* 1997). In chick, however, two synaptoporin/synaptophysin-related transcripts have been identified that are derived from the same gene and differ by their amino termini (Bixby, 1992). One of the splice forms, referred to as synaptophysin IIa, also contains two alternative polyadenylation sites (Bixby, 1992). The occurrence of two transcripts, each for murine pantophysin (Haass *et al.,* 1996) and murine mitsugumin29 (Shimuta *et al.,* 1998), may also be the consequence

of alternative polyadenylation rather than alternative intron/exon splicing and/or alternative promoter usage (Shimuta *et al.,* 1998; Leube *et al.,* unpublished observations).

Examination of transcriptional regulation of physin gene expression is still in its infancy and rudimentary information is only available for synaptophysin. It was shown by nuclear run-on experiments that its cell-type-restricted expression in neurons and neuroendocrine cells is paralleled by differential gene transcription (Bargou and Leube, 1991). However, the directly 5′ upstream region of the synaptophysin gene shows the characteristics of a housekeeping promoter lacking TATA and CAAT boxes and instead containing GC-rich sequence elements, including four Sp-binding motifs (Bargou and Leube, 1991). Accordingly, reporter assays showed that the immediate upstream region (up to 1.2 kb) is constitutively active in all cells conferring comparatively weak transcriptional activity (Bargou and Leube, 1991). Further upstream elements (a 2.6- and 1.9-kb fragment, respectively), however, were able to suppress promoter activity almost completely in synaptophysin-negative nonneuroendocrine RVF-SMC cells but only partially in neuroendocrine PC12 cells (Bargou and Leube, 1991). To our knowledge, further characterization of these activities and identification of transcription factors and their binding sites have not been carried out to date.

Posttranscriptional mechanisms have also been shown to be important for synaptophysin synthesis (Daly and Ziff, 1997). Daly and Ziff demonstrated that the increase of synaptophysin in embryonal hippocampal neurons developing in culture is largely due to an increase in the rate of translational initiation and not simply a result of an increase in mRNA level (for evidence of brain-derived neurotrophic factor-induced regulation of synaptophysin translation, see Coffey *et al.,* 1997). By using highly sensitive Northern blot hybridization protocols, synaptophysin mRNA was identified in certain nonneuroendocrine cells that do not contain detectable amounts of synaptophysin polypeptide, indicating the possibility of translational repression in these cells (Leube, 1994).

The high similarity between mRNA and polypeptide expression profiles reported so far for the other physins indicates that transcriptional regulation is also the main cause of cell-type-specific protein synthesis in these instances.

2. Gyrins

cDNAs for all four synaptogyrin paralogs have been identified in mammals, including human, rat, and mouse (Stenius *et al.,* 1995; Kedra *et al.,* 1998; Janz and Südhof, 1998; Fig. 2). Each isoform is encoded individually by a separate gene. Genes have been mapped in human to chromosome 22 for synaptogyrin 1, chromosome 17 for synaptogyrin 2/cellugyrin, and chromosome 16 for synaptogyrin 3 (Kedra *et al.,* 1998). In addition, a processed synaptogyrin 2/cellugyrin pseudogene was localized on chromosome 15 (Kedra *et al.,* 1998). These assignments have been confirmed by the Human Genome Project, which also revealed that

the synaptogyrin 4 gene is located on chromosome 19. The human synaptogyrin 1 gene locus contains six exons which are combined by alternative splicing to generate the three variants a–c (Kedra *et al.,* 1998). Each variant is encoded by four exons as shown for the predominant splice variant a in Fig. 5. The b form differs from the a form by an alternative exon 4; the c form differs from the a form by an alternative exon 1, thus resulting in a differing carboxy and amino terminus, respectively. It is noteworthy, however, that the alternative splicing affects only the variable end domains, thereby maintaining the gyrin-typical basic polypeptide domain organization. Northern blot hybridization experiments and immunoblot analyses show differential mRNA and protein expression of the individual isoforms and the type 1 splice variants (Baumert *et al.,* 1990; Janz and Südhof, 1998; Kedra *et al.,* 1998). Levels of expression regulation have not been examined systematically.

3. SCAMPs

cDNAs have been identified for five different SCAMP isoforms in mammals (Brand and Castle, 1993; Singleton *et al.,* 1997; Wen *et al.,* 1998; Fernández-Chacón and Südhof, 2000; Hubbard *et al.,* 2000). The Human Genome Project showed that they are the products of distinct genes. mRNA analyses suggest that transcriptional regulation is involved in cell-type-specific synthesis of individual isoforms (Brand and Castle, 1993; Singleton *et al.,* 1997; Fernández-Chacón and Südhof, 2000; Hubbard *et al.,* 2000).

The first characterized mammalian SCAMP gene was SCAMP1 from pig (Wen *et al.,* 1998). It is more than 70 kb and consists of nine exons. The porcine SCAMP1 gene is located on chromosome 2 and its human ortholog is on chromosome 5. In the human genome SCAMP genes are not closely linked to each other or any other TVP-encoding gene. The 5′ upstream region of porcine SCAMP1 lacks a TATA box but contains a CAAT box motif and several GC-rich regions that may act as SP1 binding sites. The functionality of these elements has not been examined.

C. Evolutionary Aspects of Gene Organization

Within the completely sequenced genome of the nematode *C. elegans* three TVP-encoding orthologs were identified. Interestingly, each is specifically related to one of the three mammalian TVP families, providing further support for the classification of the three TVP types (Figs. 1–3) which differ considerably among each other (Fig. 4). The similarities in gene organization (Fig. 5) are most striking for the physins and gyrins. All four intron positions of the nematode physin gene correspond precisely to those of its mammalian counterparts which differ only by an additional intron in the protein-coding region and, in the case of synaptophysin, by an extra intron in the 3′ noncoding region. Similarly, all three exon/intron junctions of the mammalian synaptogyrin 1 gene are also present in the corresponding positions

of the *C. elegans* gene, which contains an additional intron (Fig. 5). In contrast to the physin and gyrin situation, SCAMP exon/intron structure differs completely between pig and *C. elegans* (Fig. 5). However, based on amino acid sequence comparison (Fig. 3B) they are clearly orthologous. Given the relationship between the *C. elegans* genes and the three mammalian TVP types, we propose to name the *C. elegans* orthologous gene of synapophysin *sph-1* and that of SCAMP *scm-1* in analogy to the synaptogyrin ortholog *sng-1* that was named by Nonet (1999).

D. Transmembrane Topology

All TVPs are integral membrane proteins that can be extracted from membranes by nonionic detergents (Jahn *et al.,* 1985; Wiedenmann and Franke, 1985; Baumert *et al.,* 1990; Leimer *et al.,* 1996; Wu and Castle, 1997; Hannah *et al.,* 1998; Kupriyanova and Kandror, 2000). In addition, all TVPs contain four hydrophobic domains which are predicated to form TMRs with high probability of α-helicity. The spacing of the highly conserved four TMRs is specific for each family (Figs. 1–3 and 5). With the exception of the physin-related *C. elegans* polypeptide, no other hydrophobic domains have been identified that could transverse the membrane.

Charged amino acids within TMRs are of particular interest because these must be oriented away from the lipid environment and could thus aid in polypeptide interaction and/or formation of hydrophilic pores. An aspartic acid in TMR3 is conserved in all physins (Fig. 1). In addition, a lysine residue (arginine in the *C. elegans* ortholog) is present in TMR1 of most physins in a position that could be close to the oppositely charged aspartic acid of TMR3 (Fig. 1). Although the lysine is lacking in synaptoporin and the chicken physin ortholog (Fig. 1), mutagenesis experiments have demonstrated the importance of this residue for topogenesis of synaptophysin in transfected cells (Leube, 1995). No such function was seen for the conserved aspartic acid in the same assay (Leube, 1995). In all gyrin isoforms a charged residue is detected at the end of TMR1 that is a glutamic acid in most instances, an aspartic acid in human synaptogyrin 4, and a lysine in the *C. elegans* gyrin (Fig. 2). Remarkably, an aspartic acid is present in TMR3 of all gyrin isoforms corresponding to that conserved in the physins and thereby suggesting functional importance of this residue for both families (Figs. 1 and 2). In contrast, no conserved charged amino acids are seen within the presumptive TMRs of SCAMPs (Fig. 3).

Structure predictions which place the amino and carboxy termini of all TVPs toward the cytoplasm and the loops connecting either TMR1 and -2 or TMR3 and -4 toward the vesicle interior have been confirmed experimentally. In the case of synaptophysin, intact synaptic vesicle were treated with collagenase III which cleaved the carboxy terminus containing several collagenase recognition sites, thereby demonstrating their presence and accessibility on the vesicle exterior (Leube *et al.,* 1987). Further analyses of synaptophysin using antibodies generated against the cytoplasmic termini and the intravesicular loops in combination with

partial proteolysis corroborated the predicted transmembrane model (Johnston *et al.,* 1989b). In addition, vesicles of cDNA-transfected cells synthesizing a pantophysin mutant with a myc epitope in intravesicular loop 1 could be labeled *in vivo* by adding myc antibodies to the culture medium, thereby demonstrating transient exposition of this intravesicular epitope on the cell surface (Haass *et al.,* 1996). Further evidence for cytoplasmic location of the carboxy terminus was provided by immunoelectron microscopy. Gold particles were exclusively located on the cytoplasmic surface of vesicles when antibodies were used that react with the carboxy termini of synaptophysin (Wiedemann and Franke, 1985; Navone *et al.,* 1986; Hell *et al.,* 1988; Johnston *et al.,* 1989a; Leube *et al.,* 1989; Cameron *et al.,* 1991; Rosewicz *et al.,* 1992; Leube *et al.,* 1994), synaptoporin (Fykse *et al.,* 1993), or pantophysin (Haass *et al.,* 1996). These antibodies have also been used successfully to immunoisolate intact vesicles (Lowe *et al.,* 1988; Burger *et al.,* 1989; Leube *et al.,* 1989, 1994; Régnier-Vigoroux *et al.,* 1991; Haass *et al.,* 1996; Brooks *et al.,* 2000). Stenius *et al.* (1995) examined the topology of synaptogyrin 1 by subjecting purified synaptic vesicles to limited proteolysis and subsequent analysis of the resulting fragments in immunoblots with domain-specific antibodies. Their results are fully compatible with the proposed transmembrane arrangement with cytoplasmically exposed end domains.

The topology of SCAMP1 was examined by Hubbard *et al.* (2000) in secretory granules of rat parotid gland by also employing limited proteolysis in combination with immunoblotting using domain-specific peptide antibodies. They identified a trypsin-resistant SCAMP1 core domain corresponding to the TMRs with short, adjacent peptide segments and showed convincingly that the amino and carboxy termini face the cytoplasm. Furthermore, these authors analyzed the transmembrane topology of SCAMP1 in *Escherichia coli* by using a series of chimeras consisting of N-terminal SCAMP1 fragements fused to the PhoA indicator in alkaline phosphatase assays, fully supporting the predicted four transmembrane domain model with cytoplasmic end domains. Since the epitope of the most commonly used SCAMP antibody, SG7C12, reacts with the cytoplasmic N termini of different SCAMP isoforms (Singleton *et al.,* 1997; Hubbard *et al.,* 2000), immunoelectron microscopy (Brand *et al.,* 1991; Laurie *et al.,* 1993) and immunoisolation of intact vesicles (Brand *et al.,* 1991; Laurie *et al.,* 1993; Haass *et al.,* 1996; Brooks *et al.,* 2000) also show that this domain is exposed on the vesicular surface. Accordingly, protease treatment of zymogen granules removes the SG7C12 epitopes (Brand *et al.,* 1991).

Taken together, all available experimental evidence supports the view that TVPs are indeed integral membrane proteins with four membrane spanning segments and cytoplasmically located end domains and also that no other alternative topology exists. The high evolutionary conservation of the TMRs and the observed defects in correct topogenesis and protein stabilization of mutants lacking TMRs or containing single point mutations within these domains are strong indications of the structural and functional importance of these TVP segments (Leube, 1995).

E. Protein Modification

In view of the potential contribution of posttranslational modifications to the regulation of TVP function, we summarize experimental evidence for specific types of modification in this section.

1. *N*-Glycosylation

The physin isoforms synaptophysin, synaptoporin, and pantophysin are *N*-glycosylated as shown by their sensitivity to tunicamycin *in vivo* (Leube *et al.,* 1989), their reactivity with periodic acid Schiff in blots (Wiedenmann and Franke, 1985), and their sensitivity to endoglycosidase F and analogs *in vivo* (Johnston *et al.,* 1989a; Régnier-Vigoroux *et al.,* 1991) and *in vitro* (Johnston *et al.,* 1989a; Cutler and Cramer, 1990; Fykse *et al.,* 1993; Leube *et al.,* 1994; Brooks *et al.,* 2000). Immature (i.e., endoglycosidase H-sensitive) synaptophysin was detectable only for short periods in pulse-chase experiments (Cutler and Cramer, 1990; Régnier-Vigoroux *et al.,* 1991) but not at steady state, even in synaptophysin-overexpressing, cDNA-transfected cells (Leube *et al.,* 1994). Mutation of the N-glycosylation site in the first intravesicular loop of synaptophysin abolishes its glycosylation completely (Leube *et al.,* 1989). Sulfation of the carbohydrate moieties of synaptophysin has been used successfully in pulse-chase experiments (Régnier-Vigoroux *et al.,* 1991).

In contrast to theother physins,mitsugumin29 does not contain a *N*-glycosylation site in the first intravesicular loop and is consequently not glycosylated (Takeshima *et al.,* 1998). *N*-glycosylation is also absent in gyrins and SCAMPs, all of which lack potential *N*-glycosylation sites in the appropriate locations (Figs. 2 and 3) and have been shown to be glycosidase insensitive (Baumert *et al.,* 1990; Brand *et al.,* 1991). Taken together, *N*-glycosylation is not a common property of TVPs and does not appear to confer important functions even in the polypeptides that are subjected to it (Leube *et al.,* 1989).

2. Disulfide Linkage

Johnston and Südhof (1990) showed that the paired cysteine residues present in both intravesicular loop domains of synaptophysin form disulfide bonds within each loop and do not bind to cysteines in neighboring loop domains of either the same or adjacent molecules. They also demonstrated that these intramolecular disulfide bonds are a prerequisite for the association of synaptophysin with a low-molecular-weight protein that was later shown to be synaptobrevin 2. The paired cysteine residues are present in all mammalian physin isoforms and have been conserved throughout evolution, with the only exception being the *C. elegans* ortholog that lacks the paired cysteines in intravesicular loop domain 2 (Fig. 1). Mutation of cysteines did not alter the topogenesis of synaptophysin in

cDNA-transfected nonneuroendocrine cells, indicating that they are not needed for folding and exit of synaptophysin from the endoplasmic reticulum (Leube, 1995).

Paired cysteines are also present in the intravesicular loop 1 of all synaptogyrin para- and orthologs but not in their very short intravesicular loop 2 (Fig. 2). In addition, paired cysteines are absent in the intravesicular domains of SCAMPs (Fig. 3). It can therefore be concluded that intramolecular disulfide formation is not a common feature of all TVPs, although it appears to be of structural and/or functional importance in some cases.

3. Phosphorylation

Members of all three TVP families have been shown to incorporate phosphate. This is of particular interest since it would enable regulation of functions fulfilled by these polypeptides in membrane trafficking and complex neuronal processes including learning.

In fact, synaptophysin has been identified as one of the major tyrosine phosphorylated polypeptides of synaptic vesicles (Pang *et al.,* 1988; Baumert *et al.,* 1990; Greengard *et al.,* 1993). It is substrate of the protein tyrosine kinase $pp60^{c\text{-}src}$ that is present and active in intact synaptic vesicles, synaptosomes, and small synaptic-like microvesicles (SLMVs) of neuroendocrine cells (Pang *et al.,* 1988; Barnekow *et al.,* 1990; Linstedt *et al.,* 1992). Phosphorylation of synaptophysin by $pp60^{c\text{-}src}$ kinase and also by c-fyn-encoded kinase was shown *in vivo* in co-transfection assays of COS cells (Janz *et al.,* 1999). In addition, synaptophysin is also phosphorylated on serine residues (Pang *et al.,* 1988; Barnekow *et al.,* 1990; Rubenstein *et al.,* 1993). This phosphorylation is regulated in a Ca^{2+}-dependent manner and is mediated by Ca^{2+}/calmodulin-dependent protein kinase II, which is also present in synaptic vesicle preparations (Pang *et al.,* 1988; Rubenstein *et al.,* 1993). Interestingly, serine phosphorylation of synaptophysin is rapidly increased by depolarization (Rubenstein *et al.,* 1993). Both tyrosine and serine phosphorylation sites are located in the cytoplasmic carboxy terminus (Barnekow *et al.,* 1990; Greengard *et al.,* 1993), i.e., the most divergent physin domain (Fig. 1). Although mitsugumin29 and pantophysin lack tyrosine residues in their cytoplasmic carboxy terminus, Brooks *et al.* (2000) showed incorporation of [^{32}P]orthophosphate into pantophysin of adipocytes. Taken together, phosphorylation may be closely associated with isoform-specific functions.

Synaptogyrin 1 and synaptogyrin 2/cellugyrin are tyrosine phosphorylated on their carboxy termini [Baumert *et al.,* 1990; Janz and Südhof, 1998; Janz *et al.,* 1999; Sugita *et al.,* 1999; see also the M_r 30,000 component in Pang *et al.* (1988) and the M_r 29,000 polypeptide in Barnekow *et al.* (1990)] and are probably also serine phosphorylated [for synaptogyrin 1, see the M_r 30,000 component in Pang *et al.* (1988) and the M_r 29,000 polypeptide in Barnekow *et al.* (1990)]. Tyrosine phosphorylation of synaptogyrin 1 can be facilitated *in vitro* using intact synaptic

vesicles (Baumert *et al.,* 1990). Furthermore, similar to synaptophysin, synaptogryin 1 and synaptogyrin 2/cellugyrin are phosphorylated *in vivo* by $pp60^{c\text{-}src}$ and c-fyn tyrosine kinases after transfection of cDNA constructs into COS cells (Janz and Südhof, 1998; Janz *et al.,* 1999).

Wu and Castle (1998) identified tyrosine phosphorylation of SCAMP1 and SCAMP3 but not SCAMP2 in Chinese hamster ovary cells that were treated with the tyrosine phosphatase inhibitor vanadate. The vanadate-mediated increase in phosphorylation resulted in enhanced accumulation of SCAMP3 but not SCAMP1 in certain microdomains (“patches”) at or near the cell surface. Furthermore, it was shown that epidermal growth factor (EGF) treatment of fibroblasts overexpressing the EGF receptor induced phosphorylation of SCAMP1 and -3 but not SCAMP2 and led to enhanced colocalization of SCAMP3 and the EGFR in the cell interior, concurrent with increased association of SCAMP3 and EGFR. In support, SCAMP3 is tyrosine phosphorylated by EGFR *in vitro*. On the basis of these observations, it was suggested that phosphorylation of SCAMPs, most notably SCAMP3, and internalization/downregulation of EGFR are functionally linked (Wu and Castle, 1998). Indeed, the most likely SCAMP phosphorylation sites are strategically located in the amino terminus in close proximity to the NPF repeats that interact with EH-containing proteins known to be important for intracellular trafficking. It is of interest that in contrast to EGF, insulin-like growth factor-1, platelet-derived growth factor, and c-src did not induce SCAMP phosphorylation in several assay systems (Wu and Castle, 1998).

4. Cholesterol Binding

One of the most exciting recent findings on TVP modification was the identification of synaptophysin as a major cholesterol-binding protein in brain synaptic vesicles and in neuroendocrine PC12 cells (Thiele *et al.,* 2000; Huttner and Schmidt, 2000). Remarkably, cholesterol depletion specifically blocked the biogenesis of SLMVs in PC12 cells. It was therefore proposed that synaptophysin–lipid interactions are involved in the sorting of vesicle constituents from plasma membrane constituents by forming cholesterol-rich microdomains and that these interactions determine synaptic vesicle curvature. It will be interesting to determine whether protein–lipid interactions are also relevant for other TVPs.

5. Oligomerization

All TVPs are most likely present in multimeric complexes. First, we focus on oligomerization of TVPs either by association of identical molecules or by association with molecules of the same family. Interactions of TVPs with unrelated polypeptides are reviewed in the following section.

It is not disputed that synaptophysin and synaptoporin occur as homopolymers (Rehm *et al.,* 1986; Thomas *et al.,* 1988; Johnston and Südhof, 1990; Fykse

et al., 1993; Hannah *et al.,* 1998), and it is likely that pantophysin also occurs as a homomultimer (Haass *et al.,* 1996). It is unclear, however, whether complexes are composed of four subunits (Rehm *et al.,* 1986; Johnston and Südhof, 1990; Fykse *et al.,* 1993) or six subunits (Thomas *et al.,* 1988). Interactions between different physin isoforms have not been detected (Fykse *et al.,* 1993). Also, oligomerization of synaptogyrin isoforms has not been investigated, but SCAMP oligomers have been observed in Triton X-100 extracts of paraformaldehyde-fixed PC12 cells (Hannah *et al.,* 1998). Furthermore, cross-linking of SCAMPs has provided evidence for homomultimer formation generating up to four cross-linked SCAMP1 molecules (Wu and Castle, 1997). In immunoprecipitation experiments heteromeric complexes were identified containing SCAMP1 and SCAMP2, and in some instances even SCAMP1–3 (Singleton *et al.,* 1997; Wu and Castle, 1997).

An interesting proposal that was stimulated by the observed propensity of TVPs to form homomultimers and their similarity to connexins is that they form channels. In support, Thomas *et al.* (1988) reconstituted purified synaptophysin in lipid bilayers and demonstrated the formation of voltage-dependent pores. Furthermore, they showed 7.8-nm diameter particles with a central hollow in a negatively stained specimen of Triton X-100-solubilized synaptophysin. It should be noted, however, that the channel-forming capacity of synaptophysin and its relatives has not remained unchallenged (Südhof and Jahn, 1991).

6. Polypeptide Interactions

To understand the function of TVPs within their cellular context, the characterization of interactions with other polypeptides is of paramount interest. Synaptophysin is part of a large complex that includes several other synaptic vesicle proteins (Bennett *et al.,* 1992). Synaptophysin and two other physins, synaptoporin and pantophysin, have been shown to be associated with vSNAREs of the synaptobrevin type, of which synaptobrevin 2 [identical to vesicle-associated membrane protein 2 (VAMP2)] is apparently the major and preferred binding partner (Calakos and Scheller, 1994; Cornille *et al.,* 1995; Edelmann *et al.,* 1995; Washbourne *et al.,* 1995; Galli *et al.,* 1996; Brooks *et al.,* 2000). In the case of synaptophysin, it has been demonstrated that this association relies on the oligomeric state of the protein complex (Johnston and Südhof, 1990). Furthermore, the synaptophysin–synaptobrevin complex is Triton X-100 resistant (Calakos *et al.,* 1994; Edelmann *et al.,* 1995; Washbourne *et al.,* 1995; Galli *et al.,* 1996), it is broken by high salt concentrations (Edelmann *et al.,* 1995), it is irreversibly disrupted by low amounts of sodium dodecyl sulfate (Edelmann *et al.,* 1995), and it is sensitive to repeated freeze-thawing (Galli *et al.,* 1996). Both the amino-terminal cytoplasmic domain (Washbourne *et al.,* 1995; Cornille *et al.,* 1995) and the membrane domains (Edelmann *et al.,* 1995; Becher *et al.,* 1999) of synaptobrevin 2 have been implicated in this interaction. Also, it was shown that association of synaptobrevin with the tSNARE syntaxin I is not compatible with its interaction with synaptophysin

and vice versa (Edelmann *et al.,* 1995; Galli *et al.,* 1996). Synaptobrevin binding is specific for physins since neither synaptogyrin 1 nor SCAMPs bind to synaptobrevin 1, synaptobrevin 2, or cellubrevin *in vitro* (Edelmann *et al.,* 1995). It was hypothesized that the interaction between synaptophysin and synaptobrevin could be involved in the regulation of exocytosis (Calakos *et al.,* 1994; Edelmann *et al.,* 1995; Washbourne *et al.,* 1995). In support of this hypothesis, it was observed that the formation of the synaptophysin–synaptobrevin complex is developmentally regulated, probably by posttranslational modification of synaptophysin requiring a cytosolic factor to be present only in adult brain (Becher *et al.,* 1999). It was proposed that this maturation step is needed for high synaptic activity (Becher *et al.,* 1999). The synaptophysin–synaptobrevin complex has been shown to interact with the tail domain of brain myosin V in a Ca^{2+}-dependent fashion (Prekeris and Terrian, 1997). This interaction could be implicated in the final translocation process of synaptic vesicles to the active zone.

Release of myosin V-anchored synaptic vesicles and disassembly of the synaptophysin–synaptobrevin complex may then occur upon depolarization-induced Ca^{2+} entry into nerve endings. The synaptophysin–synaptobrevin complex also includes components of the vacuolar proton pump (V-ATPase), notably the 10-kDa c subunit (ductin and mediatophore), Ac39 (identical to physophilin), and Ac116 of the V_o sector (Thomas and Betz, 1990; Siebert *et al.,* 1994; Galli *et al.,* 1996). These associations are also Triton X-100 resistant and are disrupted by cycles of freeze-thawing (Galli *et al.,* 1996). They may be needed for recruitment of the vacuolar ATPase into synaptic vesicles.

Recently, it was reported that the cytoplasmic carboxy terminus of synaptophysin binds in a Ca^{2+}-dependent fashion to the GTPase dynamin I, which is involved in endocytotic vesicle fission from the plasma membrane (Daly *et al.,* 2000). This interaction was taken as an explanation for the observed inhibition of clathrin-independent endocytosis of synaptic vesicles in squid giant synapse preterminals that had been injected with synaptophysin peptides (Daly *et al.,* 2000).

Molecular interactions of the synaptogyrins have not been explored in detail. Fernández-Chacón *et al.* (2000) investigated whether the conspicuous NPF repeats present in the cytoplasmic amino terminus of SCAMPs1–3 (Fig. 3) interact with EH domain-containing proteins. Using the SCAMP1 amino terminus, using yeast two-hybrid assays, they identified as binding partners the EH domain proteins intersectin 1, which is involved in endocytotic budding at the plasma membrane, and γ-synergin, which may mediate vesicle budding in the trans-Golgi complex. Other EH domain proteins such as Eps 15 did not bind significantly. These results were confirmed using recombinant proteins produced in *E. coli* for identification of binding partners in rat brain. Furthermore, they showed that a SCAMP1 mutant lacking the NPF repeats inhibits endocytotic transferrin uptake almost completely. They speculated that SCAMPs participate in the recruitment of clathrin coats to the plasma membrane and the trans-Golgi network. SCAMP4 and -5, which do not have NPF repeats, may therefore act as negative regulators of these

activities. Another functionally interesting observation of SCAMP interactions is the previously-mentioned phosphorylation-dependent association of SCAMP3 with the EGFR (Wu and Castle, 1998).

III. Synthesis, Localization, and Function

A. Cell-Type-Specific Synthesis

Cell-type-specific synthesis of TVPs has been the subject of many investigations and it is not possible to give an exhaustive overview of all available literature, especially in the case of the neuronal physin isoforms. Tables II–V list mRNA and polypeptide expression profiles of the different TVPs in different cells and tissues of mammalian species, including human, cow, pig, mouse, rat, and rabbit. In most instances (except where noted), patterns of synthesis are identical in the different species examined. Furthermore, malignant transformation and *in vitro* culturing do not appear to directly affect the expression of any of the TVPs and their mRNAs. In the following sections, we note some emerging features of cell-type-specific TVP synthesis.

1. Physins

Roughly, three physin expression patterns can be distinguished: Synaptophysin and synaptoporin are detected in neuronal and neuroendocrine cells, mitsugumin29 is present exclusively in skeletal muscle and in small amounts in kidney and small intestine, and pantophysin is ubiquitously synthesized in most tissues but not all cells (Table II).

Based on its cell-type-restricted synthesis, synaptophysin has become a well-established marker for neuronal and neuroendocrine differentiation (Gould *et al.,* 1987; Buffa *et al.,* 1988; Wiedenmann and Huttner, 1989). It is indicative of the presence of the "classic" neurotransmitter-containing vesicles in synapses and of vesicles with similar appearance, the synaptic-like microvesicles (SLMVs), in neuroendocrine cells. Synaptophysin therefore represents a specific type of cellular specialization that is strictly associated with neuroendocrine differentiation, even in cases in which it might not be expected such as in platelets (Bähler *et al.,* 1990). The presence of trace amounts of synaptophysin mRNA in the absence of detectable amounts of synaptophysin polypeptide in several nonneuroendocrine cell lines may indicate the propensity/capacity of these cells for neuroendocrine differentiation (Leube, 1994). It was speculated that this feature is due to the *in vitro* cultivation which may have selected for cells with the capacity of neuroendocrine differentiation, and that this reflects a physiological process occurring also *in vivo* during times of elevated tissue proliferation in development, infection, or regeneration (Leube, 1994).

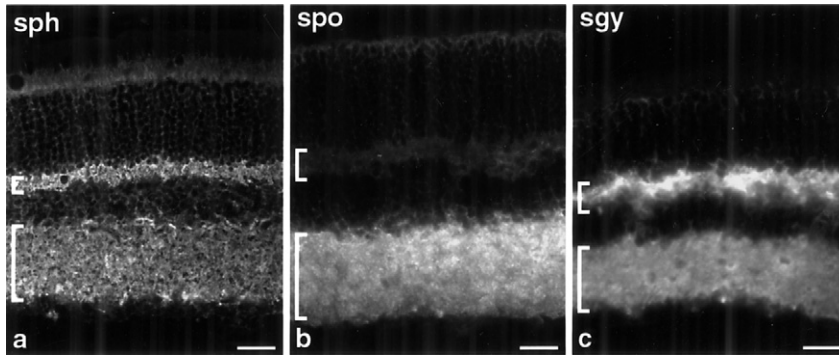

FIG. 6 Immunofluorescence microscopy of methanol/acetone-fixed (5 min methanol at −20°C; 10 min acetone at −20°C) murine retina using (a) monoclonal antibodies directed against an amino-terminal peptide of synaptophysin (kindly provided by Dr. Bertram Wiedenmann, Charite, Humboldt-University, Berlin, Germany; Eshkind and Leube, 1995; Spiwoks-Becker *et al.,* 2001), (b) polyclonal guinea pig antibodies anti-SyPo1 reacting with peptide CHSSGQRYLSDPMEKHS corresponding to a part of the cytoplasmic carboxy terminus of murine synaptoporin (the amino-terminal cysteine was added for coupling; Spiwoks-Becker *et al.,* 2001), and (c) monoclonal antibodies detecting synaptogyrin 1 (Synaptic Systems GmbH, Göttingen, Germany; Stenius *et al.,* 1995). The synapse-containing inner and outer plexiform layers are labeled by brackets. Note that synaptoporin is not detectable in the outer plexiform layer, whereas its paralog synaptophysin as well as synaptogyrin 1 are clearly detectable in both synaptic layers. Scale bars-20 μm.

In many instances, synaptophysin is coexpressed with synaptoporin (Table II and III). Synaptoporin mRNA and polypeptide expression, however, is much more restricted. Comparative examples are shown in Figs. 6–8. Figures 6a and 6b depict the complete absence of synaptoporin in the outer plexiform layer of murine retina which, in contrast, is strongly labeled by antibodies against synaptophysin. In the adrenal medulla a multipunctate staining is seen for both neuronal physin isoforms, albeit at different intensities (compare a and b in Fig. 7). Note also that the adrenal cortex is completely negative for both. In pancreas, synaptophysin and synaptoporin are not detectable in the exocrine gland but are seen exclusively in the endocrine islets of Langerhans (Figs. 8a and 8b). The signal for synaptoporin is barely above background (Fig. 8b). It has been reported that synaptoporin synthesis exceeds that of synaptophysin in a few cells (Table III), but to date there is no well-documented example of synaptoporin in the absence of synaptophysin.

Pantophysin originally obtained its name from the ubiquitous detection of its mRNA in all major tissues and their derivatives (Leube, 1994). Later, specific antibodies were developed that revealed a more complex picture. As expected, all cellular elements were labeled by pantophysin antibodies in many tissues (Haass *et al.,* 1996; Windoffer *et al.,* 1999). Figure 8 shows the ubiquitous detection of pantophysin in cells of the exocrine pancreas with prominent staining of the adluminal domain of secretory cells, and Fig. 9a presents the strong immunofluorescence of

TABLE II

mRNA and Protein Expression of Physin Gene Family Members in Mammalian Cells and Tissues[a]

	Synaptophysin		Synaptoporin		Pantophysin		Mitsugumin29	
	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein
Organs/tissues/cells								
Skin		—[9,24]			++[18]		—[28]	
Lung	—[8]	—[8,17]	—[15]		++[2]		—[28]	
Stomach		—[23,25,26]				+[32]	—[28]	—[28]
Intestine	—[8]	—[8]						
Small		—[25,26,31]				+[32]	—[28]	+[16]
Colon		—[25,26]				++[32,*e*]		
Liver	—[3,8,12,19]	—[8,14,31]	—[15]		+[2,18]	(+)[32,*e*]	—[28,29]	—[28]
Pancreas	—[8]	—[8]						
Exocrine		—[11,23,26,27,*d*]		—[*d*]		++[11,32,d]		
Endocrine		++[*b*,*d*]		++[*b*,*d*]		(+)[11,32]		
Kidney	—[8,19]	—[8,31]	—[15]		+[2]		+[28]	(+)[16,28]
Testis		—[5,22,31]			+[2]	++[32]	—[28]	
Skeletal muscle		—[6,31]			+[2]	+[32,*e*]	++[28,29]	++[1,13,16,28,29,33]
Heart muscle	—[12]	—[21]	—[15]		+[2]		—[28,29]	—[28]
Adrenal gland								
Cortex		—[*b*,*c*]		—[*b*,*c*]		+[*c*]		
Medulla	++[12]	++[*b*,*c*]	(+)[15]	++[*b*,*c*]		++[*c*]		
Brain	++[*b*]	++[*b*]	++[*b*]	++[*b*]	+[2,18]		—[28,29]	—[28]
Thymus		+[20,31]					—[28]	
Spleen	—[8]	—[8]	—[15]		+[2]		—[28]	—[28]
Adipocytes		—[4]			++[2(*g*)]	++[2(*g*)]		
Tumors								
Pheochromocytoma	+[19]	++[10,11]				+[11]		

Cell lines				
Neuroendocrine				
PC12	++[3,18,19]	+[7,11,23]	+[18]	
AtT20	++[3]	+[30]	(+)[11]	
NCI-H69	++[19]		+[18]	
NCI-H82	++[19]		+[18]	
BON	+[18]		(+)[18]	
Epithelial				
PLC	+[18]	—[18]	+[18]	++[11,f]
A431	—[18]	—[18]	++[18]	++[11]
RT-112	—[18]	—[18]	+[18]	
Mesenchymal				
SV-80	+[18]	—[18]	++[18]	

[a]Only those results are listed for which the synthesis of more than one family member was analyzed in a given cell or tissue. In addition, a complete compendium of observations and references is not given for the expression patterns of synaptoporin and synaptophysin in neuroendocrine and neuronal cells, and comparative results for both isoforms are presented in Table III. —, Not detectable; (+), weak; +, moderate; ++, high. 1, Brandt and Caswell (1999); 2, Brooks *et al.* (2000); 3, Buckley *et al.* (1987); 4, Cain *et al.* (1992); 5, Davidoff *et al.* (1993) (positive reactivity only in Leydig cells); 6, De Camilli *et al.* (1988) (positive reactivity only in nerve endings); 7, Franke *et al.* (1986); 8, Gaitanou *et al.* (1997); 9, García-Caballero *et al.* (1989); 10, Gould *et al.* (1987); 11, Haass *et al.* (1996); 12, Johnston *et al.* (1989b); 13, Jorgensen *et al.* (1990) (TS28 is identical to mitsugumin29); 14, Kanda *et al.* (1994) (positive reactivity only in nerve terminals); 15, Knaus *et al.* (1990); 16, Komazaki *et al.* (1999); 17, Lee *et al.* (1987) (positive reactivity only in neuroendocrine cells); 18, Leube (1994); 19, Leube *et al.* (1987); 20, Maggiano *et al.* (1999); 21, Metz *et al.* (1986); 22, Middendorff *et al.* (1993) (positive reactivity only in Leydig cells); 23, Navone *et al.* (1986); 24, Ortonne *et al.* (1988) (positive reactivity only in Merkel cells); 25, Portela-Gomes *et al.* (1999) (positive reactivity only in neuroendocrine cells); 26, Portela-Gomes *et al.* (2000) (positive reactivity only in neuroendocrine cells); 27, Redecker *et al.* (1991); 28, Shimuta *et al.* (1998); 29, Takeshima *et al.* (1998); 30, Tooze and Hollinshead (1992); 31, Wiedenmann and Franke (1985); 32, Windoffer *et al.* (1999); 33, Yuan *et al.* (1991) (TS28 is identical to mitsugumin29).

[b]See Table III.

[c]See Fig. 7.

[d]See Fig. 8.

[e]See Fig. 9.

[f]See Figs. 11–13.

[g]3T3-L1 derived.

TABLE III

Comparison of Synthesis of Synaptophysin and Synaptoporin in Neuronal and Neuroendocrine Cells and Tissues[a]

	Synaptophysin		Synaptoporin	
	mRNA	Protein	mRNA	Protein
Neocortex		+++[4]		+++[4]
Layers II–III	+[6]		+[6]	
Layers IV–V	+++[6]		+[6]	
Amygdala	++[6]		+[6]	
Hippocampal formation				
Prenatal		++[4]		—[4]
Postnatal		+++[4]		+++[4]
Dentate gyrus	+++[6]	+++[3,4]	++[6]	+++[3,4]
Pyramidal cell layer				
CA1/2	+++[6]	+++[4]	++[6]	—[4]
CA3	+++[6]	+++[3,4]	—[6]	+++[3,4, mossy fiber boutons]
CA4	+++[6]	+++[4]	++[6]	+++[4, mossy fiber boutons]
Hippocampal neurons *in vitro*		+++[5]		++[5]
Septum	++[6]		—[6]	
Striatum				
Prenatal (>E14)	++[7]	++[7]	(+)[7]	—[7]
Postnatal	+[6]		++[6]	
Afferent fibers		++[7]		(+)[7]
Neuronal perikarya		—[7]		++[7]
Globus pallidus	+[6]		—[6]	
Olfactory system				
Olfactory neurons		+++[1]		+[1]
Olfactory bulb		++[4]		+[4]
Periglomerula/glomeruli	++[1,6]	+++[1]	++[1,6]	+++[1]
External plexiform layer	++[6]	++[1]	++[1,6]	++[1]
Mitral cell layer	+++[1,6]	++[1]	—[1,6]	+[1, not in mitral cells]
Internal granule cell layer	+[6]	+[1]	+++[1,6]	+[1]
Cortex piriformis (layer 1a)		++[1]		—[1]
Retina		++[9, cow]		++[9, cow]
Inner plexiform layer		++[2, rat/rabbit]		+[2, rat]
		++[8,*b*, mouse]		++[2, rabbit]
				++[8,*b*, mouse]
Outer plexiform layer		++[2, rat /rabbit]		+[2 rat]
		++[8,*b*, mouse]		+[2, rabbit (only horizontal cells)]
				—[8,*b*,mouse]
Thalamus	+++[6]		—[6]	
Hypothalamus	++[6]		+[6]	
Epithalamus (medial habenula)	+++[6]		+++[6]	

(*continued*)

TABLE III (*continued*)

	Synaptophysin		Synaptoporin	
	mRNA	Protein	mRNA	Protein
Colliculus superior	++[6]		++[6]	
Colliculus inferior	++[6]		+[6]	
Substantia nigra compacta	++[6]		—[6]	
Cerebellum		++[4]		(—)[4]
Molecular cell layer	++[6]	+++[3]	—[6]	++[3, caudal >rostral]
Ganglionar cell layer	+++[6]	—[3]	—[6]	—[3]
Granular cell layer	+[6]	++[3]	—[6]	(+)[3]
Spinal cord		+[4]		—[4]
Dorsal horn	+++[6]		++[6]	
Ventral horn	+++[6]		—[6]	
Adrenal gland				
Cortex		—[b]		—[c]
Medulla		+++[c]		++[c]
Endocrine pancreas		+++[d]		+[d]

[a] Only those results are listed for which synaptophysin and synaptoporin expression were directly compared. Observations on prenatal tissues are only shown in cases of significant differences to the mature tissues. Species are only given for observations in retina since species-dependent differences were reported in this case. For further references on synaptophysin expression in neuronal and neuroendocrine tissues, see Gould *et al.* (1987), Buffa *et al.* (1988), Wiedenmann and Huttner (1989), Papierz *et al.* (1995), and Portela-Gomes *et al.* (1999, 2000). —, Not detectable; +, moderate; ++, high; +++, very high. 1, Bergmann *et al.* (1993); 2, Brandstätter *et al.* (1996); 3, Fykse *et al.* (1993); 4, Grabs *et al.* (1994); 5, Grosse *et al.* (1998); 6, Marquèze-Pouey *et al.* (1991); 7, Ovtscharoff *et al.* (1993); 8, Spiwoks-Becker *et al.* (2001); 9, von Kriegstein *et al.* (1999).

[b] See Fig. 6.

[c] See Fig. 7.

[d] See Fig. 8.

all cell elements in the colon mucosa. On the other hand, heterogeneities were noted in other tissues, especially those containing neuroendocrine and neuronal cells which appear to synthesize variable amounts of pantophysin (Haass *et al.,* 1996; Windoffer *et al.,* 1999). Another example of heterogeneity of pantophysin detection is shown in Fig. 9c, in which connective tissue elements surrounding muscle fibers are strongly positive, whereas immunofluorescence within the muscle cells is weak and restricted to certain subcellular domains that are not coincident with the mitsugumin 29-stained T tubules (Jorgensen *et al.,* 1990; Yuan *et al.,* 1991; Takeshima *et al.,* 1998; Komazaki *et al.,* 1999). In some cells, however, pantophysin immunoreactivity was not detected (Windoffer *et al.,* 1999). Strikingly, pantophysin is absent in vesicle-rich hepatocytes, as shown in the immunofluorescence micrograph of bovine liver in Fig. 9b. The fluorescence is restricted to endothelial and/or mesenchymal elements in this tissue (Windoffer *et al.,* 1999).

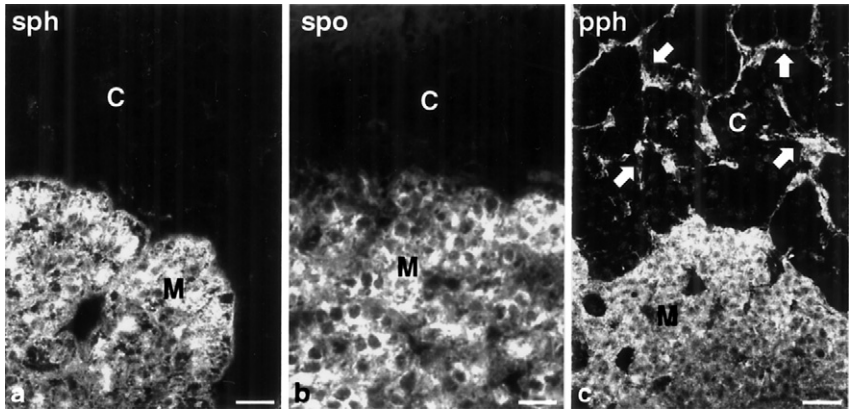

FIG. 7 Immunofluorescence microscopy of adrenal glands from mouse (a, b) and cow (c) showing the distribution of physin paralogs by detection with (a) polyclonal rabbit antibodies against a cytoplasmic carboxy-terminal synaptophysin peptide (DAKO, Glostrup, Denmark), (b) polyclonal guinea pig antibodies anti-SyPo2 against peptide CGSSGGYSQQANLG corresponding to a part of the cytoplasmic carboxy terminus of murine synaptoporin (the amino-terminal cysteine was added for coupling; Spiwoks-Becker *et al.,* 2001), and (c) polyclonal chicken antibodies ch1 against the entire cytoplasmic carboxy terminus of human pantophysin (Haass *et al.,* 1996; Windoffer *et al.,* 1999). Tissue sections in a and c were fixed with 3% formaldehyde for 20 min at 4°C and permeabilized with 0.01% digitonin for 10 min at room temperature; the section in b was fixed with methanol/acetone (see Fig. 6). Note the strong reactivity in the adrenal medulla (M) in all instances, the complete absence of synaptophysin and synaptoporin staining in the cortex (C), and the endothelium-restricted immunofluorescence for pantophysin in the cortex. Scale bars-20 μm.

Figure 7c demonstrates another example of restricted pantophysin detection. In this case, positivity is noted in the adrenal medulla and in endothelial cells of the cortex but not in cortical steroid-producing cells.

In contrast to pantophysin, mitsugumin29 is highly restricted in its distribution (Table II). Remarkably, mRNA and protein are abundantly synthesized only in skeletal muscle, and not in heart muscle, suggesting a highly specific function in these cells. In addition, mitsugumin29 is also present in small amounts in the small intestine (Komazaki *et al.,* 1999) and in kidney, in which it appears to be synthesized only in proximal and distal parts of tubules and not in collecting tubules and the glomerulus (Shimuta *et al.,* 1998).

2. Gyrins

Results reported for the detection of synaptogyrins are summarized in Table IV. Synaptogyrin 2/cellugyrin mRNA and protein exhibit the most widespread distribution and have been shown to be present in all cell types analyzed to date, albeit in differing amounts. Similar to pantophysin, its synthesis is reduced in neuronal

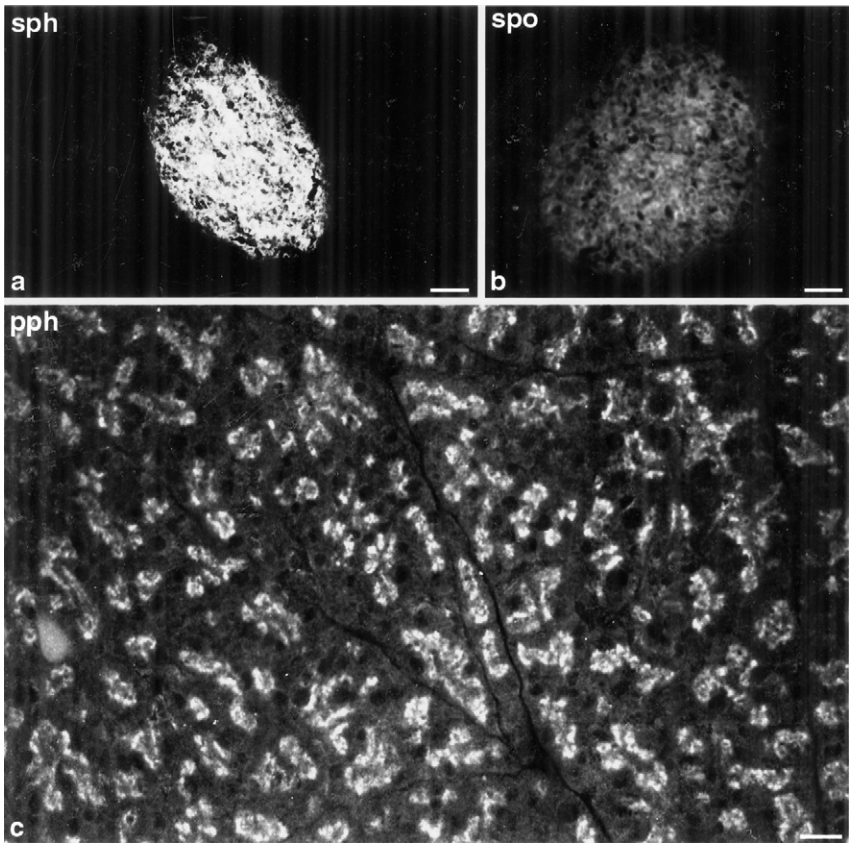

FIG. 8 Immunofluorescence microscopical detection of physin isoforms in formalin-fixed and digitonin-lysed murine (a, b) and bovine pancreas (c) using the same primary antibodies as in Fig. 7. Note the absence of fluorescence in the exocrine gland in a and b, in contrast to the staining of apical cell poles of exocrine secretory cells in c. Endocrine Langerhans islets stain strongest for synaptophysin (a), weaker for synaptoporin (b), and heterogeneously for pantophysin (not shown; compare with Haass *et al.,* 1996; Windoffer *et al.,* 1999). Scale bars-10 μm.

tissues. In contrast, synaptogyrin 1 polypeptide has only been detected in neurons and neuroendocrine cells, although mRNA expression has been observed in some nonneuroendocrine cells, most notably during development. Whether the mRNA detection of synaptogyrin 1 in nonneuronal tissues is due to the presence of neuroendocrine cells or corresponds to polypeptide expression in nonneuroendocrine cells is not clear. Figure 6c shows the restricted staining of synaptogyrin 1 antibodies in the outer and inner plexiform layer of murine retina (i.e., the regions of synaptic contact formation). In the case of synaptogyrin 3, only mRNA synthesis has been analyzed, which was shown to be restricted to placenta and brain.

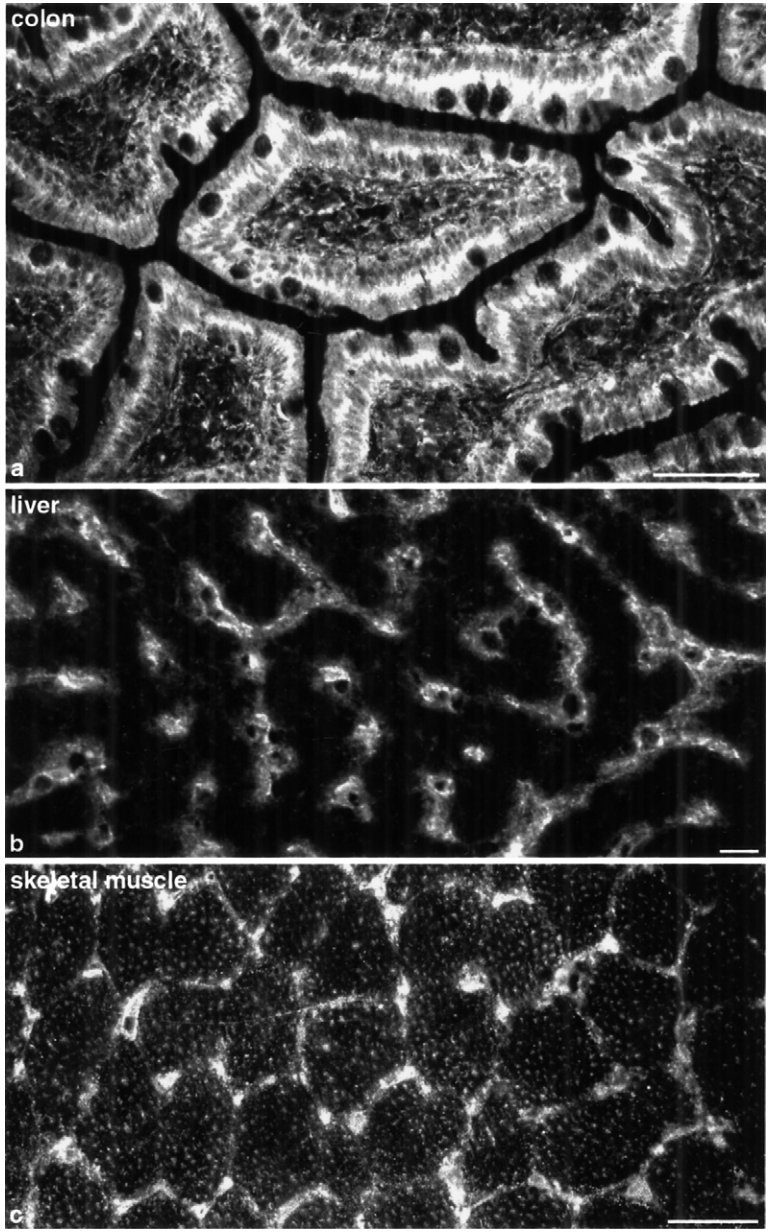

FIG. 9 Indirect immunofluorescence microscopy of formalin-fixed and digitonin-permeabilized human (a) and bovine (b, c) tissues using polyclonal antibodies ch1 against pantophysin (see Fig. 7). (a) Strong reactivity of colon mucosa, including all cells of the lamina epithelialis and lamina propria; (b) immunofluorescence of liver section with negativity in hepatocytes and punctate labeling of endothelial and/or mesenchymal cells; and (c) pantophysin-reactive stromal elements of skeletal muscle surrounding muscle fibers with restricted labeling of intracellular elements. Scale bars-50 μm in (a) 10 μm in (b) and 5 μm in (c).

TABLE IV
mRNA and Protein Expression of Gyrin Gene Family Members in Mammalian Cells and Tissues[a]

	Synaptogyrin 1		Synaptogyrin 2/ cellugyrin		Synaptogyrin 3	
	mRNA[c]	Protein	mRNA	Protein	mRNA	Protein
Organs/tissues/cells						
Liver						
Fetal	(+)[4]		++[4]			
Adult	—[2(d),4]	—[1,2]	++[2,4]	++[2,5]	—[4,10]	
Pancreas	—[4]		++[4]		—[4]	
Exocrine		—[1]				
Endocrine		++[1]				
Intestine						
Endocrine cells		++[1]				
Small intestine	(+)[4]		++[4]			
Colon	+[4]		++[4]			
Lung						
Fetal	(+)[4]		++[4]			
Adult	(+)[2] —[4]	—[1,2]	++[2,4]	++[2]	—[4,10]	
Testis	—[2,rat] +[4,mouse,human]	—[2]	+[2] ++[4]	++[2]	—[10]	
Prostate	+[4]		++[4]			
Kidney						
Fetal	+[4]		++[4]			
Adult	+[2,rat,d] —[4,mouse,human]	—[1,2]	++[2(d),4]	++[2]	—[4,10]	
Heart	—[2,rat,d] (+)[4,mouse,human,e]		++[2,4]		—[4,10]	
Skeletal muscle	—[2(d),4(e)]	—[1,g]	++[2,4]		—[4,10]	
Ovary	+[4,f]		++[4]			
Placenta	—[4]		++[4]		++[4]	
Spleen	—[2,4]	—[2]	++[2,4]	++[2]	—[10]	
Thymus	—[4]		++[4]			
Leukocyte	—[4]		++[4]			
Adipocyte				++[5]		
Central nervous system						
Fetal brain	++[4]	+[4,12]	+[4]			
Adult brain	++[2,4]	++[1–3,6,9]	(+)[2] +[4]	(+)[2]	++[4,10]	
Cortex cerebri	++[4]	++[1,3]				
Cerebellum		++[1]				
Hippocampus	++[4]	++[3,7]				
Basal ganglia	++[4]					
Hypothalamus	++[4]	++[1]				
Spinal cord	++[4]	+[1]				
Dorsal root ganglion	++[4]					

(*continued*)

TABLE IV (*continued*)

	Synaptogyrin 1		Synaptogyrin 2/ cellugyrin		Synaptogyrin 3	
	mRNA[c]	Protein	mRNA	Protein	mRNA	Protein
Hypophysis						
Neurohypophysis		++[1]				
Adenohypophysis		++[1]				
Eye						
Developing eye	++[4]					
Retina		+[13,b]				
Adrenal gland						
Medulla		++[1]				
Cortex		—[1]				
Glandula parathyroidea		++[1]				
Cells lines						
Neuroendocrine						
PC12		++[1]				
RINm5F		++[1,8]				
βTC3		++[11]				
αTC9		++[11]				

[a] No information is available for synaptogyrin 4. Species are only indicated in cases of differing results. —, Not detectable; (+), weak; +, moderate; ++, high. 1, Baumert *et al.* (1990); 2, Janz and Südhof (1998); 3, Janz *et al.* (1999); 4, Kedra *et al.* (1998); 5, Kupriyanova and Kandror (2000); 6, Maycox *et al.* (1992); 7, Mundigl *et al.* (1993); 8, Reetz *et al.* (1991); 9, Stenius *et al.* (1995); 10, Sugita *et al.* (1999); 11, Thomas-Reetz *et al.* (1993); 12, Ulfig *et al.* (2000); 13, von Kriegstein *et al.* (1999).

[b] See Fig. 6.

[c] Splice variant a.

[d] Detection of lower reactive bands that may correspond to alternative splice variants.

[e] + for splice variant b; see Kedra *et al.* (1998).

[f] + for splice variant c; see Kedra *et al.* (1998).

[g] + in nerve terminals.

3. SCAMPs

SCAMPs were originally isolated from exocrine glands as secretory vesicle components (Cameron *et al.,* 1986; Brand *et al.,* 1991; Laurie *et al.,* 1993), and it was later realized that they are also synthesized in many other cell types. Results of mRNA and protein detection are summarized in Table V. The most restricted detection is observed for SCAMP5, which is synthesized only in mature neurons and is absent even in neuroendocrine cells such as the adenohypophysis. In contrast, all other isoforms are ubiquitously expressed with some paralog-specific variability. Notably, SCAMP2 and SCAMP3 polypeptides appear not to be synthesized in appreciable amounts in adult brain.

4. Detection of Multiple TVPs and Their mRNAs

From the available information on cell-type-specific synthesis, it can be concluded that each TVP family includes members ubiquitously expressed, such as pantophysin, synaptogyrin 2/cellugyrin, and SCAMP1–4; others that are primarily restricted to neurons and/or neuroendocrine cells, such as synaptophysin, synaptoporin, synaptogyrin 1, and SCAMP5; and those that are present only in very specific cell types, such as mitsugumin29 in skeletal muscle and synaptogyrin 3 in placenta and brain. Thus, most cells contain several TVPs and in most instances at least one member of each family.

5. TVP Detection in Cells of Living Animals

Recent advances have made it possible to monitor protein expression in living organisms. An example of TVP detection is shown in Fig. 10. In this experiment, the gyrin-related *C. elegans* gene *sng-1* including 5′ upstream regulatory elements and all introns and exons up to the last amino acid-coding codon were polymerase chain reaction amplified. This segment was cloned in frame next to a cDNA coding for the enhanced yellow fluorescent protein, thereby producing a hybrid gene that codes for a fluorescent gyrin chimera. This DNA construct was injected into worms and the fluorescence detectable in one of the resulting transgenic animals is presented. This shows that gyrin synthesis is restricted to neuronal cells in which it is detectable in perikarya as well as in synapses (Nonet, 1999).

B. Subcellular Localization

The precise mapping of the subcellular localization of each TVP will certainly help in understanding the function of each member. Given experimental problems of detection and separation techniques as well as the transient presence of TVPs in various cellular membrane systems, we are far from knowing their exact distribution. Even with these limitations, it can be stated that the inner nuclear membrane, mitochondria, peroxisomes, and lysosomes are practically devoid of TVPs (Leube *et al.,* 1989; Haass *et al.,* 1996; Fig. 11c). Furthermore, only very small amounts of TVPs are found in the endoplasmic reticulum (ER) and the plasma membrane under steady-state conditions (Fig. 11a). In situations of overexpression, during certain stages of development and for certain TVP mutants, considerable accumulations have been observed in the ER (Leube, 1995; Leimer *et al.,* 1996; Komazaki *et al.,* 1999). It appears safe to conclude that the ER is the biosynthetic compartment from which fully synthesized and correctly folded polypeptides are released to the Golgi complex. Pulse-chase experiments support this view (Régnier-Vigoroux *et al.,* 1991). Similarly, in most cases the plasma membrane is only a transitory compartment to which TVPs are transported after biosynthesis and from which

TABLE V

mRNA and Protein Expression of SCAMP Gene Family Members in Mammalian Cells and Tissues[a]

	SCAMP1		SCAMP2		SCAMP3		SCAMP4		SCAMP5	
	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein
Organs/tissues/cells										
Liver	(+)[2,4,12]	+[1,2,12,13,16]	++[4] +[12]	++[2,4] +[13,16]		++[4] (+)[12]		++[4]		—[4]
Small intestine		+[16]		+[16]						
Parotid gland	+[2]	++[1,2,16,17]	++[1,2,16,17]							
Pancreas	++[12]	++[2]	++[12]	++[2]	++[12]			++[9]		
Exocrine		++[1]		++[1]						
Lung	+[4,12]	++[13]	++[4,12]	++[13]	+[4] (+)[12]		++[4]		—[4]	—[4]
Testis	(+)[4]	+[16]	++[4]	+[16]	(+)[4]		++[4]		—[4]	
Epididymis		++[16]		++[16]						
Kidney	+[2,4,12]	+[4] (+)[13]	++[4] +[12]	(+)[13]	++[4] —[12]		++[4]		—[4]	—[4]
Heart	(+)[4,15] ++[12]	++[6,13]	++[4] +[12]	++[6,13]	+[4] ++[12]		(+)[4]		—[4]	
Skeletal muscle	(+)[4] +[2] ++[12]	+[2] (+)[16]	++[4] +[12]	+[2] (+)[16]	++[4,12]		+[4]		—[4]	
White		(+)[13]		+[13]						
Red		+[13]		++[13]						
Spleen	(+)[4]		++[4]		+[4]		++[4]		—[4]	
Placenta	++[12]		++[12]		++[12]					
Fibroblast (primary)		++[1]		++[1]						
Adipocyte										
White	+[2]	+[2,11,13]		+[2,11,13]	+[12]					
Brown		++[13]		++[13]						
Neutrophil granulocyte		(+)[3]		++[3]						
Mast cell		++[7,5]		++[7]						
Red blood cell		—[13]		—[13]						
Brain										
Prenatal		++[4] +[13]		++[13]						—[4]
Postnatal (>2nd week)	++[2,4,12,15]	++[1,2,4]	++[2] —[12]	—[2,4]	+[4] —[12]	—[4]	+[4,10]		++[4]	++[4]

Cortex						$+^{10}$		
Hippocampus		$++^{4}$				$++^{10}$		$++^{4}$
Habenula						$++^{10}$		
Cerebellum		$++^{4}$						$++^{4}$
Hypothalamus						$++^{10}$		
Pituitary gland	$+^{2}$	$++^{2}$		$+^{2}$				
Neurohypophysis		$++^{4}$						$++^{4}$
Adenohypophysis		$++^{1,4}$		$++^{1}$		$++^{10}$		$—^{4}$
Retina		$++^{14}$						
Astrocytes (primary)		$+^{4}$	$+^{4}$					$—^{4}$
Adrenal gland	$+^{2}$	$++^{1,2,4}$		$++^{1,2,4}$				$—^{4}$
Tumors								
Insulinoma		$++^{1}$		$++^{1}$				
Cell lines								
Neuroendocrine								
PC12		$++^{4}$		$+^{4}$				$+^{4}$
AtT-20		$++^{4}$		$(+)^{4}$			$++^{9}$	$+^{4}$
HNT		$+^{4}$		$(+)^{4}$				$—^{4}$
Epithelial								
HeLa		$+^{12}$		$+^{12}$	$+^{12}$			
COS		$++^{4}$		$++^{4}$				$—^{4}$
Vero		$++^{17}$		$++^{17}$				
PLC		$++^{8}$		$++^{8}$				
A431		$++^{8}$		$++^{8}$				
Mesenchymal								
STO1		$+^{4}$		$+^{4}$				$—^{4}$
CHO		$++^{12,17,18}$		$++^{12,17,18}$	$+^{12,17,18}$			
3T3-L1 adipocytes		$++^{3}$		$++^{3}$				
NIH 3T3						$++^{9}$		
NRK							$++^{9}$	

[a]—, Not detectable; (+), weak; +, moderate; ++, high. 1, Brand *et al.* (1991); 2, Brand and Castle (1993); 3, Brumell *et al.* (1995); 4, Fernández-Chacón and Südhof (2000); 5, Fernández-Chacón *et al.* (1999); 6, Fischer *et al.* (1997); 7, Guo *et al.* (1998); 8, Haass *et al.* (1996) (tentative assignment of reactive bands as SCAMPs1 and -2); 9, Hubbard *et al.* (2000); 10, Krebs and Pfaff (2001); 11, Laurie *et al.* (1993); 12, Singleton *et al.* (1997); 13, Thoidis *et al.* (1993) (tentative assignment of GTV3 proteins as SCAMPs1 and -2); 14, von Kriegstein *et al.* (1999); 15, Wen *et al.* (1998); 16, Windoffer *et al.* (1999) (tentative assignment of reactive doublet as SCAMPs1 and -2); 17, Wu and Castle (1997); 18, Wu and Castle (1998).

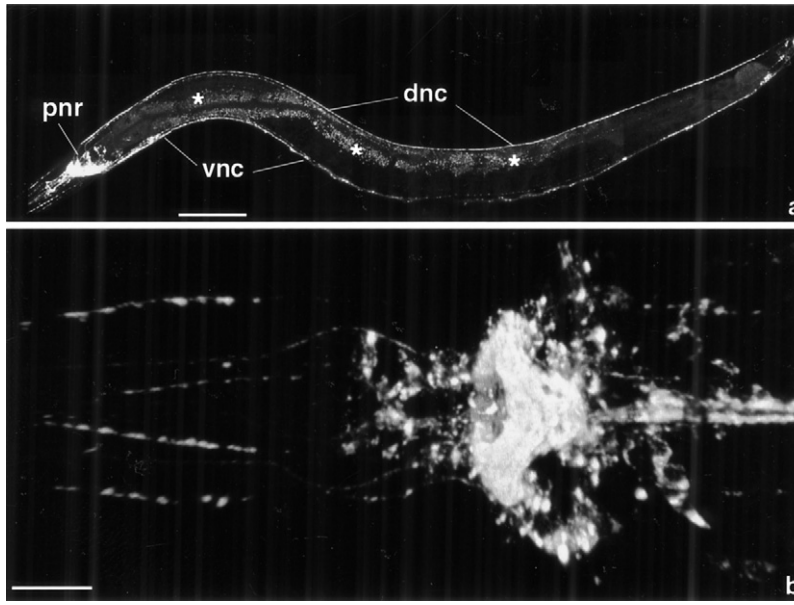

FIG. 10 Fluorescence microscopy of a live transgenic nematode expressing a fluorescent synaptogyrin chimera consisting of the entire *C. elegans* synaptogyrin that is fused to the enhanced yellow fluorescent protein. The specific fluorescence is restricted to neurons, including, most notably, the dorsal and ventral nerve cords (dnc and vnc, respectively) and the pharyngeal nerve ring (pnr) as seen in the composite micrograph in a (lateral view). Stars demarcate areas with nonspecific background fluorescence due to ingested bacteria. (b) The three-dimensional reconstruction (program Amira) shows the head region in a ventral view at higher magnification with details of the strongly fluorescent pharyngeal nerve ring with labeled perikarya, the ventral nerve cord projecting to the right with synaptic regions and dendritic processes to the left. Animation of the reconstruction is provided at *http://www.uni-mainz.de/FB/Medizin/Anatomie/Leube/movies.html*. Scale bars-100 μm in (a) and 10 μm in (b).

they are taken up into endocytotic compartments. Therefore, TVPs can be labeled or modified by extracellular agents (Johnston *et al.,* 1989a; Régnier-Vigoroux *et al.,* 1991; Green and Kelly, 1992; Schmidt *et al.,* 1997; Schmidt and Huttner, 1998). The polypeptide appears to be retained in the plasma membrane only in the case of mitsugumin29, in which it is concentrated in certain subdomains, namely the transverse tubules of skeletal muscle with highest concentrations at the curved tips that are parts of the triad junctions (Jorgensen *et al.,* 1990; Yuan *et al.,* 1991; Takeshima *et al.,* 1998; Brandt and Caswell, 1999; Komazaki *et al.,* 1999). All other remaining membrane systems contain significant amounts of TVPs. We summarize some of the emerging principles that can be formulated for the subcompartment specificity of TVPs within these systems in relation to cell-type specificity and colocalization of single family members. Results are also listed in Table VI.

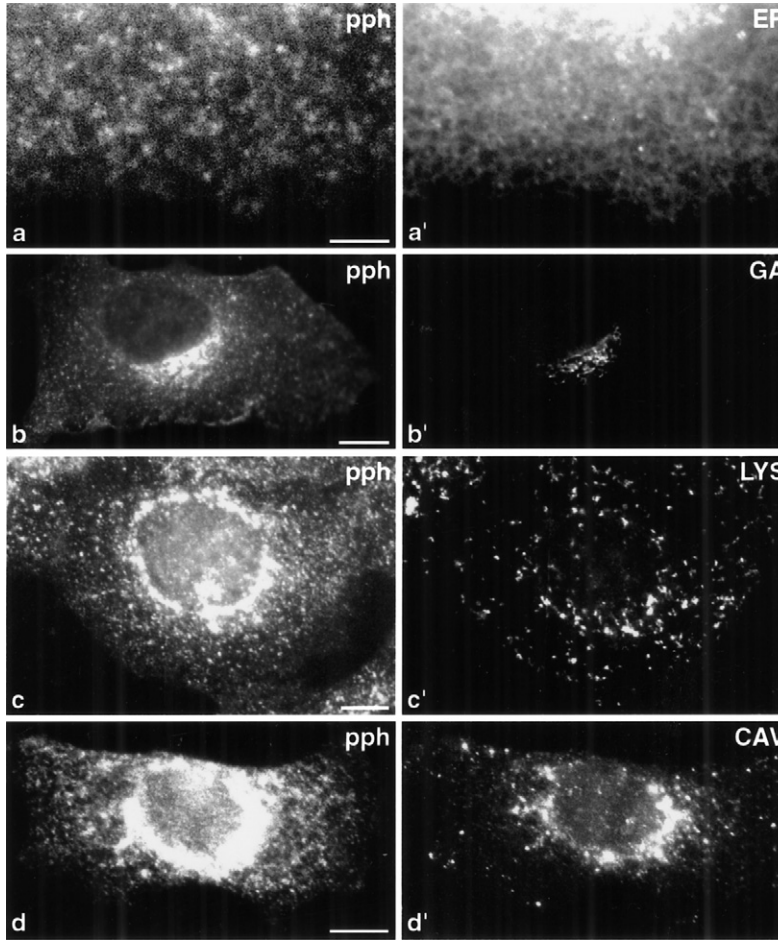

FIG. 11 Double fluorescence microscopy detecting pantophysin (pph) together with markers for the endoplasmic reticulum (ER), the Golgi apparatus (GA), lysosomes (LYS), and caveolae (CAV) in human hepatocellular carcinoma-derived PLC cells. (a, a′) Live cell fluorescence microscopy of cells cotransfected with a chimera consisting of human pantophysin and the enhanced green fluorescent protein at the carboxy terminus (a) together with a chimera consisting of the human KDEL receptor fused to the enhanced cyan fluorescent protein (b; kindly provided by Dr. Jamie White, EMBL, Heidelberg, Germany; White *et al.,* 1999). (b, b′) Fluorescence microscopy of methanol/acetone-treated cells detecting fluorescent pantophysin chimera consisting of human pantophysin and the enhanced green fluorescent protein at its carboxy terminus by direct epifluorescence in cDNA-transfected cells together with an 58-K Golgi-associated protein by indirect immunofluorescence using monoclonal antibodies (Sigma, St. Louis, MO). (c, c′) Double immunofluorescence microscopy detecting pantophysin in formalin-fixed PLC cells with chicken antibodies ch1 (see Fig. 7) together with lysosomal membrane-associated protein LAMP-2 using monoclonal antibody H4B4 (Developmental Studies Hybridoma Bank, Iowa City, IA). (d, d′) Detection of pantophysin (polyclonal antibodies ch1) together with caveolin (monoclonal antibody 2234 from Transduction Laboratories, Lexington, KY) in methanol/acetone-fixed cells. Note the different distribution patterns in all instances with some overlap in the perinuclear region between pantophysin reactivity and that of the 58-K Golgi-associated protein and caveolin, respectively. Scale bars-5 μm in (a) and 10 μm in (b–d).

TABLE VI

Identification of TVPs in Subcellular Compartments[a]

Compartment	Physins	Gyrins	SCAMPs
Endoplasmic reticulum	sph[40,c]		
Rough endoplasmatic reticulum	sph[50(weak!)]		
Sarcoplasmic reticulum	mgu[34 (during development)]		
Golgi complex	pph[25,75]	sgy1[4,46]	sca1[7(only very little)]
	sph[31,41(b),50,60,67]		sca2[7]
	spo[53]		
Trans-Golgi system	sph[24]		
Transport vesicles			sca1-3[72]
Ubiquitous microvesicles	pph[25]		
	sph[12(b),16(b),28(b),40(c),41(b),42(b)]		
Neuronal transport vesicles	sph[43,44,52,74]		
GLUT4 vesicles	pph[10]	sgy2[36]	sca1,2[19,32,38,61]
Regulated secretory vesicles			
Synaptic vesicles	sph[1,5,14,20,26,31,35,39,50,51,60,67,69,71]	sgy1[4,27]	sca1[7,17,65]
	spo[5,8,22,23,33]		sca5[17]
Large, dense core granules			sca1[17]
			sca1,2[7]
Small, dense core vesicles	sph[3]		
SLMVs	sph[12,15,21,28,42,50,55,57,64,66]	sgy1[4,66]	
SLMV donor compartment	sph[58,59]		
Parotid and pancreatic secretion granules			sca1,2[7,72]
Neutrophil secondary, tertiary and secretory granules			sca2[11]
Mast cell granules			sca1[18]
Serotonin granules from platelets	sph[2]		

Endosomes			
Early	sph[37]		
Late	sph[24]		
Tubular (BFA treatment)	sph[48,68]		
Fluid phase	sph[28,28(*b*),37,41(*b*),68]		
Receptor mediated	pph[25]	sgy[62 *b*]	sca1–3[6]
	sph[12,12(*b*),13,16(*b*),22(*b*),28(*b*),42(*b*),45,45(*b*),48]		sca1–2[72]
	spo[22*b*]		
Recycling	sph[65]		sca[65]
Clathrin coated	sph[20,47,49,54,70 (however, see 41*b*)]	sgy1[47]	sca[65]
Caveolae	mgu[9]		
Tubules	sph[12,12(*b*)]		
Plasma membrane	pph[25]		
	sph[29,58,59,67]		
T tubule	mgu[9,30,34,63,73]		

[a] mgu, mitsugumin29; sca, SCAMP; sgy, synaptogyrin; sph, synaptophysin; spo, synaptoporin. 1, Ahnert-Hilger *et al.* (1996); 2, Bähler *et al.* (1990); 3, Bauerfeind *et al.* (1995); 4, Baumert *et al.* (1990); 5, Bergmann *et al.* (1993); 6, Brand and Castle (1993); 7, Brand *et al.* (1991); 8, Brandstätter *et al.* (1996); 9, Brandt and Caswell (1999); 10, Brooks *et al.* (2000); 11, Brumell *et al.* (1995); 12, Cameron *et al.* (1991); 13, Chilcote *et al.* (1995); 14, Colasante and Pécot-Dechavassine (1996); 15, Cutler and Cramer (1990); 16, Feany *et al.* (1993); 17, Fernández-Chacón and Südhof (2000); 18, Fernández-Chacón *et al.* (1999); 19, Fischer *et al.* (1997); 20, Fischer von Mollard *et al.* (1994); 21, Franke *et al.* (1986); 22, Fykse *et al.* (1993); 23, Grabs *et al.* (1994); 24, Green and Kelly (1992); 25, Haass *et al.* (1996); 26, Jahn *et al.* (1985); 27, Janz and Südhof (1998); 28, Johnston *et al.* (1989a); 29, Johnston *et al.* (1989b); 30, Jorgensen *et al.* (1990); 31, Kagotani *et al.* (1991); 32, Kandror *et al.* (1995); 33, Knaus *et al.* (1990); 34, Komazaki *et al.* (1999); 35, Kretzschmar *et al.* (1996); 36, Kupriyanova and Kandror (2000); 37, Lah and Burry (1993); 38, Laurie *et al.* (1993); 39, Leclerc *et al.* (1989); 40, Leimer *et al.* (1996); 41, Leube *et al.* (1989); 42, Leube *et al.* (1994); 43, Li (1996); 44, Li *et al.* (1996); 45, Linstedt and Kelly (1991); 46, Matteoli *et al.* (1991); 47, Maycox *et al.* (1992); 48, Mundigl *et al.* (1993); 49, Nakata *et al.* (1998); 50, Navone *et al.* (1986); 51, Navone *et al.* (1989); 52, Okada *et al.* (1995); 53, Ovtscharoff *et al.* (1993); 54, Pfeffer and Kelly (1985); 55, Reetz *et al.* (1991); 56, Régnier-Vigoroux *et al.* (1991); 57, Rosewicz *et al.* (1992); 58, Schmidt and Huttner (1998); 59, Schmidt *et al.* (1997); 60, Schmied and Holtzman (1989); 61, Sevilla *et al.* (1997); 62, Stenius *et al.* (1995); 63, Takeshima *et al.* (1998); 64, Tao-Cheng *et al.* (1995); 65, Thoidis *et al.* (1998); 66, Thomas-Reetz *et al.* (1993); 67, Tixier-Vidal *et al.* (1988); 68, Tooze and Hollinshead (1992); 69, van Lookeren Campagne *et al.* (1990); 70, Walch-Solimena *et al.* (1995); 71, Wiedenmann and Franke (1985); 72, Wu and Castle (1997); 73, Yuan *et al.* (1991); 74, Zimmermann (1996); 75, Fig. 11.

[b] cDNA-transfected cells.

[c] Insect cells infected with recombinant baculovirus and overexpressing synaptophysin.

1. Physins

The subcellular localization of the neuroendocrine physin isoforms synaptophysin and synaptoporin has been subject of extensive studies and the reader is referred to results and references that are listed in Table VI. Their main localizations in neurons are the small 30- to 80-nm, electron-lucent neurotransmitter-containing vesicles. The sheer abundance of both neuronal physin isoforms in these vesicles and the unusually high specificity of localization suggest an important function of these molecules for synaptic vesicles. Prior to neuron maturation and the formation of these specific vesicles, significant amounts of synaptophysin are detected in the Golgi complex (Tixier-Vidal *et al.,* 1988). In mature neurons carriers containing synaptophysin have been identified that may transport the molecule toward the synapse, where mature synaptic vesicles are formed (Okada *et al.,* 1995; Li, 1996; Li *et al.,* 1996). Mature synaptic vesicles go through several rounds of recycling and can therefore be labeled by extracellular tracers (Mundigl *et al.,* 1993). Interestingly, in neuroendocrine cells synaptophysin is also primarily localized to a small vesicle type that is similar in appearance and molecular composition to synaptic vesicles and is therefore referred to as synaptic-like microvesicle (SLMV). Despite earlier controversies (Lowe *et al.,* 1988; Obendorf *et al.,* 1988; Schilling and Gratzl, 1988), it is now accepted that synaptophysin is not present or only present in insignificant levels in the much larger dense-core vesicles containing peptidergic transmitters (Navone *et al.,* 1986; Fischer von Mollard *et al.,* 1990; Reetz *et al.,* 1991). In neuroendocrine cells, synaptophysin vesicles can also be labeled by extracellular tracers and colocalize, at least in part, with markers of the endosomal recycling system (Table VI). Subcellular localization of synaptophysin and synaptoporin has been examined by transfecting cDNAs into nonneuroendocrine cells. In these instances, a significant colocalization was observed between synaptophysin/synaptoporin and markers of the endosomal recycling system (Johnston *et al.,* 1989a; Cameron *et al.,* 1991; Linstedt and Kelly, 1991; Fykse *et al.,*1993; Leube *et al.,* 1994). However, we have also noted that a large percentage of synaptophysin segregates away from endogenous membrane proteins into a special synaptophysin-rich small vesicle type (Leube *et al.,* 1989, 1994; Leimer *et al.,* 1996). A high degree of compartmental specificity was also observed for mitsugumin29 in skeletal muscle, in which it is restricted to the tips of T tubules and certain subplasmalemmal vesicles that may be caveolae near the triad junction (Jorgensen *et al.,* 1990; Yuan *et al.,* 1991; Takeshima *et al.,* 1998). In addition, during development and under conditions of overexpression mitsugumin29 is present in cisternae of the smooth/sarcoplasmic ER (Brandt and Caswell, 1999; Komazaki *et al.,* 1999). In contrast to the other physin isoforms, pantophysin is a component of many different types of transport vesicles (Haass *et al.,* 1996). Typical examples of pantophysin immunofluorescence microscopy are shown in Fig. 12, demonstrating the characteristic multipunctate cytoplasmic distribution with perinuclear accumulation in epithelial and endothelial cells. Immunoelectron

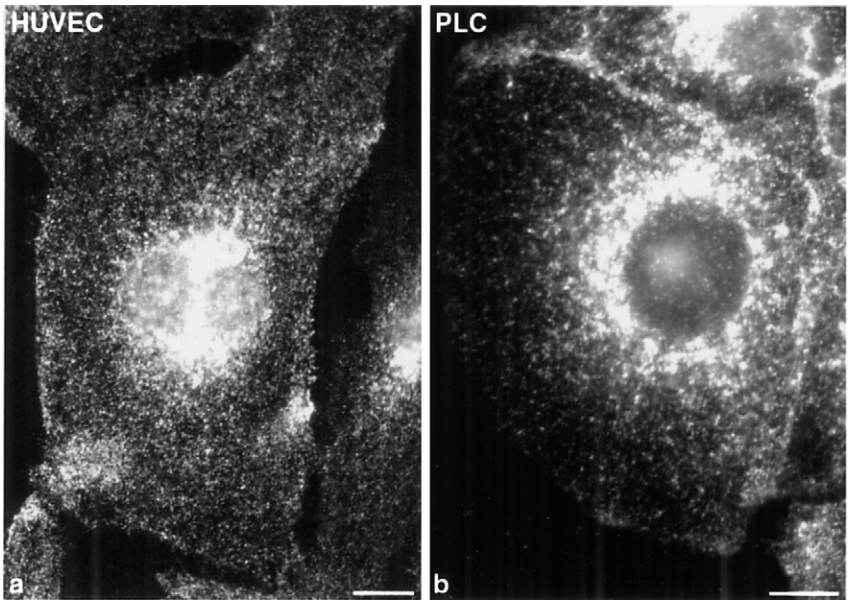

FIG. 12 Indirect immunofluorescence microscopy of formalin-fixed and digitonin-treated cells showing the reaction of polyclonal ch1 antibodies directed against pantophysin (see Fig. 7) in human umbilical vein endothelial cells (HUVEC; a) and human hepatocellular carcinoma-derived PLC cells (b). Note the multipunctate fluorescence throughout the cytoplasm and the perinuclear enrichment of staining. Scale bars-10 μm.

microscopy identified these puncta as small vesicles (Haass *et al.,* 1996). In most instances, a perinuclear concentration of pantophysin immunoreactivity is noted in the Golgi region (Haass *et al.,* 1996; Figs. 11b and 11b′). Figure 11 demonstrates that practically no overlap is observed between pantophysin and lysosomal membrane proteins (Figs. 11c and 11c′) and between pantophysin and caveolin-labeled caveolae (Figs. 11d and 11d′). Of particular interest is the presence of pantophysin in GLUT4 vesicles (Brooks *et al.,* 2000), especially since its paralog synaptophysin does not colocalize with GLUT4 in cDNA-transfected cells (Hudson *et al.,* 1993; Herman *et al.,* 1994).

2. Gyrins

Synaptogyrin 1 is found in the same synaptic vesicles and SLMVs as the neuronal physin isoforms (Baumert *et al.,* 1990; Janz and Südhof, 1998). Furthermore, transfection and expression of synaptogyrin 1 cDNA constructs in nonneuroendocrine cells revealed a similar colocalization with the receptor recycling pathway as for the neuronal physin isoforms (Stenius *et al.,* 1995). Like pantophysin, synaptogyrin

1 codistributes with the ubiquitous vSNARE cellubrevin (Stenius *et al.,* 1995; Haass *et al.,* 1996; Brooks *et al.,* 2000). Furthermore, synaptogyrin 2/cellugyrin seems to codistribute with pantophysin in certain compartments, including GLUT4 vesicles (Brooks *et al.,* 2000; Kupriyanova and Kandror, 2000), although detailed studies are lacking.

3. SCAMPs

Synaptic vesicles also contain SCAMPs (i.e., SCAMP5 and SCAMP1; Brand *et al.,* 1991; Thoidis *et al.,* 1998; Fernández-Chacón and Südhof, 2000). However, their expression pattern differs from that of the other synaptic vesicle TVPs: SCAMP1 is universally distributed in other vesicles and SCAMP5 is lacking in SLMVs of neuroendocrine cells (Table VI). In general, SCAMPs exhibit a much broader subcompartmental distribution than the other TVPs. Thus, SCAMPs are also components of larger vesicles and may be present in all secretory vesicles, including the constitutive vesicles and regulated vesicles such as the large dense-core vesicles of neuroendocrine cells, the GLUT4 vesicles of adipocytes and cardiomyocytes, and the secretion granules of the parotid gland and the pancreas, mast cell granules, and neutrophil granules (Table VI). Furthermore, they are also located in the endosomal compartment because they are prominent components of recycling vesicles (Brand and Castle, 1993; Wu and Castle, 1997).

4. Subcellular Colocalization of TVPs

Table VII lists observations of subcellular colocalization of different TVPs. It can be concluded from these data that different combinations are possible and that more than one member of a single family and members of different families may be present in the same membrane compartment in a given cell (Figs. 13a and 13a′). On the other hand, differential distributions of individual polypeptides have been observed in the same cell (Brand *et al.,* 1991; Singleton *et al.,* 1997; Fernández-Chacón and Südhof, 2000; Figs. 13b and 13b′).

5. TVPs in Living Cells

By expressing fluorescent TVP chimeras it is possible to study their distribution and dynamic behavior in living cells. An example is displayed in Figs. 13b and 13b′. In this case, the cDNAs coding for the *C. elegans* gyrin- and SCAMP-related polypeptides were modified in such a way that they code for chimeras in which either the enhanced cyan fluorescent protein (gyrin construct) or the enhanced yellow fluorescent protein (SCAMP construct) are at the respective carboxy termini. These constructs were placed next to CMV promoter elements and DNA was transfected into epithelial PLC cells. By specifically eliciting and detecting the blue or the yellow fluorescence with appropriate filter combinations, either the

TABLE VII
Colocalization of Different TVPs in Subcellular Compartments[a]

TVPs	Cell/tissue type	Method	Result
sph/spo	Brain	Immunoblot of synaptic vesicle fraction	Codistribution[9]
		Immunoblot of immunoisolated vesicles	Coisolation[6]
		Immunoblot of immunoprecipitates	No coprecipitation[6]
sph/pph	PLC cells stably transfected with rat sph cDNA	Immunofluorescence microscopy	Colocalization[7]
	A-431 cells stably cotransfected with rat sph cDNA and human pph cDNA with myc-tag	Immunoblot of immunoisolated vesicles	Coisolation[7]
	Human pheochromocytoma	Immunoblot of immunoisolated vesicles	Partial coisolation[7]
	PC12 cells stably transfected with human pph cDNA with myc-tag	Immunoblot of sucrose gradient fractions	Codistribution[7]
		Immunoblot of immunoisolated vesicles	Coisolation[7]
sph/sgy1	Brain	Immunoblot of synaptic vesicle fraction	Codistribution[1]
	PC12 cells	Immunoblot of immunoisolated vesicles	Coisolation[1]
	RINm5F cells	Immunofluorescence microscopy	Colocalization[12]
		Immunoblot of sucrose gradient fractions	Codistribution[12]
	βTC3 cells	Immunofluorescence microscopy	Colocalization[15]
		Immunoblot of immunoisolated vesicles	Coisolation[15]
	CHO cells cotransfected with rat sph and rat sgy1 cDNA	Immunofluorescence microscopy	Colocalization[14]
pph/sca1,2	PLC cells	Immunofluorescence microscopy	Colocalization[7, 17]
	A-431 cells stably cotransfected with rat sph cDNA and human pph cDNA with myc-tag	Immunoblot of immunoisolated vesicles	Coisolation[7]
sgy1/sgy2	Brain	Immunoblot of synaptic vesicle fraction	Detection of sgy1 but not sgy2[8]
	COS cells cotransfected with rat sgy1 and rat sgy2 cDNA	Immunofluorescence microscopy	Colocalization[8]

(*continued*)

TABLE VII (*continued*)

TVPs	Cell/tissue type	Method	Result
sgy2/sca1,2	Adipocytes	Immunoblot of immunoisolated vesicles	Coisolation[10]
sgy/sca	PLC cells cotransfected with DNAs coding for fluorescent sca.ce and sgy.ce chimeras	Epifluorescence microscopy of living cells	Partial colocalization[18]
sca1/sca2	Pancreas	Immunoblot of purified storage granule membranes	Codistribution[2]
	Parotid gland	Immunoblot of purified storage granule membranes	Codistribution[2,16]
		Immunoblot of immunoprecipitates	Partial coprecipitation[16]
	Liver	Immunoblot of Golgi fraction	Detection of sca2 but only little sca1[2]
	Adrenal medulla	Immunoblot of chromaffin granule fraction	Codistribution[2]
			Detection of sca1, but only little sca2[5]
		Immunoblot of microsomal fraction	Codistribution[5]
	Neutrophils	Immunoblots of secondary, and secretory granule fractions tertiary,	Detection of sca2, but only little sca1[3]
	Cardiomyocytes	Immunoblot of GLUT4-enriched vesicles	Codistribution[4]
	Adipocytes	Immunoblot of GLUT4-enriched vesicles	Codistribution[11]
sca1/sca5	Brain	Immunoblot of synaptic vesicle fraction	Codistribution[5]
sca1/sca2/sca3	CHO cells	Immunoblot of subcellular fractions	Codistribution[16]
		Immunoblot of immunoprecipitates	Partial coprecipitation[13,16]
	HeLa cells	Immunofluorescence microscopy	Colocalization with slight differences for sca3[13]
		Immunoblot of immunoprecipitates	Coprecipitation[13]

[a] Abbreviations used: ce, *C. elegans*; pph, pantophysin; sca, SCAMP; sgy, synaptogyrin; sph, synaptophysin; spo, synaptoporin. 1, Baumert *et al.* (1990); 2, Brand *et al.* (1991); 3, Brumell *et al.* (1995); 4, Fischer *et al.* (1997); 5, Fernández-Chacón and Südhof (2000); 6, Fykse *et al.* (1993); 7, Haass *et al.* (1996); 8, Janz and Südhof (1998); 9, Knaus *et al.* (1990); 10, Kupriyanova and Kandror (2000); 11, Laurie *et al.* (1993); 12, Reetz *et al.* (1991); 13, Singleton *et al.* (1997); 14, Stenius *et al.* (1995); 15, Thomas-Reetz *et al.* (1993); 16, Wu and Castle (1997); 17, Figs. 13a and 13a′; 18, Figs. 13b and 13b′.

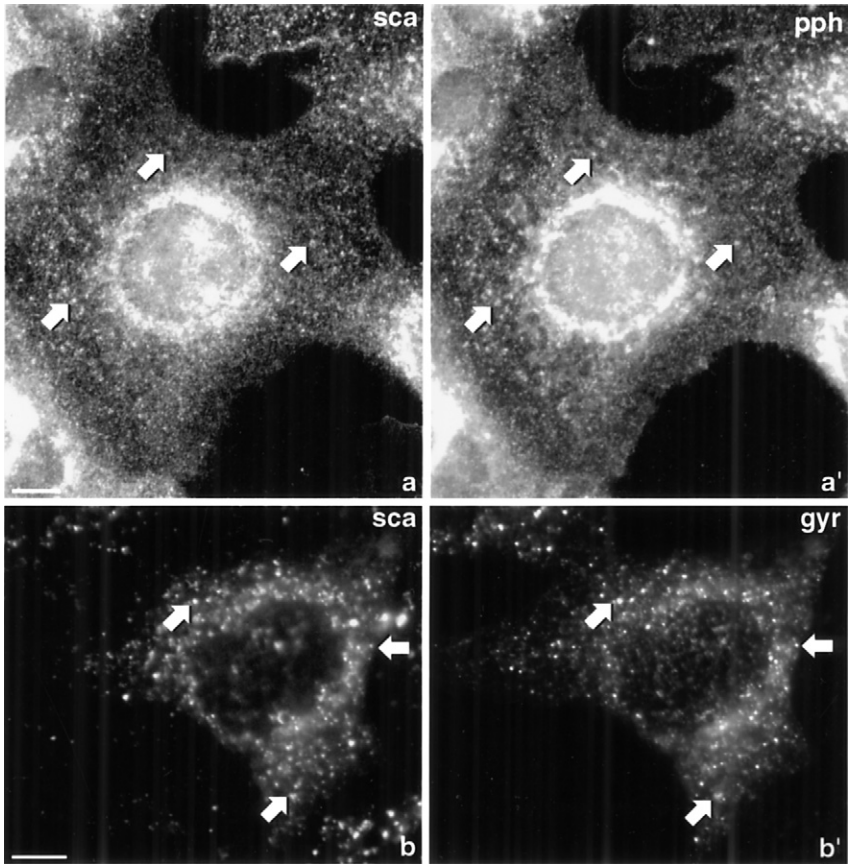

FIG. 13 Double fluorescence microscopy of tetraspan vesicle proteins in human hepatocellular carcinoma-derived PLC cells. (a, a′) The distribution of SCAMPs (sca) as detected by monoclonal antibody SG7C12 (kindly provided by Dr. D. Castle, University of Virginia, Charlottesville, VA; Brand *et al.,* 1991) together with pantophysin (pph) using polyclonal antibodies ch1 (see Fig. 7). Cells were fixed with formalin and lysed with digitonin prior to antibody incubation. (b, b′) The distribution of fluorescent protein chimeras consisting either of the entire *C. elegans* SCAMP and the enhanced yellow fluorescent protein at its carboxy terminus (b; sca) or the entire *C. elegans* synaptogyrin and the enhanced cyan fluorescent protein at its carboxy terminus (b′; gyr) after cotransfection of suitable expression constructs into the same cells. The chimeras were detected by direct epifluorescence microscopy in living cells. Note the significant colocalization in a and a′ and the partial colocalization in b and b′ (arrows). Scale bars-10 μm.

gyrin or the SCAMP chimeras were visualized in the same living cells. Note that in both cases a multipunctate fluorescence pattern is seen indicative of a vesicular localization and that many puncta colocalize (arrows). In these cells it is possible to examine changing distribution patterns and the fate of individual carriers by time-lapse fluorescence microscopy. Figures 11a and 11a′ demonstrate that this

approach is also promising in the case of physins. In this instance, a fluorescent pantophysin chimera (blue) was synthesized together with a KDEL receptor fusion protein (yellow) in transfected PLC cells. The resulting multipunctate vesicular fluorescence of the pantophysin construct does not overlap noticeably with that of the ER/cis-Golgi-restricted KDEL construct.

Nakata *et al.* (1998) examined transport of a synaptophysin-enhanced green fluorescent protein chimera in axons of dorsal root ganglion neurons. They showed that it is present in tubulovesicular organelles of various sizes and shapes that circulate within axons from branch to branch and change the direction of movement. By photobleaching they identified retrogradely moving larger endosomal and anterogradely moving smaller tubulovesicular carriers. Stimulation of exocytosis by laterotoxin resulted in redistribution of the chimeras onto the plasma membrane that was seen as an increase of surface area of varicosities with fluorescence accumulations.

C. Functions

Circumstantial evidence as summarized previously provides a strong indication for the importance of TVPs for cellular membrane trafficking and membrane biogenesis. In this section, we summarize some of the results obtained by gain- and loss-of-function experiments with emphasis on four major aspects of TVP function, namely participation in exocytosis, endocytosis, vesicle biogenesis, and cell-type-specific processes.

1. Exocytosis

To investigate the contribution of synaptophysin to neurotransmitter exocytosis, Alder *et al.* (1992a) first injected total rat cerebellar mRNA into oocytes of *X. laevis,* thereby establishing calcium-dependent glutamate secretion. Coinjection of either synaptophysin antisense oligonucleotides or synaptophysin antibodies led to a reduction of secretion in this system. The same effects were observed on acetylcholine secretion when mRNA from the electric lobe of *Torpedo californica* was injected together with antisense oligonucleotides and specific antibodies for synaptophysin. Shibaguchi *et al.* (2000) demonstrated in a similar assay using rat brain mRNA and a synaptophysin antibody that synaptophysin contributes to calcium-dependent dopamine release. Consequently, overexpression of synaptophysin enhances neurotransmitter secretion at *X. laevis* neuromuscular synapses (Alder *et al.,* 1995). Conversely, antibodies to synaptophysin inhibited transmitter secretion in the same system (Alder *et al.,* 1992b). Exactly when interference with synaptophysin function affects exocytosis is not clear and it is not known whether synaptophysin function is directly linked to transmitter secretion by forming a fusion pore as proposed by Thomas *et al.* (1988), which has been challenged

by some (Südhof and Jahn, 1991) and has not been further substantiated. It is not readily apparent how the described inhibition and overexpression experiments can be reconciled with observations in neuroendocrine PC12 cells in which either synaptophysin or synaptogyrins 1–3 were overexpressed, thereby leading to strong inhibition of calcium-dependent exocytosis of cotransfected human growth hormone (Sugita *et al.,* 1999). It is possible that these effects are not directly due to TVP-induced inhibition of secretion but are the indirect consequence of removal of essential exocytosis factors from human growth hormone-containing secretion vesicles which may not normally contain these TVPs. Indeed, no evidence was provided that human growth hormone vesicles and synaptophysin/synaptogyrin vesicles coincide (for differential distribution, see Schweitzer and Paddock, 1990).

Fernández-Chacón *et al.* (1999) examined the consequence of SCAMP1 ablation on the degranulation of mast cells. They showed that exocytosis could still be reliably triggered by GTPγS in SCAMP1-deficient mast cells but that the final cell capacitance measured after completion of exocytosis was significantly smaller in cells obtained from SCAMP1-deficient than in wild-type cells. Furthermore, an increased proportion of reversible fusion events was observed. They concluded that SCAMP1 is not essential for exocytosis but participates in the formation of fusion pores and/or endocytotic uptake of membrane after fusion pore formation.

2. Endocytosis

The presence of TVPs in vesicles that participate in receptor-mediated endocytosis and nonspecific fluid uptake suggest that they may also be of importance for this membrane compartment. To examine the function of synaptophysin in the synapse, Daly *et al.* (2000) injected peptides corresponding to the cytoplasmic carboxy terminus of synaptophysin into squid giant terminals. These peptides, which presumably represent domains that interact with dynamin, led to a reduction in the number of synaptic vesicles and resulted in a significant increase in clathrin-coated vesicles. It was therefore suggested that synaptophysin is a crucial component of a clathrin-independent recycling pathway of synaptic vesicles. Support for this interpretation has recently been obtained in our laboratory, in which we observed a reduced number of synaptic vesicles and an increased number of clathrin-coated vesicles in photoreceptors of synaptophysin knockout mice (Spiwoks-Becker *et al.,* 2001).

Furthermore, based on the interaction of the amino-terminal NPF repeats of SCAMPs with the EH-containing protein intersectin that is involved in endocytosis, Fernández-Chacón *et al.* (2000) overexpressed SCAMP1 and certain fragments thereof in COS cells and investigated its effect on transferrin endocytosis. Full-length SCAMP1 inhibited endocytosis partially, an amino-terminally truncated SCAMP1 lacking the NPF repeats inhibited endocytosis almost completely, but an amino-terminal soluble SCAMP1 fragment did not affect endocytosis. These

results were interpreted as an indication of the importance of NPF repeats for coupling to EH domain proteins while the transmembrane regions perform separate, unknown functions.

3. Vesicle Biogenesis

Given the cell-type-restricted synthesis of synaptophysin, a simple way to examine its contribution to membrane trafficking and vesicle formation is to express it in nonneuroendocrine cells, i.e., cells that are devoid of synaptophysin and other neuronal/neuroendocrine polypeptides. Although all studies are in agreement that a large percentage of synaptophysin is targeted to vesicles that partake in receptor-mediated vesicle recycling (Johnston *et al.,* 1989a; Cameron *et al.,* 1991; Linstedt and Kelly, 1991; Leube *et al.,* 1994), our analyses have demonstrated that a considerable amount of synaptophysin is sorted away from other preexisting membrane compartments into a small vesicle type that is particularly rich in synaptophysin (Leube *et al.,* 1989, 1994; Leimer *et al.,* 1996). From these observations it was proposed that synaptophysin participates in vesicle formation. Support for this idea was recently obtained by detailed analyses of synaptophysin knockout mice (Spiwoks-Becker *et al.,* 2001). Assuming that synaptoporin compensates for the loss of synaptophysin, we searched for alterations in photoreceptors, a neuronal cell population that in normal circumstances synthesizes only synaptophysin but no synaptoporin. We found that in these completely physin-deficient cells of synaptophysin knockout mice overall vesicle density was reduced, and it was most pronounced during periods of high recycling activity (i.e., during the dark period). Furthermore, regulation of vesicle diameter differed significantly during the dark period from that of control animals. These findings indicate a contribution of synaptophysin to vesicle biogenesis and vesicle morphogenesis. It is not clear how this function is accomplished but the presence of synaptophysin in a multiprotein complex and its direct interaction with the lipid environment (Thiele *et al.,* 2000) may be underlying principles. The observed inhibition of SLMV biogenesis in cholesterol-depleted PC12 cells provides further evidence for the importance of protein–lipid interactions in this process (Thiele *et al.,* 2000).

4. Cell-Type-Specific Functions

A surprising finding was that ablation of synaptophysin, synaptogyrin 1, or SCAMP1 alone did not result in severe neuronal deficiencies in knockout mice (Eshkind and Leube, 1995; McMahon *et al.,* 1996; Fernández-Chacón *et al.,* 1999; Janz *et al.,* 1999), except for the previously mentioned, comparatively mild alterations in photoreceptors of synaptophysin-deficient mice (Spiwoks-Becker *et al.,* 2001). Janz and colleagues (1999) therefore produced double knockout mice lacking synaptophysin and synaptogyrin 1. Remarkably, even these mice were fertile without significant morphological or biochemical alterations (no significant

compensatory changes). Furthermore, they observed normal glutamate release from synaptosomes and normal EPSPs in CA1 pyramidal cells after stimulation of Schaffer collateral/commissural fibers. Only electrophysiological measurements of the hippocampal CA1 region revealed deficiencies in short- (paired pulse facilitation and posttetanic potentiation) and long-term (long-term potentiation) synaptic plasticity but normal synaptic depression during repetitive stimulation and normal probability of transmitter release. Janz *et al.* (1999) concluded that both polypeptides together are essential for synaptic plasticity. The underlying mechanisms for the observed phenotype are unknown, although it is attractive to speculate that phosphorylation and/or changes in protein synthesis play a role (Mullany and Lynch, 1997, 1998).

Cell-type-specific functions of mitsugumin29 were also explored in murine knockout experiments, which showed that the loss of mitsugumin29 results in abnormalities of membrane structures of the triad junction in skeletal muscles and is associated with lower twitch force and faster decrease of twitch tension under Ca^{2+}-free conditions in the extensor digitorum longus muscles (Nishi *et al.,* 1999). Recent publications further support the importance of mitsugumin29 for muscle function (Nagaraj *et al.,* 2000). It was observed that muscle strength (tetanic force and twitch force) was normal but that muscle fatigue increased, resulting in lower twitch forces, lowering of the force–frequence relationship, a reduced recovery, and reduced calcium release in response to caffeine in fast- and slow-twitch muscles but not in mixed fiber muscles. It was concluded that the phenotype is due to altered excitation–contraction coupling and may be a consequence of altered intracellular calcium homeostasis.

IV. Concluding Remarks

It should be kept in mind that TVPs are only one type of integral membrane proteins with four membrane-spanning segments and cytoplasmically located end domains. Other types include junctional proteins of either the gap junction (connexins) or tight junction (claudins and occludin); proteins of the urothelial crusta (uroplakins); components of the myelin sheath (e.g., proteolipid protein and peripheral myelin protein 22); several neurotransmitter receptors of the postsynaptic density (e.g., acetylcholine receptor, GABA receptor, and glycine receptor); peripherin/*rds,* a component of the peripheral rim of the membrane stacks in photoreceptors; and the plasma membrane-bound tetraspanins that are involved in proliferation, adhesion, and differentiation (Taylor *et al.,* 1995; Conti-Fine *et al.,* 1996; Maecker *et al.,* 1997; Dimou *et al.,* 1999; Sun *et al.,* 1999; Tsukita and Furuse, 1999; White and Paul, 1999; Falk, 2000; Wrigley *et al.,* 2000). One of the most remarkable similarities of all these diverse polypeptides, in addition to their shared transmembrane topology, is their tendency to occur as homomultimers in specialized membrane

domains in which they are enriched in dense molecular aggregates. It is therefore attractive to think that the accumulation of these polypeptides in their functionally and structurally distinct membranes is in each case intricately interwoven with the particular function of the membrane domain at hand and its formation. With respect to TVPs, it will therefore be important to provide further evidence that they contribute to the morphogenesis of cytoplasmic transport vesicles or, in the case of mistugumin29, the highly curved membrane tip of T tubules in the triad junction and to determine the tasks that they fulfill. We are convinced that exciting new insights will soon emerge that will help us to understand isoform-specific TVP function in the context of constitutive and regulated vesicle-mediated trafficking.

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Dynamic Changes and the Role of the Cytoskeleton during the Cell Cycle in Higher Plant Cells

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In higher plant cells microtubules (MTs) show dynamic structural changes during cell cycle progression and play significant roles in cell morphogenesis. The cortical MT (CMT), preprophase band (PPB), and phragmoplast, all of which are plant-specific MT structures, can be observed during interphase, from the late G_2 phase to prophase, and from anaphase to telophase, respectively. The CMT controls cell shape, either irreversibly or reversibly, by orientating cellulose microfibril (CMF) deposition in the cell wall; the PPB is involved in determining the site of division; and the phragmoplast forms the cell plate at cytokinesis. The appearance and disappearance of these MT structures during the cell cycle have been extensively studied by immunofluorescence microscopy using highly synchronized tobacco BY-2 cells. Indeed, these studies, together with visualization of MT dynamics in living plant cells using the green fluorescent protein, have revealed much about the modes of MT structural organization, for example, of CMTs at the M/G_1 interphase. The microfilaments which also show dynamic changes during the cell cycle, being similar to MTs at particular stages and different at other stages, appear to play roles in supporting MTs. In this article, we summarize our ongoing research and that of related studies of the structure and function of the plant cytoskeleton during cell cycle progression.

KEY WORDS: BY-2 cells, Cell cycle, Cellulose microfibril, Green fluorescent protein, Microfilament, Microtubule, Microtubule organizing center.

I. Introduction

The microtubule (MT) and actin microfilament (MF), which are fibrous structures in eukaryotic cells, together with species-specific intermediate filaments constitute the "cytoskeleton." The MT, a hollow tube with a 24–25 nm diameter, consists of α- and β-tubulins that continuously expand and contract by polymerization and disassembly of the tubulin molecules in living cells. The MT is essential for cell division, movement, and morphogenesis, especially in higher plant cells. In contrast, the MF is a twisted fiber of 5–6 nm diameter, which is also involved in such cell phenomena. The intermediate filament is a generic name for fibrous structures that are about 10 nm in diameter.

The structure and role of the MTs and MFs may differ between plant and animal cells. During cell division and morphogenesis, for example, MTs play a major role in plant cells, whereas MFs are the major contributors in animal cells. Hence, at cytokinesis, higher plant cells are divided centrifugally by the cell plate made in the phragmoplast of MTs, whereas animal cells are constricted centripetally by contractile rings of MFs. Furthermore, whereas the aster and contractile ring are not observed in higher plant cells, the plant-specific cortical MT (CMT), preprophase band (PPB), and phragmoplast are never found in animal cells.

These cytoskeletal structures form and disappear at specific stages of the cell cycle (Staiger and Lloyd, 1991; Lambert and Lloyd, 1994). In higher plant cells, the CMT, PPB, and phragmoplast, which are involved in cell morphogenesis, in the determination of the division site, and in cell plate formation, appear in interphase, in the late G_2 phase, and at telophase, respectively. These structures are also known to be involved in various phenomena specific to higher plant cells, such as cell elongation, cytokinesis, and differentiation.

In this article, the changes and roles of these MT structures are described in order of cell cycle progression in higher plant cells. The changes and functions of other cytoskeletal structures are also described in relation to such MT structures.

II. Dynamic Changes of Cytoskeletal Structures during Cell Cycle Progression

A. Dynamics of MTs and Changes in MT Organizing Center Sites during Plant Cell Cycle

Although there are no permanent MT structures as observed in the nerve axon or cilia of animal cells, the transient MT structures observed in plant cells are more diverse than those in animal cells. The arrangement of MTs is known to change dynamically during the cell cycle of higher plant cells (Lambert and Lloyd, 1994; Kumagai and Hasezawa, 2001), as exemplified by a series of MT structures in a vacuolated tobacco BY-2 cell (Fig. 1). At the G_1 phase, MTs are not observed

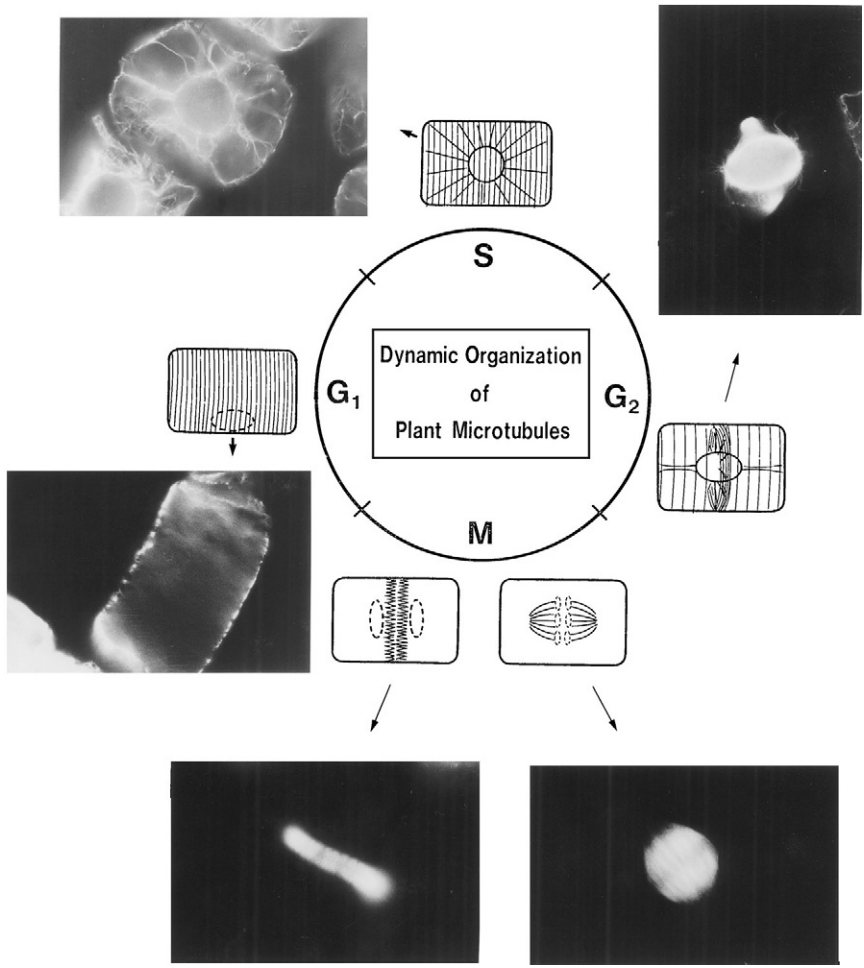


FIG. 1 Dynamic organization of plant microtubules observed in a vacuolated cell, such as a BY-2 cell, during cell cycle progression. The CMTs, cytoplasmic radial MTs, PPB, mitotic spindle, and phragmoplast appear in order at the G_1 phase, S phase, late G_2 phase, metaphase, and telophase, respectively. The CMTs, PPB, and phragmoplast are MT structures specific to higher plant cells.

around the small and uneven nucleus, located at the cell periphery, whereas well-developed CMTs can be identified in the cell cortex. When DNA synthesis begins at S phase, MTs nucleate from the round nucleus through the cytoplasmic strands to the cell cortex. Accompanied by the formation of a radial MT network, the nucleus becomes round in shape and moves to the central region of the cell. As a result, at the S–G_2 phase, radial MTs nucleating from the nucleus are observed in the cytoplasmic strands, with CMTs on the cell cortex. At the late G_2 phase, when CMTs gradually disappear from both edges of the cell, the PPB becomes identifiable as

a ring of thick MT bundles surrounding the nucleus. At prophase–prometaphase the mitotic spindle is formed with the collapse of the PPB. At metaphase, the chromosomes become arranged on the equator plane by the kinetochore MTs. At anaphase–telophase, the phragmoplast, in which the cell plate is later formed, appears in the central area after chromosomal segregation by the kinetochore MTs. At cytokinesis, the CMTs become reorganized when the phragmoplast collapses at the completion of the cell plate. At the next G_1 phase, the reformed nucleus moves to the cell periphery and again only the CMTs can be observed in the cell cortex.

In eukaryotic living cells, one (minus) end of the MT is connected to the microtubule organizing center (MTOC) so that the MT elongates by polymerization of tubulin at the other (plus) end. The MTOC is identified as the centrosome in animal cells, in which electron microscopic observations have revealed that the MT minus end is buried within a high electron-dense amorphous material that surrounds the centriole. This surrounding material is known to include MTOC-related proteins, such as elongation factor (EF)-1α, pericentrin, and NuMA, although their exact functions have yet to be clarified (Anderson, 1999). Furthermore, at the MTOC–MT junction site in animal cells, a ring of γ-tubulin couples the two structures (Oakley, 1995).

During the cell cycle of higher plant cells, the sites of MTOC appear to change with the type of MT structures (Lambert, 1993). Five MT structural types are observed at the various stages of the cell cycle, namely, the CMTs, radial cytoplasmic MTs, PPB, mitotic spindle, and the phragmoplast. Therefore, the sites of MT organization may depend on the particular MT structures and vary with the different stages of the cell cycle. However, it is difficult to identify the MTOC site of each MT structure since higher plant cells do not possess distinct MTOCs as in animal cells. In order to characterize the MTOC site of each MT structure, the MTs were completely destroyed by a combined cold and drug treatment at each stage of the cycle in synchronized BY-2 cells (Hasezawa *et al.,* 1997). The reorganization of MTs could then be observed after washing and reculturing of the cells. The MTOC sites at each stage were identified as the cell cortex and nuclei, the mitotic apparatus, the nuclei (or the nuclei and cell cortex), and the cell cortex at the S–G_2 phase, M phase, M/G_1 interface, and the G_1 phase, respectively (Fig. 2). These results suggested that the MTOC activity of nuclei and cell cortex of plant cells function in a cell cycle-dependent manner, and that a certain factor(s) that provides them with MTOC activity changes their location during the cell cycle.

B. MTOC-Related Proteins in Higher Plant Cells

In animal cells, the MTs are organized from the pericentriolar material (PCM) of the centrosome. Toriyama *et al.* (1988) showed that aster-like structures could be formed *in vitro* when a partially purified fraction of the mitotic apparatus from sea urchin was incubated with purified porcine tubulin. After purification

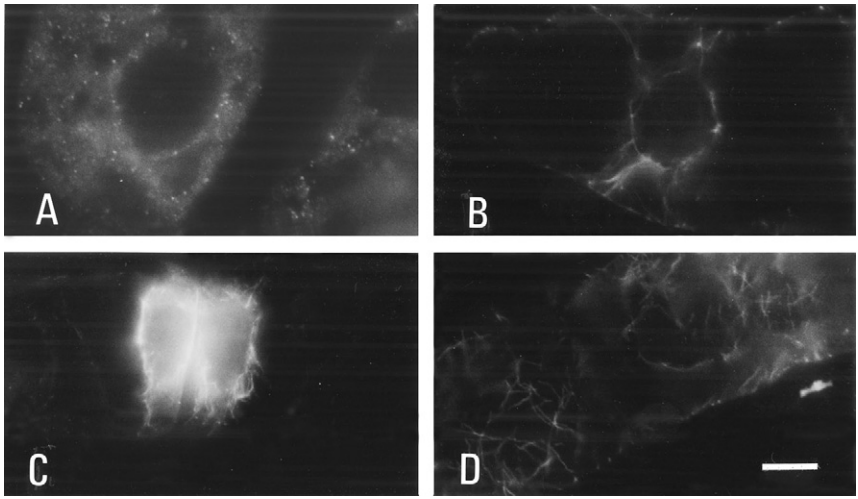


FIG. 2 Reorganization of MTs after disruption of MTs at different stages of the cell cycle. MTs of BY-2 cells were destroyed by a combined cold and drug treatment (A). Subsequently, the cells were washed and cultured at 30°C and stained with anti-tubulin antibodies. The MTs were reorganized in the perinuclear region (B, C) and the cell cortex (B, D) at the S phase (B), M/G_1 interphase (C), and G_1 phase (D). Scale bar-10 μm.

by phosphocellulose column chromatography, this aster-forming activity was attributed to a 51-kDa protein that was located in the cores of reconstituted aster-like structures, as determined by immunofluorescence microscopy.

In tobacco BY-2 cells, a 49-kDa protein, a counterpart of the 51-kDa protein from sea urchin, was identified using monoclonal antibodies raised against the 51-kDa protein (Hasezawa and Nagata, 1993). The 49-kDa protein was observed in the PPB, mitotic spindle, and phragmoplast at the M phase, as well as in the cell cortex at the G_1 phase and in the cell cortex and perinuclear region at the S–G_2 phase. Therefore, the 49-kDa protein was considered to be located at the MTOC sites of plant cells as described previously.

The BY-2 cDNA encoding the 49-kDa protein revealed that it encoded the protein synthesis EF-1α (Kumagai *et al.,* 1995, Lambert 1995). On the other hand, a fraction that was partially purified from evacuolated BY-2 protoplasts (Jiang *et al.* 1992) by passage through a phosphocellulose column and was eluted with 0.5 *M* KCl contained numerous particles with diameters of about 1 μm after desalting by dialysis. When the fraction was incubated with purified porcine tubulin, the MTs became radially elongated from the particles and formed structures similar to the asters of animal cells (Fig. 3A; Kumagai *et al.,* 1999). By fluorescence microscopy, the 49-kDa protein was shown to be located at the centers of these "asters," suggesting that EF-1α plays a role in MT organization of both animal and plant cells (Figs. 3C and 3D).

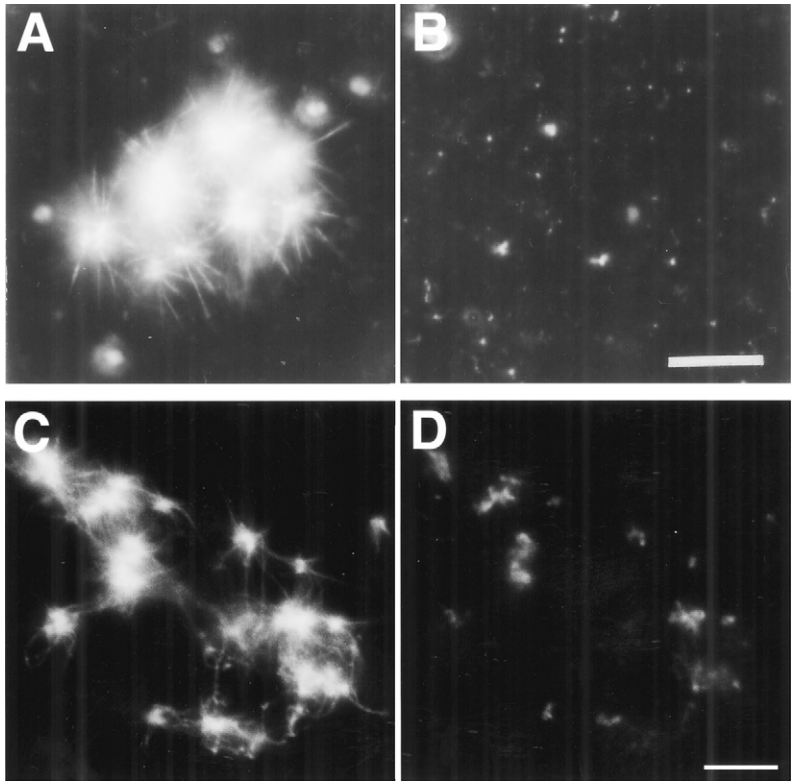


FIG. 3 Aster-like structures formed by a plant protein fraction. The BY-2 proteins were applied onto a phosphocellulose column and eluted stepwise with increasing KCl concentrations. When the protein fractions included a 49-kDa protein, identical to EF-1α, the *in vitro* formation of aster-like structures was observed by darkfield microscopy (A). MT structures were never formed by fractions lacking the 49-kDa protein (B). The "asters" were double stained with anti-tubulin antibody (C) and anti-49-kDa protein antibody (D). The 49-kDa protein was localized to the central regions of the "asters." Scale bar-20 μm.

From studies of animal cell centrosomes, several MTOC-related proteins have been identified and characterized, particularly pericentrin (Doxsey *et al.,* 1994), NuMA (Gaglio *et al.,* 1995), MPM-2 antigen (Centonze and Borisy, 1990), and γ-tubulin (Félix *et al.,* 1994; Stearns and Kirschner, 1994). In higher plant cells, however, the nuclear envelope appears to possess MTOC activity, at least at the S–G_2 phase (Lambert, 1993; Lambert and Lloyd, 1994; Vaughn and Harper, 1998); therefore, some investigations into plant MTOC-related proteins have been performed using plant nuclei. For example, when frozen–thawed BY-2 nuclei, or nuclear particles obtained by gentle homogenization, were incubated with purified porcine tubulin or tobacco tubulin, the MTs nucleated and became radially elongated from them (Mizuno, 1993). The main component of the nuclear sap

fraction was found to be a 58-kDa protein. Furthermore, isolated maize nuclei were also able to nucleate MTs when they were incubated with purified porcine tubulin (Stoppin *et al.,* 1994). A monoclonal antibody, mAb6C6, raised against an animal pericentriolar antigen, was found to immunostain the surface of isolated maize nuclei and to recognize a 100-kDa polypeptide in the nuclear extract. Furthermore, the microtubule-associated protein (MAP) fraction of carrot cells was reported to have aster-forming activity, and an antibody raised against a 120-kDa protein of carrot MAP fraction could stain the nuclear surface (Chan *et al.,* 1996). Although these putative MTOC-related proteins of higher plant cells are very interesting, they have not been well characterized and so their functions remain unclear.

γ-Tubulin, a member of the tubulin superfamily, was originally identified in *Aspergillus nidulans* and has since been established as a key component of the MTOC complex (Oakley and Oakley, 1989; Oakley *et al.,* 1990). The protein is highly conserved in eukaryotic cells and is primarily localized to animal centrosomes or to fungal spindle pole bodies. There, γ-tubulin appears to connect the minus end of MTs to the MTOCs, where, together with other proteins, it forms a ring-like complex in animal cells (Oakley, 1995; Jeng and Stearns, 1999). γ-Tubulin, which has also been identified in higher plant cells, has been localized to the mitotic spindle and phragmoplast, and the localizations of γ-tubulin slightly shifted to the putative MTOC sites (Liu *et al.,* 1993, 1994). However, another report suggested that the MTs and γ-tubulin were perfectly colocalized, and that γ-tubulin has no relationship with plant MTOCs (Panteris *et al.,* 2000). γ-Tubulin has also been identified at the prekinetochores before prophase in *Vicia faba* cells (Binarová *et al.,* 2000). We have also obtained similar results to those of Liu *et al.,* as shown in Fig. 4 (F. Kumagai *et al.,* unpublished observation), but more detailed observations are needed to determine its localization. γ-Tubulin has the ability to stabilize preformed MTs by binding to their minus ends (Wiese and Zheng, 2000), and the high-molecular-weight complexes of γ-tubulin have also been found in higher plant cells (Stoppin-Mellet *et al.,* 2000). Although the localization of γ-tubulin is not restricted to the immediate minus ends of MTs in plant cells, it is conceivable that it plays a similar role as that in animal cells.

C. Dynamics of MFs during the Plant Cell Cycle

MFs are known to be involved in cytoplasmic streaming (Kuroda, 1990; Valster *et al.* 1997), in the movement and positioning of organelles such as chloroplasts (Williamson, 1993), and in apical growth such as that of the pollen tube (Taylor and Hepler, 1997). MFs are also involved in the control of cell shape, although by a process different from that of MTs, and their distribution is always varied in living cells. MFs show dynamic changes during cell cycle progression in higher plant cells (McCurdy and Gunning, 1990). The dynamics of MFs, observed in BY-2 cells during the cell cycle, are described here.

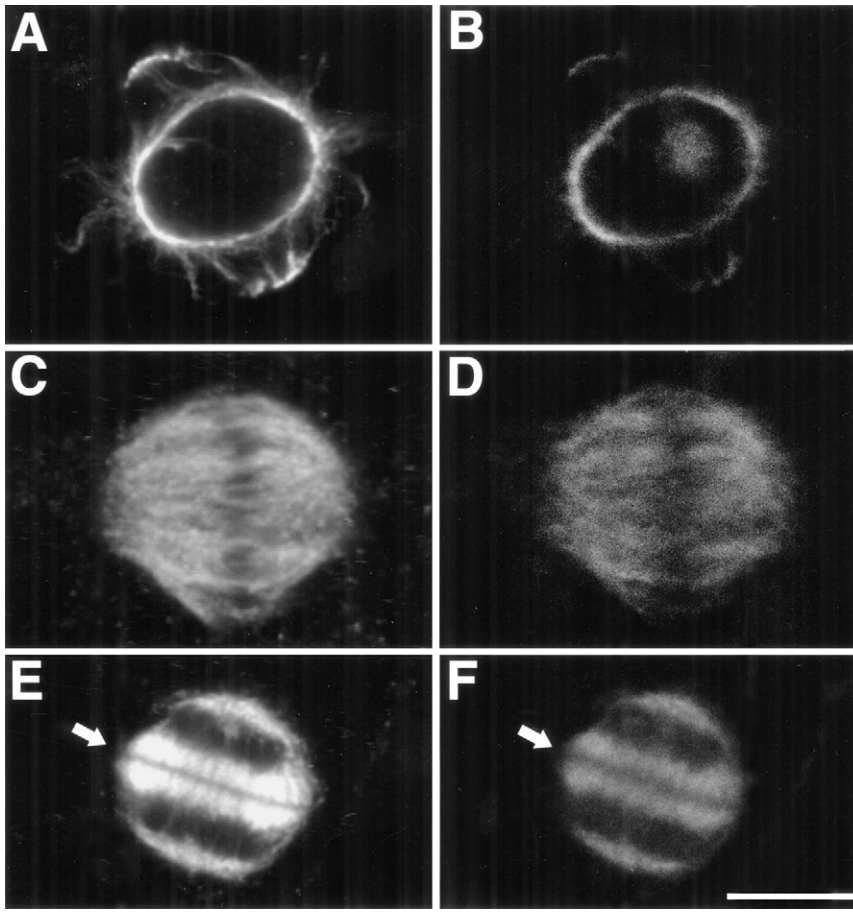


FIG. 4 Localization of γ-tubulin in representative MT structures of BY-2 cells. BY-2 cells were double stained with antibodies against α- and β-tubulins (A, C, E) and γ-tubulin (B, D, F). In a late G_2 phase cell (A, B), γ-tubulin appeared to accumulate at the perinuclear region rather than at the PPB (B). In a metaphase cell (C, D), γ-tubulin was biased toward the poles of the mitotic spindle (D). In a telophase cell (E, F), the dark midzone (arrow) in F was wider than that in E. Scale bar-10 μm.

Although the distribution patterns of MFs may be very similar to those of MTs at certain stages of the cell cycle, they may be quite different at other stages (Traas *et al.,* 1987; Cleary *et al.* 1992). At the S–G_2 phase, most MFs run parallel with MTs and show similar patterns as MTs in both the cytoplasmic strands and the cell cortex (Figs. 5A–5C). At the M phase, MFs markedly colocalize with MTs at the PPB and phragmoplast during prophase and telophase, respectively (see Figs. 7A–7F). MFs, however, become localized to the cytoplasm surrounding the mitotic spindle of MTs at metaphase (Figs. 5D–5F). Note that an actin-depleted zone (ADZ; Fig. 5D, arrowheads) is also observed in the cortex around the chromosomes. At

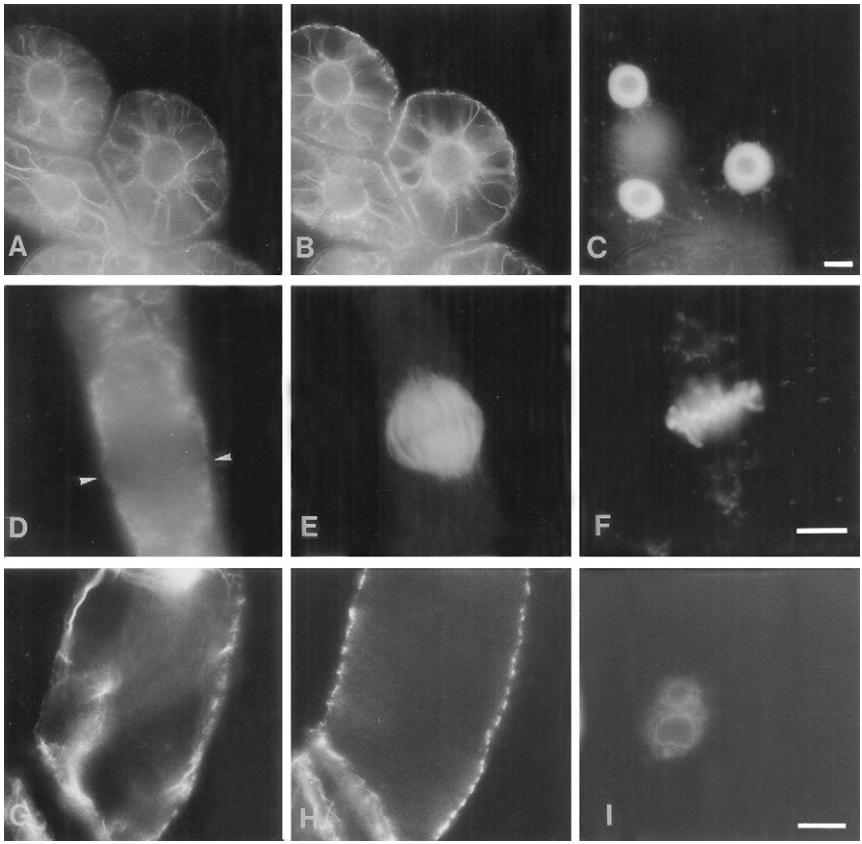


FIG. 5 Typical organization of MFs, MTs, and nuclei (or chromosomes) at S phase (A–C), metaphase (D–F), and G_1 phase (G–I). The BY-2 cells were triple stained with rhodamine–phalloidin (A, D, G), anti-tubulin antibody (B, E, H), and DAPI (C, F, I). The patterns of MFs and MTs were similar (A, B), partially similar (G, H), or different (D, E) at S phase, G_1 phase, and metaphase, respectively. Arrowheads (D) represent the ADZ. Scale bars-10 μm.

the G_1 phase, the MFs that originate from the nucleus become clearly observable, whereas the cytoplasmic MTs from the nucleus can be only scarcely identified (Figs. 5G–5I). In contrast, MFs show similar distribution patterns to MTs in the cell cortex.

D. Intermediate Filaments in Plant Cells

Networks of 10-nm intermediate filaments can also be identified under the electron microscope. It has been reported that the CMT, PPB, mitotic spindle, and phragmoplast of tobacco cells could be stained by the ME101 antibody, which

recognizes a consensus sequence of animal intermediate filaments (Fairbairn *et al.,* 1994). Using antibodies raised against animal proteins, some plant proteins have been proposed as possible components of plant intermediate filaments, for example, a keratin-like protein found in several plant species (Yang *et al.,* 1992) and a lamin-like protein identified in the nuclear matrix of plants (Frederick *et al.,* 1992). These proteins, which appear to be related to plant intermediate filaments, are known to differ between plant species; however, their structures and functions remain unresolved.

E. Use of Tobacco BY-2 Cells for Studies of Cell Cycle Events

As described previously, the tobacco BY-2 cell line has been employed in investigations into the plant cytoskeleton during cell cycle progression. One of the primary reasons for the frequent use of the BY-2 cell line is that it is the only plant cell line that can be highly synchronized. In this synchronization, 7-day-old BY-2 cells are transferred to a modified Linsmaier and Skoog (1965) medium with aphidicolin, an inhibitor of DNA synthesis, for 24 h of culture, after which they are washed and recultured with fresh medium without inhibitor (Nagata *et al.,* 1982). The cells then begin their progress through the cell cycle and enter into the S phase. A peak mitotic index (MI) of approximately 70% is observed about 8.5–9.5 h after release from aphidicolin (Fig. 6, ○). The BY-2 cells can be more highly synchronized by sequential treatments of aphidicolin and propyzamide (Kakimoto and Shibaoka, 1988; Hasezawa and Nagata, 1991) (Fig. 6, ●). In this case, the cells begin the cycle from metaphase and show a MI of more than 90% 1 h after release from propyzamide. In addition to their highly synchronous character, BY-2 cells are rich in cytoskeletal proteins, and their cytoskeletal structures can be easily observed by immunofluorescence microscopy. Moreover, the cells show rapid growth and are also suitable for biochemical analyses using evacuolated protoplasts (Jiang *et al.,* 1992). For these reasons, the BY-2 cell line has been extensively used in studies of the various aspects of plant cell biology, including gene expression, cyclin, organellar, and cell plate formation (Nagata *et al.,* 1992; Nagata and Kumagai, 1999). The current users of this cell line are distributed in more than 20 countries.

III. Events at Distinct Transition Points during the Cell Cycle

A. Roles of MTs and MFs in the Migration of Nuclei at the G_1/S Interface

In higher plant cells, dynamic changes in MT configuration are observed during the cell cycle, especially at the interfaces of G_1/S, G_2/M, and M/G_1. These changes

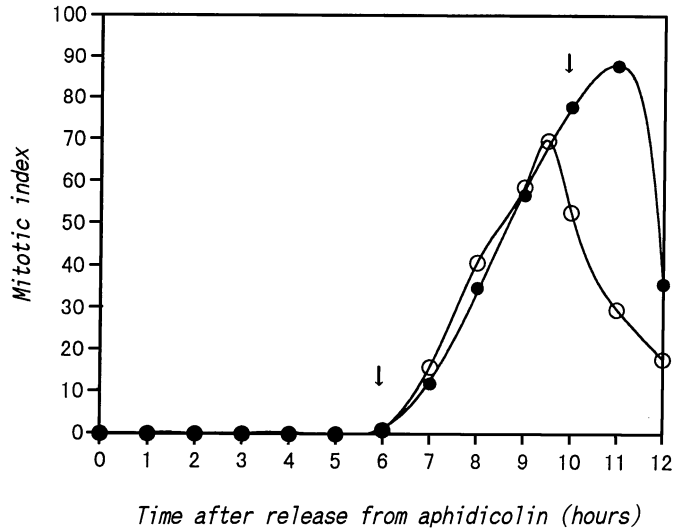


FIG. 6 Synchronization of tobacco BY-2 cells. The BY-2 cells, at the stationary phase, were subcultured for 24 h with aphidicolin and then washed and recultured in fresh medium. Changes in the mitotic index (MI) were observed after release from aphidicolin treatment (○). In a sequential synchronization procedure, the cells were treated with propyzamide 6 h after release from aphidicolin (first arrow), cultured for 4 h, and finally washed and recultured (second arrow). The peak MI was as high as 90% (●).

in MTs are accompanied by distinct changes in the configuration of MFs, which frequently colocalize with MTs in a cell cycle-dependent manner (Fig. 7). At the G_1/S interface of BY-2 cells, MTs were developing in the perinuclear regions and elongating toward the cell cortex at the start of DNA synthesis. During this period, MTs seemed to elongate along the previously developed cables of MFs, which seemed much like scaffolding [Miyake *et al.,* 1997; Figs. 7G–7I, 8C and 8D (see color insert for Figs. 8C and 8D)]. The migration of the cell nucleus to the center of the cell during the G_1 and S phases is an important event in symmetric cell division since the PPB will, in the future, appear around the migrated nucleus and play a key role in determining the division site. Because both MT elongation and nuclear migration are inhibited when MFs are disorganized by treatment with cytochalasins, which are inhibitors of MFs, nuclear migration at the G_1/S interface appears to be determined mainly by MTs rather than by MFs.

B. PPB Formation and Disappearance at the G_2/M Interface

The PPB is formed when, at the late G_2 phase, CMTs begin to disappear from around the edge of the cell. The PPB is initially observed as a wide bundle of

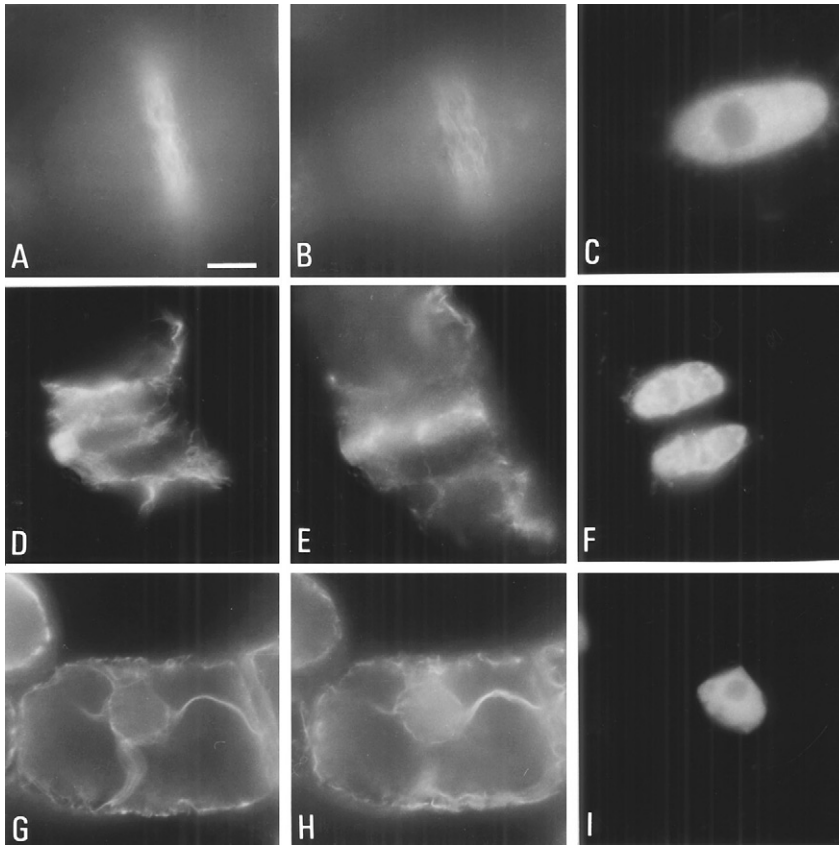


FIG. 7 Distribution patterns of MTs and MFs at distinct transition points of G_2/M (A–C), M/G_1 (D–F), and G_1/S (G–I) interfaces. The BY-2 cells were triple stained with anti-tubulin antibody (A, D, G), rhodamine–phalloidin (B, E, H), and DAPI (C, F, I). The patterns of MTs and MFs are similar but not completely identical. Scale bar-10 μm.

CMTs around the ellipsoid nucleus, but then it gradually develops into a narrow ring. The PPB appears to be formed by newly developed CMTs in the central region of the cortex rather than by the migration of predeveloped CMTs (Cleary *et al.,* 1992). MFs colocalize with MTs through the S phase to the G_2 phase even in the PPB. At the late G_2 phase, the MF arrangement becomes similar to the PPB of MTs, but the PPB-like structure of MFs is always wider than that of MT PPB (Figs. 7A–7C). In this process, MFs appear to play a role in the formation of the narrow PPB since cytochalasin prevents this narrowing (Mineyuki and Palevitz, 1990). Although the PPB disappears with spindle formation at prometaphase, the cell plate becomes scheduled to coalesce with the cell wall where the PPB existed.

Furthermore, because the ADZ appears in the area occupied by the narrow PPB, the ADZ is considered to be a candidate structure that inherits the memory of the future division site determined by the PPB (Figs. 5D–5F) (Cleary *et al.,* 1992; Liu and Palevitz, 1992; Cleary, 1995).

C. Phragmoplast Formation and Collapse at Anaphase–Telophase

At anaphase in both higher plant and animal cells, the chromatids are pulled and gathered to the two poles of the mitotic spindle by the shortening of the kinetochore MTs. Furthermore, in plant cells, after segregation of the chromatids, the new short MTs of the phragmoplast become organized first as a disk and then as a circle within which the cell plate is later formed. The phragmoplast, in which MTs are arranged with their plus ends interdigitated near the equatorial plane (Euteneur *et al.,* 1982), continues to grow outwards by the formation of external MTs and the disruption of internal MTs (Yasuhara *et al.,* 1993). The materials of the cell plate, contained in Golgi-derived vesicles, may be transported to the equatorial plane by the kinesin-like motor proteins (Asada and Shibaoka, 1994; Asada *et al.,* 1997). The exact start of the phragmoplast is unclear, even by real-time observations using microinjected fluorescent tubulin, since the changes from spindle to phragmoplast are consecutive (Lambert and Lloyd, 1994; Pickett-Heaps *et al.,* 1999). The phragmoplast continues to enlarge and to form the cell plate within it until it reaches the plasma membrane (Samuels *et. al.,* 1995). Finally, the phragmoplast begins to collapse where the cell plate coalesces with the preexisting walls at the particular sites. In the phragmoplast, the arrangement of MFs is similar to that of MTs (Figs. 7D–7F, 8A). MFs appear at the equator at late anaphase before the formation of the MT phragmoplast (Schmit and Lambert, 1988). Although cytochalasins do not completely inhibit cytokinesis, microinjection of profilin, which binds to G-actin and thus leads to MF depolymerization, results in malformation of the cell plate (Valster *et al.,* 1997). The function of the phragmoplast MFs, which are shorter than MTs, is still unclear.

D. Reorganization of CMTs at the M/G_1 Interface

When the phragmoplast collapses at late telophase, the CMTs begin to be reorganized. Using synchronized BY-2 cells, we have demonstrated the following order of processes in CMT reorganization (Fig. 9). First, short MTs are formed in the perinuclear region and then elongate to the cell cortex. Second, MTs grow on the cell cortex, parallel to the long axis, toward the distal end of the cell. Third, around the time when these parallel MTs reach the distal end, MTs that are transversely oriented to the long axis of the cell are formed on the cell cortex near the division

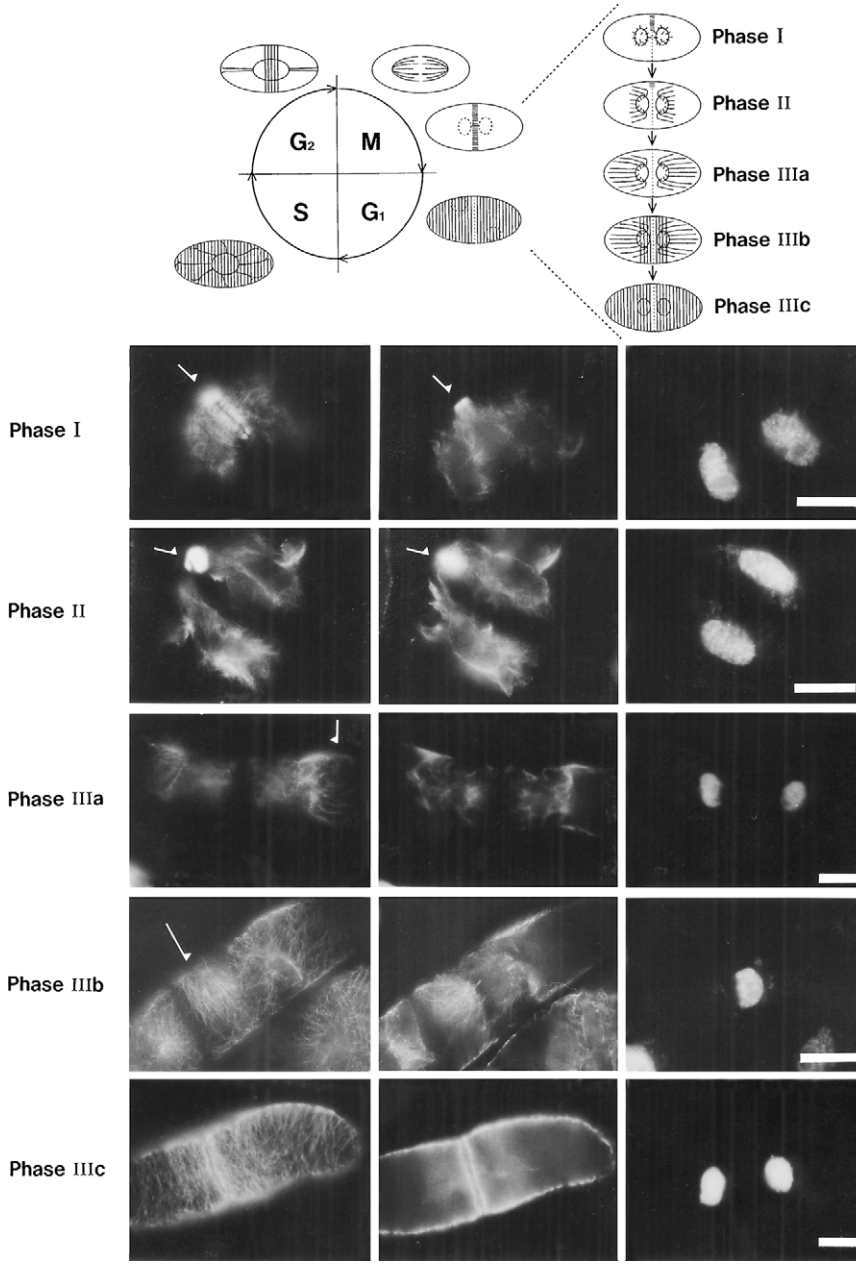


FIG. 9 Schematic diagram and micrographs of CMT reorganization at the M/G_1 interface. The short MTs first formed in the perinuclear regions (phase I), elongated to reach the cell cortex (phase II), and subsequently grew parallel to the long axis toward the distal ends of the cells (phase III_a). When these MTs reached the distal ends, the MTs that were transversely oriented to the long axis of the cells appeared near the division site (phase III_b) and then spread throughout the cell cortex (phase III_c). The right, middle, and left columns of micrographs represent the MTs on the cell cortex, the MTs in the midplane, and the daughter nuclei, respectively. Scale bars-10 μm.

site. Fourth, the transverse CMTs spread throughout the whole cell while the parallel MTs gradually disappear. Finally, in the G_1 phase, only the transverse CMTs can be observed in the cell cortex. The question that arises is why such parallel MTs are transiently observed before the formation of typical transverse MTs. One possibility is that such parallel-oriented MTs could provide a measure of cell length. Indeed, as soon as the tips of the parallel MTs reach the distal end of cells, the formation of transverse MTs begins. Such a mechanism is not inconceivable, especially since similar MTs have been proposed to serve as a measure of cell size in fission yeast (Brunner and Nurse, 2000). Another possibility is that the parallel MTs could, by their decomposition, supply tubulins and other materials required for formation of the transverse MTs.

MFs are not involved in the first process (Fig. 8A). However, when the parallel MTs are elongating in the second process, they are associated with the predeveloped parallel MFs (Fig. 8B). Furthermore, when the transverse MTs become organized in the third and fourth processes, MTs closely associate with MFs. The disorganization of MFs at the M/G_1 interface prevents the organization of CMTs and results in the appearance of abnormal MT configurations (Hasezawa *et al.,* 1998). These results suggest that MFs are involved in the organization of CMTs by determining the orientation of MTs on the cell cortex.

E. Interaction of MTs and MFs during the Cell Cycle

As mentioned previously, MFs are involved in the organization of MT structures during the cell cycle, and the relationship between these two fibrous structures appears to be closer in higher plant cells than in animal cells. In interphase, MFs are present in the cell cortex, sometimes very close to CMTs (Collings *et al.,* 1998), and from the S phase to G_2 phase MTs and MFs are also colocalized in the cytoplasmic strands (Hasezawa *et al.,* 1991). Therefore, there is some clear interaction between MTs and MFs. Although disruption of MFs results in abnormalities in the PPB, phragmoplast, and cytoplasmic MTs, the PPB, mitotic spindle, and phragmoplast can still be organized, at least in the case of BY-2 cells (S. Hasezawa and F. Kumagai, unpublished observation). Furthermore, cell division can still occur when MFs are disrupted by cytochalasins (Molé-Bajer and Bajer, 1988). Therefore, in plant cells, MFs appear to play roles that support the function of MTs in cell division and cell cycle progression.

Interactions between MTs and MFs, at distinct transition points in the plant cell cycle, appear to be mediated by specific proteins that can bind both MTs and MFs. Indeed, EF-1 α and some MAPs, for example, have the capacity to connect MTs and MFs (Itano and Hatano, 1991; Sattilaro, 1986; Pedrotti *et al.,* 1994; Igarashi *et al.,* 2000), and some motor proteins can run on both fibers (Huang *et al.,* 1999; Leung *et al.,* 1999). Although such proteins are considered as candidate cross-linkers of MTs and MFs, their precise functions have yet to be clarified.

IV. Time Sequence Observations of the Cytoskeleton in Living Plant Cells

A. Observations by Microinjection Techniques

Changes in the distribution of MTs and MFs have been mainly studied by fluorescence microscopy using fixed plant cells (Lloyd, 1987; Goddard *et al.,* 1994). However, in order to gain deeper insights into cytoskeletal dynamics during cell cycle progression, it is necessary to establish systems in which the cytoskeleton can be observed in a single living cell. Microinjection of rhodamine-labeled phalloidin for MFs or fluorescent-labeled tubulin for MTs has been employed in such a system, using *Tradescantia* stamen hair cells and the developing stomata cells, in order to examine the changes during mitosis (Zhang *et al.,* 1990, 1993; Cleary *et al.,* 1992; Cleary, 1995). Consequently, it was shown that the dynamic changes in MFs are similar to those of MTs, but that MFs appear not to be completely the same as MTs. In the course of these early studies, ADZ was first discovered.

In cortical or epidermal plant cells, changes in MT arrangement are known to be induced by plant hormones (Shibaoka, 1994). The number of CMTs, which are transversely oriented to the elongation axis, is increased and the rate of cell elongation is accelerated by gibberellins and auxins. In contrast, the number of parallel- or obliquely oriented CMTs is increased and the rate of cell elongation is reduced by ethylene and abscisic acid. In living plant cells, such MT changes can be monitored by time sequence observations following microinjection of fluorescent-labeled tubulins (Wasteneys *et al.,* 1993, Yuan *et al.,* 1994). In the case of pea stem epidermal cells, changes in CMTs were monitored following microinjection of rhodamine-labeled porcine tubulin (Yuan *et al.,* 1995). Time-lapse CLSM observations have revealed that the changes in CMTs occur not as a whole but in partial steps (Fig. 10). Hence, in the outer tangential walls of epidermal cells, the differently oriented CMTs first appear at the edge and then expand throughout the whole cell.

B. Observations Using the Green Fluorescent Protein

Reorientation of CMTs in living pea epidermal cells and *Tradescantia* stamen hairs could, as mentioned previously, be examined following microinjection of tubulin conjugated with fluorescent dyes. However, this technique requires special skills and can be applied to only a limited number of materials. Recently, a more convenient and generally applicable technique, using the green fluorescent protein (GFP), has been developed to observe MT dynamics (Cubitt *et al.,* 1995; Ludin and Maturs, 1998). The use of GFP as a marker has been employed in fava bean cells by transient expression of a GFP-MBD (microtubule binding domain of MAP4) fusion

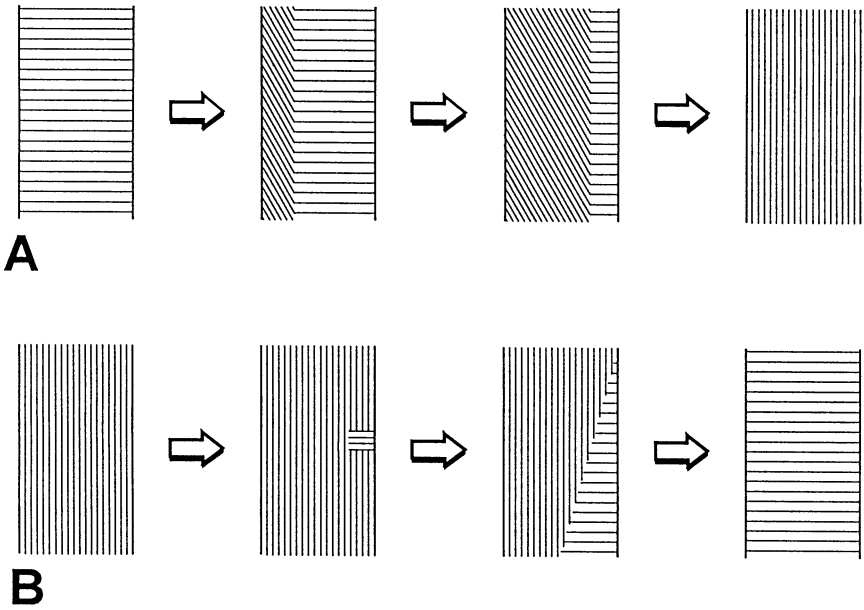


FIG. 10 Schematic diagrams of CMT reorientation in living pea cells. The changes in CMT arrays were followed by microinjecting rhodamine-conjugated tubulin into living pea stem epidermal cells. Reorientation from transverse to longitudinal (A) and from longitudinal to transverse by gibberellin (B) appear to be initiated by the appearance of CMTs organized in different alignments. The diagrams are based on the reports of Yuan *et al.* (1994, 1995).

protein (Marc *et al.,* 1998) and in *Arabidopsis thaliana* cells by stable expression of a GFP-TUA6 fusion protein (Ueda *et al.,* 1999). More recently, for the first time in either higher plant or animal cells, we successfully followed MT dynamics of living *Arabidopsis* cells through mitosis, from the late G_2 to early G_1 phase (Hasezawa *et al.,* 2000). Time-lapse observations of these *Arabidopsis* cell suspensions which stably express GFP-TUA6 revealed that at the M/G_1 interface, the CMTs were first reorganized in the perinuclear regions and then in the cortex (Fig. 11). This process has also been confirmed in transgenic tobacco cells expressing the GFP-MBD fusion protein (Granger and Cyr, 2000). Most recently, we have successfully developed transgenic BY-2 cells expressing a GFP–α-tubulin fusion protein (Fig. 12) and investigated the detailed reorganization of cortical MTs from telophase to the early G_1 phase (Kumagai *et al.,* 2001). Hence, at the M/G_1 interface, the cortical MTs are initially organized in the perinuclear regions, but then they elongate to reach the cell cortex where they form dense MT structures seen as "bright spots." Subsequently, the first cortical MTs rapidly elongate from these spots and become oriented parallel to the long axis toward the distal end of the cells. At approximately the time when the tips of the parallel MTs reach the distal end, the formation of transverse cortical MTs

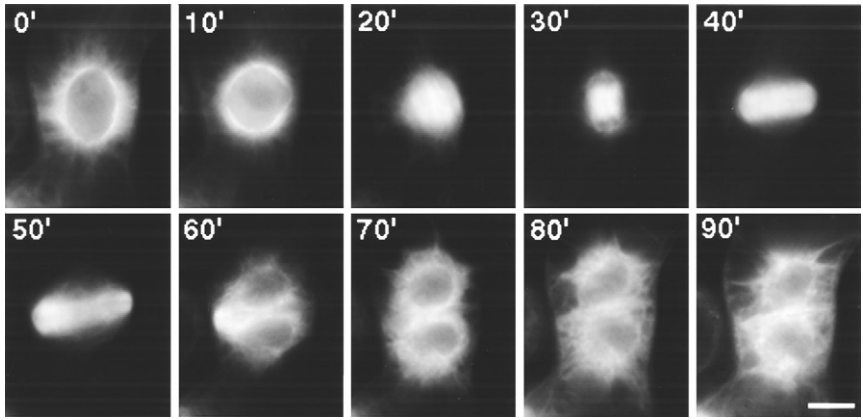


FIG. 11 Time sequence observations of MT dynamics throughout mitosis in a living *Arabidopsis* cell. The dynamic changes of MTs in a single AGT cell (*Arabidopsis* cell suspension expressing GFP–tubulin fusion protein) were observed using a time-lapse observation system with a cooled CCD camera at 10-min intervals. Time 0–20 min, disappearance of PPB and development of the mitotic spindle; 30–50 min, growth of phragmoplast; 60–90 min, decomposition of phragmoplast, nucleation of nascent MTs from perinuclear regions, and subsequent organization of CMTs on the cell cortex. Scale bar-10 μm.

begins in the cortex near the division site, as we previously hypothesized (Hasezawa and Nagata, 1991; Nagata *et al.,* 1994).

V. Modes of Control of Cell Shape by the Plant Cytoskeleton

A. Role of MTs in the Orientation of CMF Deposition

Generally, plant cells are not as flexible as animal cells since their rigid cell walls, which primarily consist of cellulose microfibrils (CMFs), restrict their morphological alterations. During interphase, the growth of plant cells is basically unidirectional. When the cells expand, CMFs function as a fence that prevents the cells from elongating in a direction parallel to CMFs so that the cells necessarily elongate in a direction at right angles with CMFs. In this context, the MT is considered a critical and decisive structure in the formation of plant cell shape since arrays of CMTs determine the direction of CMFs in the primary cell walls, where cellulose constitutes the major component. On the plant cell surface, the rosette-shaped molecular machinery (cellulose synthesizing complex; CSC) is believed to convert glucose residues, such as UDP-glucose, into fibers of β-1,4-D-glucan (Delmer and Amor, 1995). Several candidate genes encoding enzymes involved in cellulose

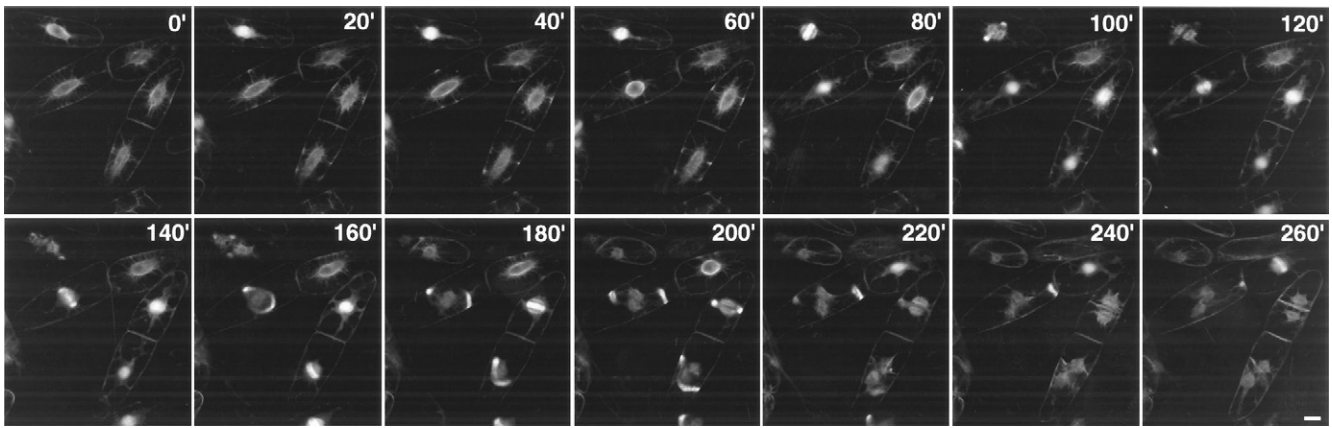


FIG. 12 Time sequence observations of MT dynamics in synchronized living BY-2 cells. The scans were performed at 20-min intervals from 6 h after the release from aphidicolin. Most of the BY-GT cells (BY-2 cells expressing GFP–tubulin fusion protein) were at the G_2 phase, with the PPB and long nuclei at 0 min. In each cell, the development and disappearance of PPB, mitotic spindle, phragmoplast, and the reorganization of CMTs were observed in order. Most cells completed cytokinesis by 260 min. Scale bar-10 μm.

biosynthesis have been characterized (Pear *et al.,* 1996; Arioli *et al.,* 1998), and one of these enzymes is localized in the CSCs (Kimura *et al.,* 1999). It is generally accepted that CMTs influence CMF orientation and regulate the orientation of CMF deposition by guiding CSCs in the fluid bilayer membrane (Ledbetter and Porter, 1963; Heath, 1974). However, it is uncertain whether CMTs are directly connected with CSCs; therefore, the function of MTs in orienting CMF deposition remains unclear. Two possible mechanisms have been proposed to explain how CMTs control the direction of CMF deposition (Figs. 13A and 13B). From studies of *Closterium* cells, Giddings and Staehelin (1988, 1991) stated that because the rosettes were found not directly on top of CMTs but rather between them or sometimes adjacent to them, and because of the distances between the rosettes and MTs, their findings argue against a mechanical linkage between them but support a model in which CSCs travel in membrane channels delineated by CMTs. Although this model is persuasive and has been widely accepted, there are obvious differences between algae and higher plant cells. For example, CMTs of *Closterium* appear only in semicells at the elongating stage, immediately after cell division (Hogetsu and Oshima, 1985), unlike CMTs of higher plant cells which are consistently observed during interphase. We have investigated the mechanism of CMF deposition using cultured tobacco protoplasts derived from taxol-treated BY-2 cells (taxol protoplasts) (Hasezawa and Nozaki, 1999). The BY-2 protoplasts, both nontreated and taxol-treated, regenerated patches of β-1,3-glucan (callose) and fibrils of β-1,4-glucan (cellulose). The taxol protoplasts possessed the same ordered MT arrays as the original cells and regenerated CMFs with patterns that were almost coincidental with MTs (Figs. 13C and 13D). Electron microscopy further revealed that on the surface of cultured taxol protoplasts, each CMF bundle was deposited on each CMT (Figs. 13E and 13F), suggesting that in higher plant cells MTs may be directly attached to CSCs by some unknown linkage(s) and thus regulate the movement of CSCs much like trolley buses (Fig. 13A). To date, putative cross-linkers between CMTs and CSCs have not been identified.

B. Cytoskeletal Regulation of Irreversible Changes in Plant Cell Shapes

In contrast to the diffuse growing cells described previously, pollen tubes and root hairs elongate by tip growth. In these cells, thick MF bundles run longitudinally but there are fewer MFs in the tip region, thus creating clear caps where cell wall components and membranes are actively secreted (Cai *et al.,* 1997; Taylor and Hepler, 1997; Kropf *et al.,* 1998). Such an organization of MFs has been considered to play a role in cytoplasmic streaming and in growth. However, the following examples imply a direct interaction between the cytoskeleton and determination of cell polarity. In growing *Vicia sativa* root hairs, net axial MFs were observed in subapical regions. After lipochitooligosaccharide (LCO) application, the tips of root hairs that were terminating growth swell, and a new outgrowth appeared

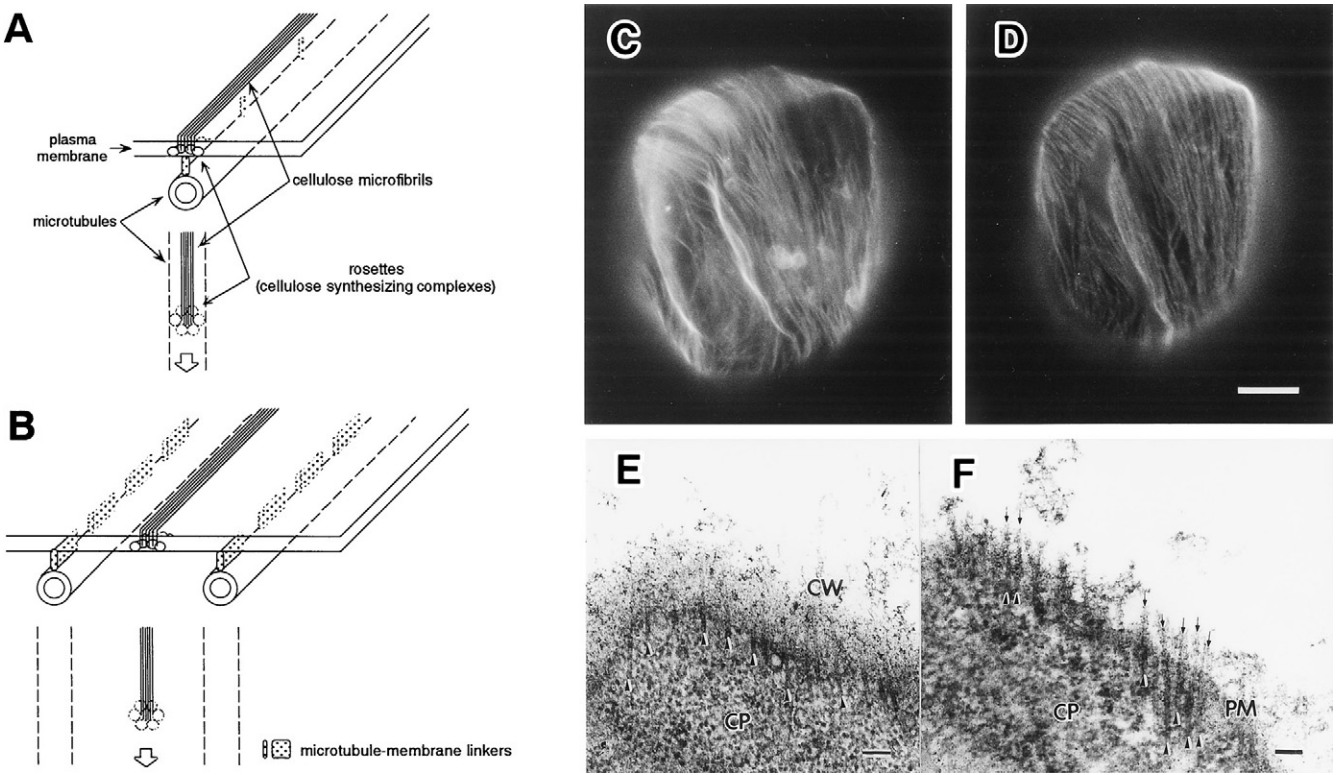


FIG. 13 Two models for CMT-mediated control of CMF deposition and the actual CMF deposition observed in taxol protoplasts. The CSC is thought to be controlled either like a trolley bus connected to CMTs by some linkages (A) or like a car surrounded by barriers attached to the CMTs (B). The pattern of regenerated CMFs (C) almost coincides with the pattern of CMT distribution (D) in cultured taxol protoplasts. (E, F) Tangential sections (viewed by transmission electron microscopy) of cultured protoplasts without (E) or with (F) taxol treatment for 1.5 h. Each putative CMF bundle appears to elongate only on each CMT in F. Arrows, CMFs; arrowheads, CMTs; CW, cell wall; CP, cytoplasm; PM, plasma membrane. Scale bars-200 nm.

from a site in the swelling with reappearance of the net axial MFs. Cytochalasin D, at levels below that required to stop cytoplasmic streaming, did not inhibited LCO-induced swelling but inhibited LCO-induced outgrowth from swelling and changes in MF configuration. Therefore, the organization of net axial fine bundles of MF in the subapical region appears to be necessary for polar growth of root hairs (Miller *et al.,* 1999). MF reorientation has also been well studied in animal cells, and one of the Rho-like small GTPases, Rac, has been implicated in this process. When constitutively active Rac was transiently expressed in a pollen tube, the cell could not undergo polar growth but rather formed a balloon-shaped cell with abnormally running MFs (Kost *et al.,* 1999). These data indicate that MFs in tip-growing cells may not only be used as "highways" of cytoplasmic streaming but also may play critical roles in the regulation of the growing direction. On the other hand, root hairs treated with MT-stabilizing or -destabilizing reagents were found to form multiple growth points (Bibikova *et al.,* 1999). Thus, the dynamic nature of MTs appears to be critical for the directionality of root hair growth. Alternatively, MT–MF interactions may be significant in determining cell growing polarity since MTs have been found associated with MFs at the cortical or subcortical regions of tip-growing cells (Tominaga *et al.,* 1997).

As described previously, it appears that CMTs play significant roles in regulating diffuse growth, whereas MFs are involved in tip growth. A trichome cell, which develops several branches within a single cell, apparently grows by combined and more complex mechanisms (Marks, 1997; Hülskamp *et al.,* 1999). The significance of MTs in trichome branching is corroborated by the observation that mutations in the *Arabidopsis ZWISCHEL* gene, which encodes a kinesin-like calmodulin binding protein, result in reduced branched trichomes (Oppenheimer *et al.,* 1997). The study of Mathur and Chua (2000) further suggests that the reorientation of CMTs is a critical step in the branching initiation of trichomes in transgenic *Arabidopsis* expressing GFP-MBD. When such reorientation occurred, dense, stable MT structures could be identified. In our study of BY-2 cells at the M/G_1 transition stage similar dense MT structures, which we call "bright spots," could be observed where MTs from daughter nuclei began to change in a direction that was parallel to the long axis of the cell (Kumagai *et al.,* 2001). From these observations, it may be reasonable to assume that MT aggregation is a general feature of MT reorientation, although the precise mechanism remains to be determined. After branching initiation, the actin MFs appear to play important roles in the establishment and maintenance of mature trichomes (Mathur *et al.,* 1999; Szymanski *et al.,* 1999).

C. Cytoskeletal Regulation of Reversible Changes in Plant Cell Shape

Reversible changes in cell shape are also observed in higher plants, such as petiole bending and stomatal movement. MTs and MFs are also involved in such changes.

When *Mimosa pudica* L. is touched, its leaves close and its petiole points downward; this is a phenomenon in which MFs have been implicated since the MF inhibitors cytochalasin B and phalloidin prevent petiole bending (Fleurat-Lessard *et al.,* 1988). Bending of the petiole is caused by a rapid shrinking of the lower side of the motor organs, called the main pulvinus, in which the arrangement of MFs changes before and after bending. Kameyama *et al.* (2000) demonstrated that the actin of *M. pudica* was heavily tyrosine phosphorylated, and that reduced levels of phosphorylation were correlated with the degree of petiole bending. Hence, about 80% of actin molecules in the pulvinus were phosphorylated before bending, but the level of phosphorylation decreased after bending. A specific inhibitor of tyrosine phosphatases, phenylarsine oxide, was able to inhibit both actin dephosphorylation and petiole bending. Therefore, at least in this case, tyrosine phosphorylation of actin is thought to control changes in plant cell shape.

MTs have also been implicated in stomatal movement during the diurnal cycle. Generally, stomata open in the daytime and close at night, so they appear to have a diurnal rhythm, and they also open under light conditions and close under dark, dry, or high-CO_2 environments. Stomata regulate the rate of photosynthesis and transpiration in response to many external and internal signals. Stomatal movement is thought to be controlled by osmoregulatory changes in the volumes of guard cells, which involve changes in their relative turgor pressure and control by the mechanical properties of their cell walls.

Recently, CMTs were also found to be involved in changes in the guard cells of *V. faba* L. (Jiang *et al.,* 1996; Fukuda *et al.,* 1998). CMTs began to be organized in a radial array at dawn and increased in number in the morning following the increase in stomatal aperture size (Fig. 14). Thereafter, MTs became localized near the nucleus and subsequently began to be destroyed from evening to midnight following the decrease in stomatal aperture size. These diurnal changes in MT organization were observed even 2 days after transfer from natural light conditions to total darkness and were accompanied by corresponding changes in stomatal aperture. The increase in stomatal aperture size in the early morning was inhibited by propyzamide, which destroys MTs, whereas the decrease in aperture size in the evening was suppressed by taxol, which stabilizes MTs. Moreover, diurnal changes in the electrophoretic profiles of total proteins, extracted directly from the guard cells were also observed (Fukuda *et al.,* 2000; Fig. 15A). Immunoblot analysis also demonstrated changes in α-tubulin and β-tubulin contents with the diurnal cycle (Fig. 15B). Both tubulins were abundant at dawn and at noon but were almost undetectable at midnight. Although treatment with actinomycin D or cycloheximide at dusk inhibited neither the radial organization of CMTs nor stomatal opening, treatment at dawn inhibited both. These results suggest that the dynamic diurnal changes in the organization of MTs in guard cells and the stomatal movement of *V. fava* may at least partially be regulated by *de novo* synthesis and decomposition of tubulin molecules in guard cells.

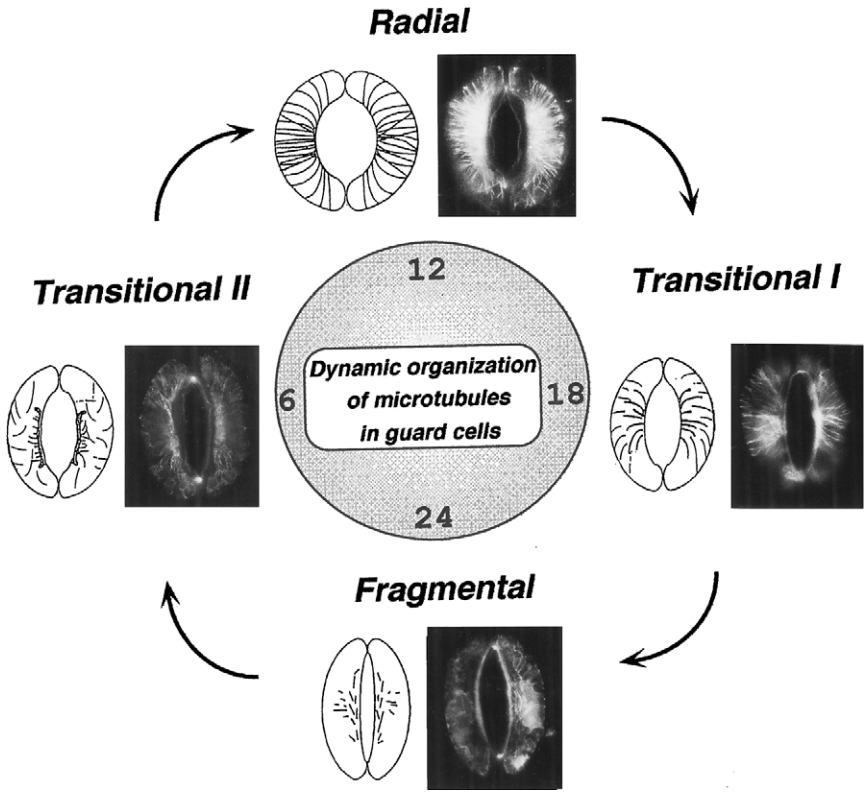
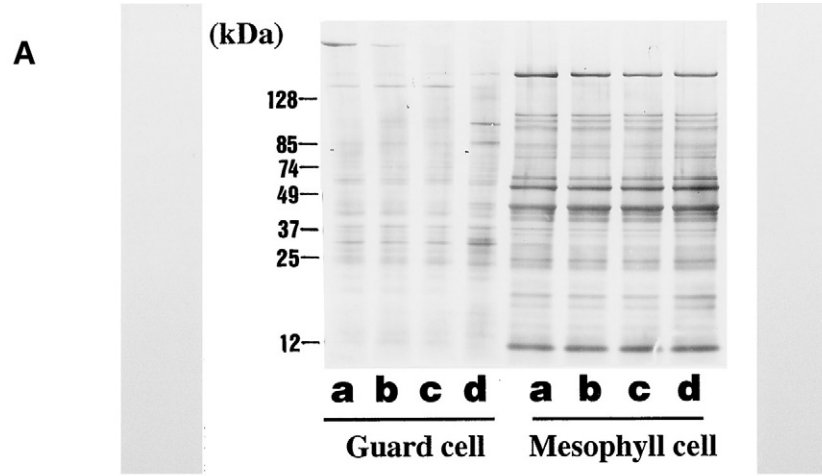


FIG. 14 Schematic diagram of typical CMT organization patterns in guard cells during the diurnal cycle. The CMTs begin to be organized in a radial array at dawn and increase in numbers until noon, following the increase in the stomatal aperture size. Thereafter, the CMTs become localized near the nucleus at dusk and then begin to be destroyed until midnight, following the decrease in stomatal aperture size. (Fukuda *et al.,* 1998).

VI. Concluding Remarks

We sometimes hear the phrase, the "diversity of life." Plant forms are also diverse, and such variation in shape can be compared to a three-dimensional jigsaw puzzle in which each cell represents one piece. Plant cells have limited flexibility to change their shapes since they are surrounded by a rigid cell wall consisting mainly of CMFs. Therefore, there are only three means by which plant cells can irreversibly change their shapes, namely, by cell expansion, cell elongation, or cell death. The cytoskeleton is involved in all these means of plant morphogenesis. The plant cytoskeleton is also involved in reversible changes of cell shape, as observed in the specific cases of the stomata and main pulvinus discussed earlier.



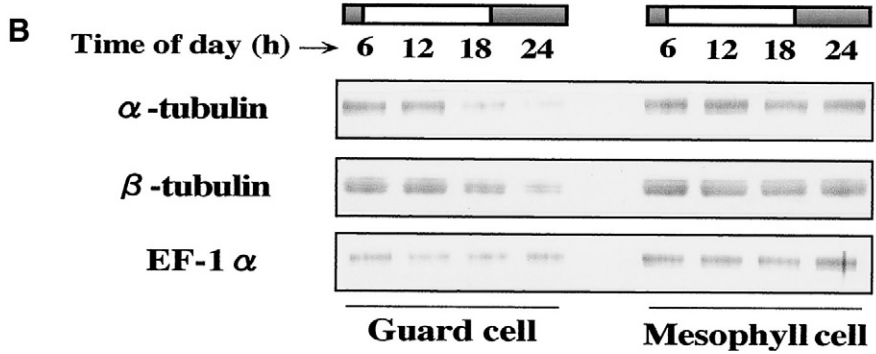


FIG. 15 Changes in the total protein composition and in the levels of tubulins in guard cells during the diurnal cycle. (A) Biotin-blot analysis of total proteins extracted from guard cells and mesophyll cells at midnight, down, noon, and evening, respectively. Lane a, 24:00 $\pm$ 1 h; lane b, 06:00 $\pm$ 1 h; lane c, 12:00 $\pm$ 1 h; lane d, 18:00 $\pm$ 1 h. (B) Levels of α-tubulin and β-tubulin in guard cells and mesophyll cells at 6, 12, 18, and 24 h. In guard cells, but not in mesophyll cells, diurnal changes in both total protein composition and in the levels of tubulins could be clearly observed. (Fukuda *et al.,* 2000).

Thus, the cytoskeleton is related to all phenomena that are involved in the control of plant cell shape.

Studies of the fibrous structures of the plant cytoskeleton have been conventionally performed by electron microscopy or immunofluorescence microscopy. Recently, the dynamics of MTs and MFs have been observed in living plant cells by microinjection of fluorescent-labeled tubulin or phalloidin. More recently, time-lapse observations of the plant cytoskeleton have been performed by the transient or stable expression of fusion proteins between GFP and various cytoskeletal proteins. We have also succeeded in developing cultured cell lines of *Arabidopsis* and tobacco BY-2 that stably express GFP tubulin, with which we have clarified some of the MT-related events of the cell cycle. These technical advances may contribute to the study of the plant cytoskeleton.

In this article, we demonstrated the dynamics of MTs during cell cycle progression in higher plant cells, with several examples obtained using mainly highly synchronized tobacco BY-2 cells. We also presented examples of the relationship between MTs and MFs during the cell cycle. The distribution patterns of these two fibrous structures are very similar at certain stages but different at other stages of the cycle, as summarized in Fig. 16 (see color insert). From the viewpoint of MTs, MFs may be required at certain stages for progress to the next step of the cycle. Our observations have further shown that there may be close relationships between MTs and MFs, not observed in animal cells, at the distinct transition points of the G_1/S and M/G_1 interfaces. Our continued focus on the plant cytoskeleton is based on the consideration that unraveling such plant-specific relationships will provide clues in elucidating the mechanisms of plant cell morphogenesis.

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Sexual Dimorphism in the Central Nervous System of Marsupials

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It is now evident that gonadal steroids, acting within a limited critical period during fetal or neonatal life, bring about sexual differentiation of both the reproductive tract and the central nervous system (CNS) in eutherians. This results in structural dimorphism in several regions of the brain and spinal cord and the programming of future patterns of adult reproductive behavior. At birth the CNS of marsupials is very underdeveloped and debate continues as to the importance of hormones in its sexual differentiation. Nevertheless, some sexually dimorphic regions have been identified, including the lateral septal nucleus in the hypothalamus and the spinal nucleus of the bulbocavernosus and dorsolateral nucleus in the spinal cord, but interestingly not the cremasteric nucleus, which is dimorphic in eutherians. To date, no apparent sex differences in estrogen and androgen receptor-immunoreactive structures have been detected in the marsupial brain; however, higher levels of aromatase activity during early development in male opossums have been reported. Sex differences have been identified in the localization of cholecystokinin-immunoreactive structures in the marsupial brain indicating that the expression of this neuropeptide is differentially regulated in each sex. A sex difference also exists in the density of arginine vasopressin-immunoreactive fibers. Arguments continue as to whether sexually dimorphic behavior in marsupials, as in eutherians, is largely predetermined by hormones acting on the CNS early in development or if it is entirely dependent on the adult steroid hormonal environment.

KEY WORDS: Marsupials, Sexual dimorphism, Brain, Spinal Cord, Hormones, CNS.

I. Introduction

In eutherian mammals several regions of the central nervous system (CNS) exhibit morphological sex differences, including variations in brain weight, the size of specific regions, the number of nerve cells present, the patterns of synaptic connections, and the distribution and content of neurotransmitters and neuropeptides. Such observed sexual dimorphisms may result from cell division and migration, programmed cell death, and growth or synaptogenesis. Research, largely on rodents, has indicated that the brain is inherently female and remains so unless exposed to gonadal steroids from the fetal or neonatal testis during a limited critical period in development, some time after differentiation of the reproductive tract.

Initially, embryos of both sexes develop in a similar manner. However, in the male activation of the sex determining region (SRY) gene in the gonadal ridge results in the formation of a testis. This produces testosterone and Müllerian inhibitory substance which masculinize the genitalia. Until recently, it was assumed that female development of the reproductive system was the default pathway and would take place unless functional embryonic testes were present. However, it now appears that this is an oversimplification (Mackay, 2000). Research by Vainio *et al.* (1999) has indicated that a signaling molecule (designated Wnt-4) not only maintains the Müllerian duct and represses the Wolffian duct but also prevents the development of Leydig cells and maintains postmeiotic ovarian development. Support for this also comes from the finding that an X-linked gene is required for ovarian development and must be silenced in the male (Bardoni *et al.,* 1994; Zanaria *et al.,* 1994, 1995). A SRY-homologous gene has been identified on the marsupial Y chromosome (Foster *et al.,* 1992), but it has not been firmly established whether it plays a role in gonadal sex determination. It is even possible that phenotypic sexual differentiation may differ between eutherians and marsupials. Interestingly, however, work by Watson *et al.* (2000) suggests that failure to transcribe sufficient amounts of the SRY binding protein during the critical time for testis differentiation led to reduced testosterone production and consequent gonadal dysgenesis in two bandicoot siblings.

The timing of the sexual differentiation of the CNS depends on the maturity of the animal when born. In small altricial mammals such as the rat, it occurs during the perinatal period (shortly before birth and during the first few days thereafter). In larger mammals differentiation of the CNS is completed entirely during intrauterine life. Only during a limited window in development is neuronal tissue sufficiently plastic to respond irreversibly to the gonadal steroids or to environmental contaminants that mimic their action. In the male, androgens act in an inductive capacity on the undifferentiated brain to bring about its masculinization. This long-term organizational action appears to take place at the molecular, ultrastructural, and cellular levels. It is brought about by alterations in levels of the gonadal steroids, the numbers of receptors available, and the presence of the appropriate metabolizing enzymes in the relevant brain areas. However, it should

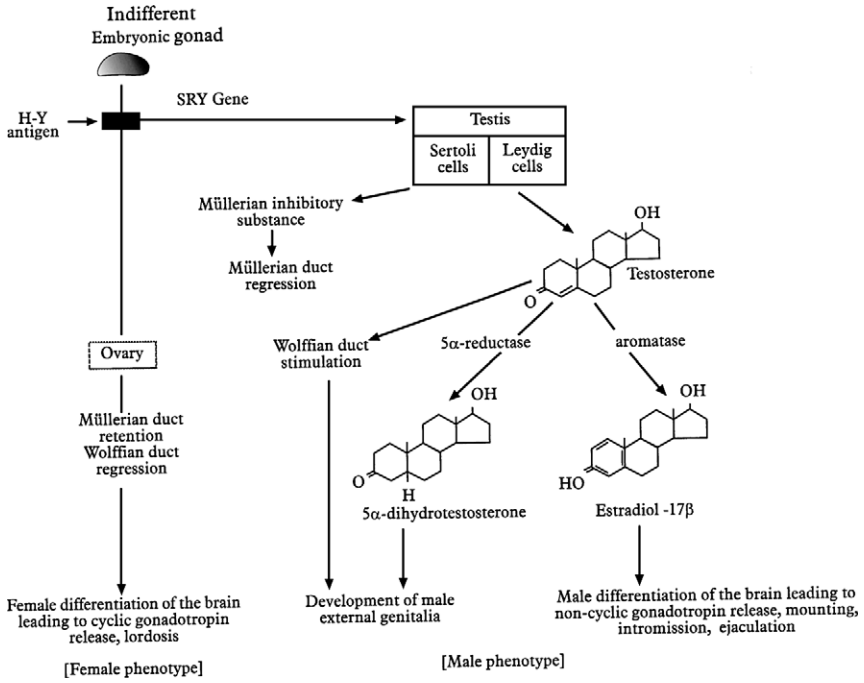

FIG. 1 A diagram summarizing the hormonal involvement in sexual differentiation of the reproductive tract and CNS in eutherians and possibly also in marsupials.

be emphasized that the critical period is an empirical concept and does not represent a clearly defined stage of development. It cannot be assumed that all sexually differentiated CNS structures are maximally sensitive to androgens at exactly the same time and therefore the critical period may vary temporally for disparate sexually dimorphic traits. Sexual differentiation of the CNS is obviously more subtle than that of the reproductive system but is of equal importance. As a consequence, the brain becomes programmed for patterns of behavior appropriate to one sex or the other. Later, following puberty, the gonadal steroids act on the CNS in an excitatory or inhibitory manner to influence gonadotropin secretion and the expression of sexual behavior that has been predetermined weeks, months, or even years previously. These actions may be direct or take place through the modulating effects on various neurotransmitter and neuropeptide systems in the CNS (Fig. 1).

Considerable research has been undertaken on various aspects of the biology of many Australian and American marsupials, in particular the tammar wallaby (*Macropus eugenii*), the brush-tailed possum (*Trichosurus vulpecula*), the stripe-faced dunnart (*Smithopsis macroura*), the Virginia opossum (*Didelphis virginiana*), and the gray short-tailed opossum (*Monodelphis domestica*). Of these, the tammar,

the dunnart, and *Monodelphis* have become established as self-sustaining laboratory animals in many centers. In Glasgow, we have set up a colony of *M. domestica,* and we have been undertaking research on reproductive development (Xie *et al.,* 1996, 1998; Van der Schoot *et al.,* 1999).

Newborn marsupials are particularly small in comparison to adults, with their weight being four or five orders of magnitude less. It is only at approximately 16 days of age, for example, that the young of *Monodelphis* are able to detach and reattach to the teats, having reached a level of development approximating that of the newborn rat (Fadem *et al.,* 1993). Moreover, in marsupials many organ systems are very undeveloped at birth, including the CNS, which is extremely immature—equal to an early fetal stage in eutherians (Saunders, 1997). Consequently, the majority of CNS development, including the whole of neocortical maturation, occurs *ex utero,* so it is relatively accessible for experimental manipulation. This is especially true for *Monodelphis* which, unlike most marsupials, lacks a pouch. *Monodelphis* pups are born after a gestation length of only 14 or 15 days (Mate *et al.,* 1994). At this time the neocortex is still at the two-layered "embryonic" stage of development, consisting of only a narrow outer primordial plexiform layer and an inner, much deeper ventricular zone. The cortical plate does not commence to appear until 3–5 days following birth (Saunders *et al.,* 1989). However, the general pattern of neocortical development is similar in both marsupials and eutherians. Krause and Saunders (1994), who studied brain growth and neocortical development in 245 specimens of the opossum *D. virginiana,* noted that there was progressive growth throughout the postnatal period with no apparent differences with regard to the sex of the animals.

II. Structural Dimorphism in the CNS

A. Eutherian CNS

1. The Brain

In 1973, Raisman and Field reported the existence of morphological sex differences, in terms of the pattern of the termination of afferent synaptic nerve endings from a nonamygdaloid source, within the medial preoptic area (MPOA) of the rat hypothalamus. Since that time other areas in the adult eutherian CNS also found to exhibit sex differences include the amygdala; the vomeronasal organ; the accessory olfactory bulb; the anterior hypothalamus; the hypothalamic supraoptic (SON), suprachiasmatic (SCN), arcuate (ARN), and ventromedial (VMHN) nuclei; the bed nucleus of the stria terminalis (BNST); the corpus callosum; vocalization centers in the forebrain and brain stem; and several motoneuron groups in the lumbosacral spinal cord (Cooke *et al.,* 1998; Payne, 1996). In rodents the MPOA is a crucial area for the control of mating behavior in males, and in females it has been implicated in the induction of lordosis and maternal behavior and cyclic

gonadotropin release (Cooke *et al.,* 1998). It receives inputs from many regions of the brain and sends projections back to these regions. Within the MPOA is a region of densely packed cells known as the sexually dimorphic nucleus of the medial preoptic area (SDN-MPOA). This SDN-MPOA has been identified in many species, including the human, in which there are twice as many neurons present in men than in women until middle age; the sex difference then reverses so that women possess more neurons in this region (Swaab and Hoffman, 1988). Much of our knowledge about the SDN-MPOA was obtained from the research undertaken on rats by Gorski and colleagues (Gorski *et al.,* 1978, 1980). These workers demonstrated that the SDN-MPOA is permanently altered by the organizational effects of androgens acting during the limited critical perinatal period, becoming between 2.5 and 5 times larger in the male than in the female. It is thus an extremely important morphological example of the organizing action of androgens on the male brain.

Although the SDN has been put forward as a model system for the study of sexual differentiation, there is no absolutely clear knowledge about its function. The absolute volume of the nucleus is not correlated with ovulatory function or with lordosis responsiveness. Lesions of the SDN do not disrupt male copulatory behavior, although small lesions just dorsal to it do interfere with such activity (Arendash and Gorski, 1983; De Jonge *et al.,* 1989). When the SDN nuclear tissue is punched out of the brains of newborn male rats and transplanted into their female littermates (stereotaxically inserted into the MPOA), the females (when adult) display enhanced behavioral responses in terms of both masculine and feminine copulatory behavior (Arendash and Gorski, 1982). Transplant volume is increased by treatment of the recipient with testosterone propionate. Thus, a tropic influence of gonadal steroids on the SDN is apparent. It is therefore evident that, at least in rodents, the gonadal steroids alone can determine the volume of the SDN (Gorski, 1988). The interaction between the gonadal steroids and those processes which lead to the formation of the sexually dimorphic brain regions may be quite complex, even for the small cluster of neurons that comprise these regions. As noted by Toran-Allerand (1985), it appears that steroids prevent some neuronal death during early development; affect protein synthesis, cell division, neuronal growth, axonal branching, and synaptic remodeling; and influence the migration of neurons and bring about their aggregation in sexually dimorphic regions.

2. The Spinal Cord

In addition to the situation in the brain, it has been demonstrated that in the rat and hamster there are particular groups of motoneurons in specific areas of the spinal cord which innervate muscles that are sexually dimorphic. These target muscle groups are believed to have a tropic effect on their innervating motoneurons, thus increasing the synaptic input (Lowrie and Vrbová, 1992). Sex differences are evident in the numbers of neurons comprising the spinal nucleus of bulbocavernosus (SNB) and the dorsolateral nucleus (DLN) These nuclei contain motoneurons

supplying the perineal muscles involved in penile reflexes and erection and are located in cord segments L5–S1. They are thought to be homologous with Onuf's nucleus present in the spinal cord of cats, dogs, and primates (including the human). In the adult male rat SNB motoneurons are approximately five times more numerous than those in the female, in which they are also only half as large (Breedlove and Arnold, 1980; Jordan *et al.,* 1982; Tobin and Payne, 1991).

The perineal muscles innervated by the DLN and SNB are the ischiocavernosus, the bulbocavernosus, the levator ani, and the anal sphincter. Although these muscles are present at birth in equal numbers in both sexes, they atrophy in the female early in postnatal life due to a lack of circulating androgens (Nordeen *et al.,* 1985). Manipulation of androgens during the perinatal period in rats can alter these numbers (Cowburn and Payne, 1992). Masculinized females possess a male-typical motoneuron number and retain the perineal musculature that would otherwise have atrophied (Breedlove, 1992; Jordan *et al.,* 1997). In rats, 5 days prior to birth no sex difference is apparent in neuron numbers in the previously mentioned nuclei, although only a fraction of neurons are in place. Subsequent migration of neurons to the nucleus results in numbers in both sexes being higher even than in adult males by 1 day prior to birth (Nordeen *et al.,* 1985; Sengelaub and Arnold, 1986). A period of cell death in early postnatal life reduces SNB neurons to adult levels by Day 10; the degree of loss is significantly greater in females. It appears that by preventing atrophy of the target muscles androgens also indirectly preserve the neurons supplying these muscles.

In contrast to the situation with regard to the DLN and SNB, other motoneuron groups present in the spinal cord fail to show any significant sex differences in number. These include the retrodorsolateral nucleus, and the ventromedial nucleus, which innervate the hindlimbs. However, the cremasteric nucleus, located in segments L1 and L2 of the rat spinal cord, is also sexually dimorphic in most mammals. This group of motoneurons projects exclusively via the genitofemoral nerve to innervate the cremaster muscle (Nagy and Senba, 1985; Payne, 1996; Van der Schoot *et al.,* 1999) and is organized into a centromedially located column (Kojima and Sano, 1984). In female eutherians the cremaster muscle usually atrophies after birth, whereas in the male it is mainly involved in supporting and in raising and lowering testes in response to temperature changes. The adult male rat has approximately 260 cremasteric motor neurons in the spinal cord, compared to only 70 in the female (Barthold *et al.,* 1994). These remaining cremasteric motoneurons in the female innervate a small patch of abdominal musculature on either side of the clitoris.

B. Marsupial CNS

Marsupials pose a number of challenges to the views on sexual differentiation gleaned from studies on eutherians. For instance, the development of both the gonads and reproductive tract generally occurs postnatally, but the formation of

several sexually dimorphic structures, such as the pouch, scrotum, mammary primordium, gubernaculum, and processus vaginalis, appear to take place prior to differentiation of the gonad and their early development may thus be independent of hormones (O *et al.,* 1988; Renfree and Short, 1988; Renfree *et al.,* 1995, 1997; Ullmann, 1993). Sharman *et al.* (1990) proposed that genes on the X chromosome may be the primary determinants of scrotal formation. Moreover, it is unclear exactly how virilization of the reproductive tract takes place. Wilson *et al.* (1999), who investigated this process in detail in the tammar wallaby, concluded that it could not result from the transport of androgens to the urogenital sinus in the general circulation. Their study and earlier ones on *Monodelphis* by Fadem and Harder (1992) and Xie *et al.* (1998) failed to detect sex differences in circulating gonadal hormone levels until puberty. Wilson *et al.* postulated that masculinization of the marsupial reproductive tract may be mediated either by the transport of androgens to this region directly via the Wolffian ducts or by the conversion of circulating prohormones to androgens in the target tissues.

There is also controversy regarding whether sexual dimorphism is widespread within the marsupial CNS, when it might take place, and what role, if any, the gonadal steroids might play in this process. Johnson (1977) provided a very detailed account of the anatomy and functioning of the brain and spinal cord in marsupials, compiling information from all the material published between 1890 and 1975 and comparing the situation with that existing in eutherians. Anatomically, the brain of marsupials exhibits several differences from that of eutherians such as the rat; the most obvious ones are the absence of a corpus callosum and its replacement by a prominent interhemispheric pathway, the anterior commissure, carrying fibers connecting virtually all parts of the neocortex. In many marsupials, including the tammar wallaby (*M. eugenii*), these commissural fibers form a bundle (the fasciculata aberrans) that radiates to the cortex via the internal instead of the external capsule. The fasciculata aberrans thus represents a more direct pathway from one half of the dorsal neocortex to the other than that seen in eutherians. Despite these obvious differences, it is clear that the general pattern of neocortical development is similar in both marsupials and eutherians.

The anatomy of the marsupial nervous system was also reviewed in detail by Barbour (1977), who drew on a wealth of earlier studies. Between the different marsupial groups there is a large variation in the structure of the CNS. In general, however, the cerebral hemispheres are comparatively small and not prolonged backward over the cerebellum. The cerebellum, although also relatively small and simple, is notable for the large size of the vermiform lobe compared to the lateral lobes. The olfactory bulbs are large (Figs. 2 and 3). Work carried out on *D. virginiana* by Voris (1982) shows a generally typical mammalian spinal cord.

Very little information exists on sex differences in the marsupial brain and their genesis. Sexually dimorphic structures, such as the lateral septal nucleus (LSN), have been identified in the marsupial CNS (Iqbal and Jacobson, 1995a), although this dichotomy is not attributable to hormone action during development.

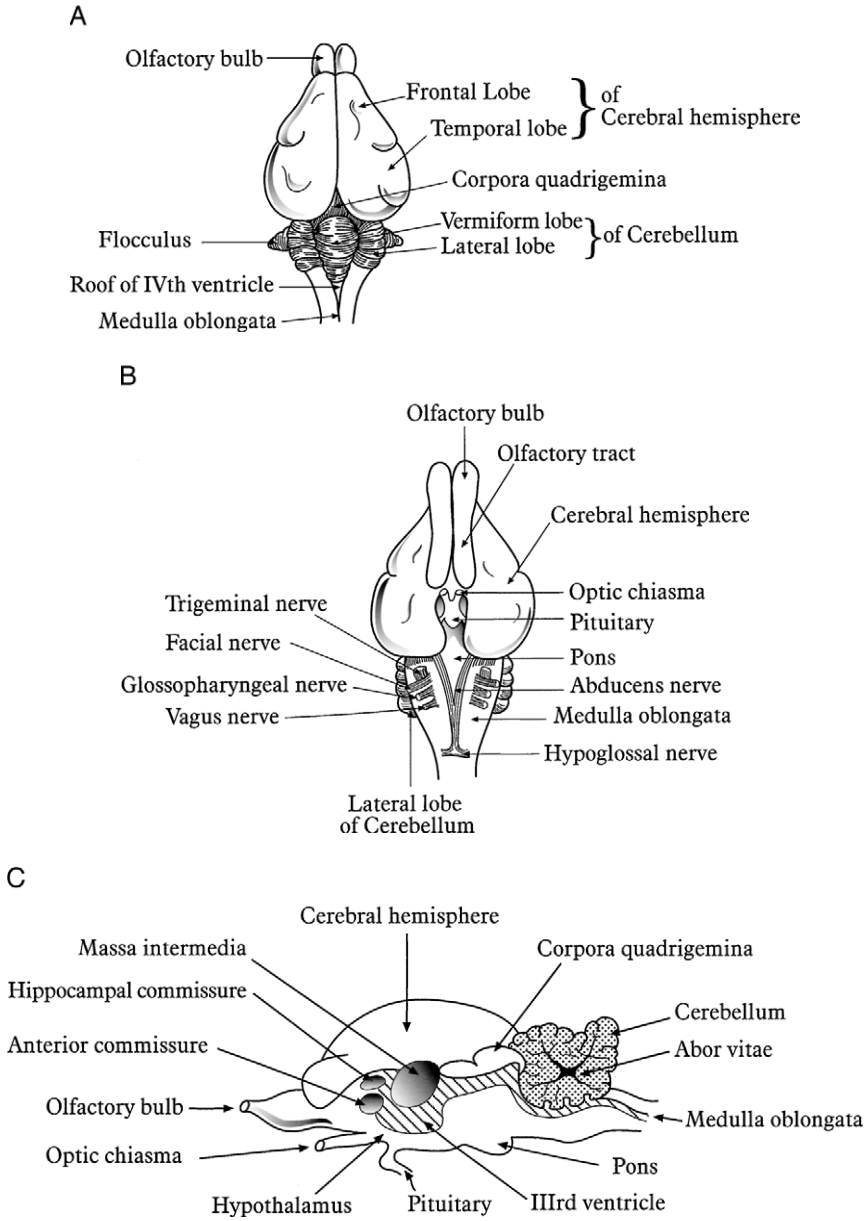

FIG. 2 Dorsal (A), ventral (B), and saggittal (C) views of a marsupial brain typified by the Australian brush-tailed possum, *Trichosurus vulpecula* (adapted from Gilmore and Da Costa, 1995).

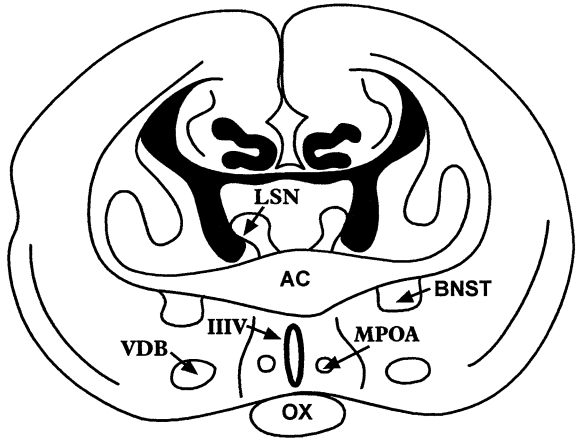

FIG. 3 Diagrammatic representation of a coronal section of a marsupial brain (*Monodelphis domestica*) in the region of the anterior hypothalamus. III V, third ventricle; AC, anterior commissure; BNST, bed nucleus of stria terminalis; LSN, lateral septal ventral nucleus; MPOA, medial preoptic area; OX, optic chiasma; VDB, nucleus of the vertical limb of the diagonal band.

Rudd (1994) found no evidence of any sexually dimorphic brain nuclei in the hypothalamic MPOA nor in the accessory olfactory bulb of the tammar brain, in contrast to their presence in many eutherians.

Sexual dimorphism of the vomeronasal system is very widespread in eutherians (Cooke *et al.,* 1998), and one might therefore expect to find the same situation in marsupials. Several detailed studies have been undertaken on the vomeronasal organ and its associated structures in the opossums, *Didelphis marsupialis* and *M. domestica* (Shammah-Lagnado and Negrão, 1981; Jackson and Harder, 1996; Jia and Halpern, 1998; Poran, 1998), but no sex differences have been reported. However, pheromones delivered from male to female opossums via the vomeronasal organ will accelerate reproductive maturation and induce estrus (Fadem, 1985, 1987; Stonerook and Harder, 1992; Jackson and Harder, 1996, 2000), indicating that it has a major reproductive role in marsupials as well as in eutherians.

Recently, we examined possible sex differences in the spinal cord nuclei of *M. domestica*. Initially, adults of both sexes were euthanized and perfused transcardially with 3% gluteraldehyde and 1% formaldehyde. Laminectomies were then performed to expose the spinal cord and its associated nerves. Spinal cords were removed and the dorsal root ganglia identified according to the level of the vertebral column from which they exited. Segments containing the four groups of motoneurons mentioned previously were removed, sectioned on a cryostat, and stained with Toluidine blue (Fig. 4). Cell numbers in the four regions were counted and averaged. It was found that, as in rodents, sex differences were apparent in the SNB and DLN, but not in the RDLN and VMN. More than twice as

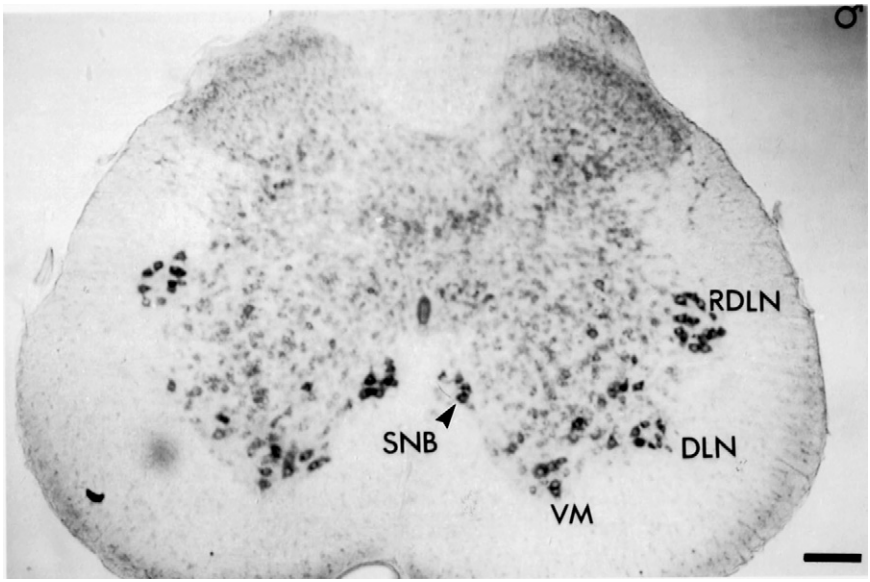

FIG. 4 Transverse section of a male opossum (*Monodelphis domestica*) spinal cord stained with Toluidine blue to illustrate the motoneurons comprising the retrodorsolateral nucleus (RDLN), dorsolateral nucleus (DLN), spinal nucleus of the bulbocavernosus (SNB), and ventromedial nucleus (VM). Scale bar = 180 μm.

many motoneurons were present in the male SNB and DLN than in these areas in the female opossum.

We then studied the motoneurons supplying the cremasteric muscles (Allen *et al.,* 2000). The marsupial cremaster muscle presents an anomaly compared to eutherians in that it is well developed in both sexes. In the female, the muscle is attached to the mammary glands and is commonly considered to retract them—a useful feature in both pouched marsupials (in which it retains the young in the pouch) and in pouchless ones (in which it keeps the attached young off the ground). In the male *Monodelphis* the cremaster muscles are compact and circular in outline and are located superficially in the ventral inguinal region close to the testes where they are associated with the spermatic cord/scrotal sac. However, although containing about 30% less fibers, the muscles are more diffuse and widespread in the female, spanning over the dorsal surface of part of the mammary gland and attaching to the base of the uterine round ligament (Van der Schoot *et al.,* 1999) (Fig. 5). As already mentioned, in eutherians retention of both the cremaster muscles and their innervating neurons are androgen dependent, but it is unknown if this is also the case in marsupials. To identify exactly which spinal cord ganglia were innervating the cremaster muscles in *Monodelphis,* a retrograde neuronal tracer, cholera B toxin (CBT), was utilized. Adult opossums of both sexes were anesthetized with

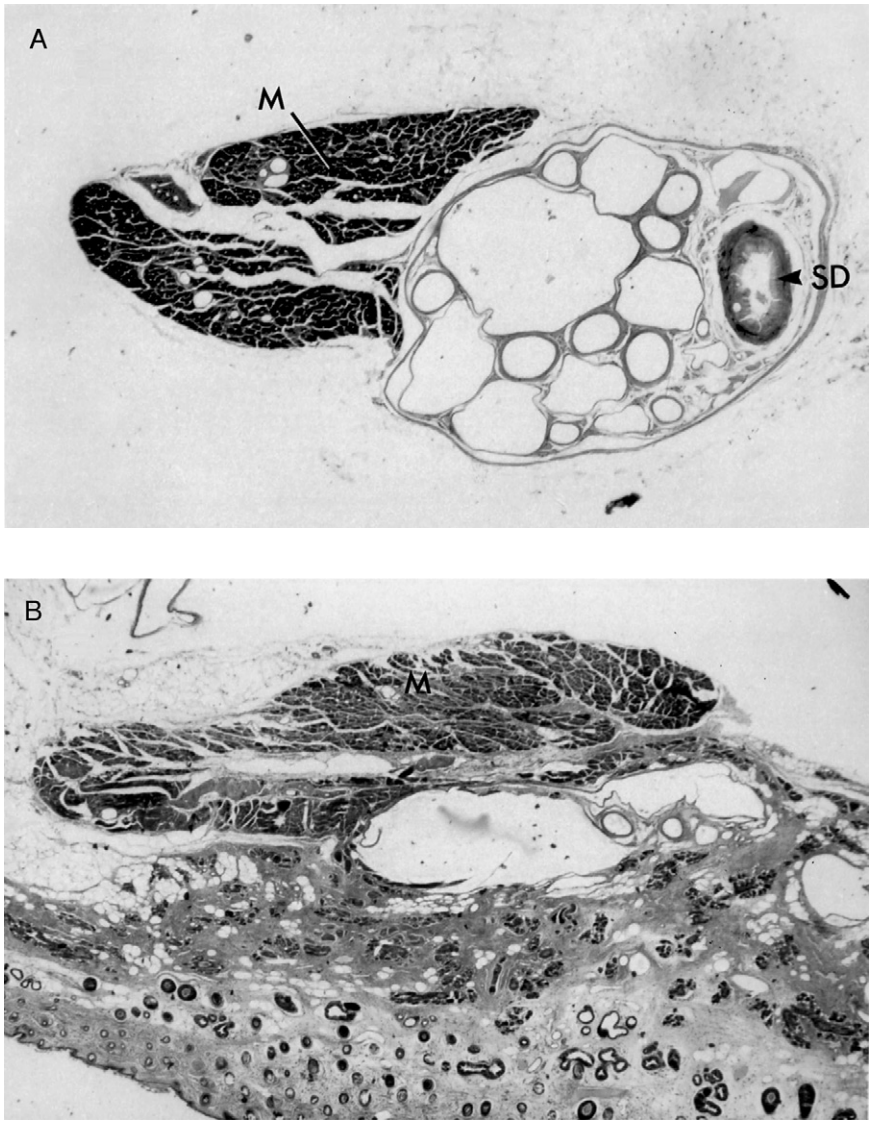

FIG. 5 Transverse sections of (A) male and (B) female *Monodelphis domestica* cremaster muscles (M). In the section showing the male muscle the spermatic duct (SD) is visible on the right along with a plexus of blood vessels and associated adipose tissue. The female cremaster muscle is larger and broader than that in the male and spreads across the dorsal surface of the mammary gland. Magnification × 75.

halothane and the cremaster muscle located just beneath the skin of the inguinal region. Several microinjections of CBT were made along the length of each left cremaster muscle, and the animals were allowed to recover for a week to enable the toxin to travel up the axons supplying the muscles and accumulate in the neuronal cell bodies. After this time the opossums were euthanized and perfused, and the spinal cords were removed and sectioned as described previously. They were then stained with antibodies to CBT using routine immunocytochemistry. Stained neurons were readily visible within the ventral gray horn forming a relatively long cremasteric nucleus. Motoneurons occurred principally in segments L2–L4, with a few in T13, L1, and L5. Almost all were ipsilateral to the injection sites, but a small number of contralateral neurons were also stained [Figs. 6 and 7 (see color insert for Fig. 7)].

There were no sex differences in the rostrocaudal extent of stained neurons, their distribution between individual segments, or their overall numbers. Counts averaged 1.42 cells per section in the males and 1.34 in the females. It is unclear if the retention of the cremaster muscle in both sexes in marsupials is due to a nonhormonal mechanism or to the absence of androgenic cues at the appropriate critical period of development. Nevertheless, a corresponding lack of sexual dimorphism in the cremasteric neuron pool could be expected and this indeed was confirmed.

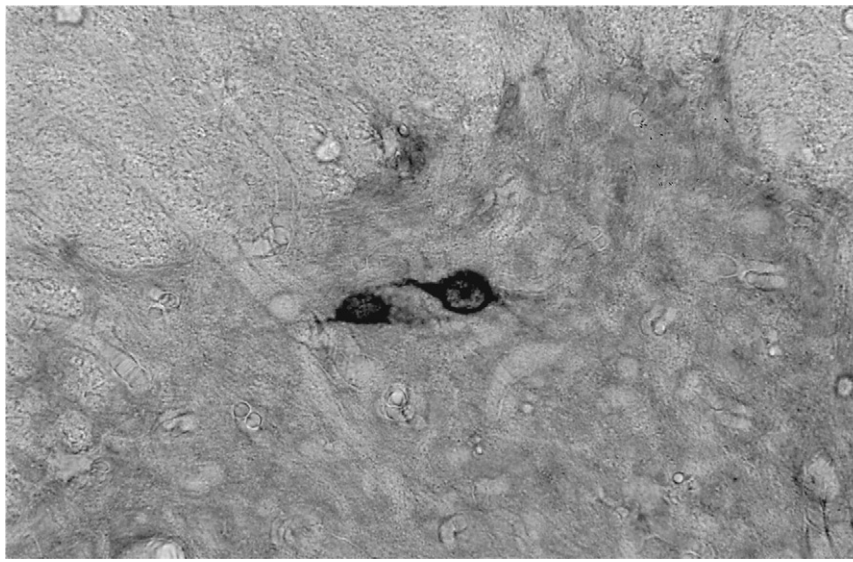

FIG. 6 Motoneurons stained by immunocytochemistry in the cremasteric nucleus of the opossum, *Monodelphis domestica,* spinal cord showing uptake of the retrograde tracer cholera B toxin. Scale bar = 30 μm.

III. Gonadal Steroids

A. Gonadotropin-Releasing Hormone System

The release of the gonadotropins follicle-stimulating hormone and luteinizing hormone (LH) from the anterior pituitary is regulated by gonadotropin-releasing hormone (GnRH), also known as luteinizing hormone-releasing hormone. The GnRH system thus plays a crucial role in regulation of the hypophyseal–gonadal axis and is involved in the control of reproductive behavior as well. GnRH neurons are widely distributed throughout the olfactory system and basal forebrain, but in eutherians they are concentrated in certain hypothalamic regions. In rodents, these are the preoptic area (POA) and anterior hypothalamic area; in primates, the GnRH cell bodies are located in the medial basal hypothalamus. GnRH fibers project extensively within the brain to terminate in such areas as the median eminence (ME). It has been shown that the neurons arise in the region of the medial olfactory placode and migrate along branches of the terminal and vomeronasal nerves to reach the basal forebrain (Schwanzel-Fukuda and Pfaff, 1989; Wray *et al.,* 1989). Cell migration from the developing olfactory neuroepithelium to the brain was studied by Tarozzo *et al.* (1994) in tissue from mouse embryos and neonatal opossums (*M. domestica*) maintained in culture. Many of the migrating cells were found to contain GnRH. Other cells, with different functional commitments, were seen to move along the same pathway. GnRH immunoreactive cells were identifiable in the newborn opossums and by Day 7 had already reached the POA, with developed fibers projecting toward the ME. Cummings and Brunjes (1995) observed that the CNS protein S100 apparently acts as a guidance molecule for migrating GnRH neurons in *M. domestica* because it is closely associated with the hormone along the entire GnRH migratory route from the vomeronasal organ to the septo-POA as early as the day of birth.

Schwanzel-Fukuda *et al.* (1987, 1988) used immunocytochemistry to detect the presence of GnRH in cells and fibers of the brain and nervus terminalis of both neonatal and adult opossums (*M. domestica*). Many of the cells and fibers in the nervus terminalis, which runs between the nasal mucosa and the septal and preoptic areas of the forebrain, are close to the blood vessels and cerebrospinal fluid suggesting that its pathways may be involved in the mediation of the endocrine control of mating behavior or in reproduction (Schwanzel-Fukuda *et al.,* 1988). It was found that the pattern of distribution of GnRH immunoreactivity differed from that seen in eutherians in that the ganglion cells and fibers of the nervus terminalis appeared to be the principal source of GnRH in both the neonate and the adult. Few immunoreactive cells were detected in other areas of the forebrain or in the hypothalamus. However, GnRH fibers were identified in these regions and, as might be expected, were especially numerous in the ME. No sex differences were apparent.

In eutherians abnormalities in the GnRH system can affect the synthesis and release of the gonadal hormones, and alterations in gonadal steroid levels during early development can affect the secretion of GnRH in adulthood. Fadem and Schwanzel-Fukuda (1990) investigated development of the GnRH system in the brain of adult male opossums (*M. domestica*) that had been treated with estradiol benzoate at 1 and 3 days of age. Application of this estrogen to the neonatal male opossum completely blocks testicular development. However, the distribution of GnRH immunoreactive cells and fibers was found to be similar in both treated opossums and their controls, indicating that growth of the GnRH system in this animal progresses normally even in the absence of testes during development.

B. Roles of Gonadal Steroids in Sexual Differentiation of the CNS

During development androgens act as signal transduction molecules, neurotropic factors, and/or neuromodulators by binding to specific intracellular receptors and thereby altering gene expression and/or transcriptional factors within the target cell and affecting protein synthesis in neuronal tissue (Evans, 1988; Miesfeld, 1989; McEwen, 1991). Likewise, estrogens are known to attach to specific intracellular receptors in the brain, transforming them so that they can then bind DNA and alter the expression of estrogen-sensitive genes (Walters, 1985; Parker, 1990). It should be noted that in many of the species examined, the early organizational effect of androgens on the CNS is actually via their aromatization to 17-β estradiol within target cells present in specific regions, including the MPOA, ARN, ventromedial hypothalamus, and amygdala.

Within the perinatal period in rats testicular androgens appear to alter functional neuronal development in three ways: (i) by changing the number of receptors for the different neurotransmitters or by affecting the enzymes controlling synaptic transmission, (ii) by interference with maturational or metabolic aspects of the receptor system, and (iii) by an alteration in membrane properties.

1. Steroid Receptors in the Marsupial Brain

A considerable amount of research has been undertaken on the presence of steroid receptors in the brain. Although much of this work has been carried out on rodents, the opossum, *M. domestica,* has also been the subject of several investigations. In both sexes of all vertebrates studied, steroid binding has been demonstrated in the hypothalamus, the POA, and parts of the limbic system and mesencephalon. Female sexual behavior is most effectively induced by the administration of estrogen to the VMHN, whereas the MPOA is known to be the best site for the action of testosterone in stimulating male sexual behavior (Fabre-Nys, 1998). Other areas, such as the amygdala and the septum, bind steroids as well and are

also implicated in the control of sexual behavior. The existence of steroid receptors has been demonstrated within the opossum brain by many groups, including Etgen and Fadem (1989), Iqbal *et al.* (1995), Fox *et al.* (1991b,c); and Handa *et al.* (1991). Many similarities have been found to the situation in the rat. However, little research has been carried out on other areas of the CNS, including the spinal cord.

a. Estrogen Receptors Like androgens, estrogens appear to influence development of neural systems involved in the control of reproductive behavior. Moore and Thurstan (1990) demonstrated the capacity of male opossums (*M. domestica*), treated for varying periods for up to 1 month from the day of birth with estradiol benzoate, to show estrous behavior in the presence of untreated males. This suggested that brain sexual differentiation in these marsupials had been altered by the exogenous estrogen applied early in life. Etgen and Fadem (1989) investigated the ontogeny of estrogen binding sites in mixed-sex opossum (*Monodelphis*) pups ranging from newborn to 63 days of age. The methodology employed was a modification of that of Ginsburg *et al.* (1974) and involved a measurement of the degree of binding of tritiated estradiol to brain cytosols. In 1-, 4-, and 8-day-old pups the whole brains were removed and pooled; in animals aged 16 and 63 days the tissue taken comprised the hypothalamus together with the POA and that of the remainder of the brain. The authors made no attempt to analyze male and female brains separately because their earlier work (Etgen and Fadem, 1987) indicated there were no sex differences in estrogen-binding macromolecules in the adult opossum hypothalamus and POA. Although no estrogen binding was detectable in the brains of newborns, by 4 days high-affinity estrogen binding sites had appeared and from that time onward these were detectable but remained low in number. In contrast, the number of estrogen binding sites in the hypothalamus–POA increased substantially from Day 16 to reach 50% of adult levels by 2 months of age.

A much more detailed study on the ontogeny of cells containing estrogen receptor-like immunoreactivity was undertaken by Fox *et al.* (1991b). This group identified such immunoreactivity in many areas of the opossum brain from 16 days of age; the distribution was broadly similar to that seen in the adult as reported by Fox *et al.* (1991c). The areas containing estrogen-like immunoreactive cells included the LSN, MPOA, periventricular POA, lateral ventromedial hypothalamus, the premammillary nucleus, medial subdivision of the BNST, the dorsomedial nucleus (DMN), posterior cortical amygdaloid nucleus, and midbrain central gray matter. Moreover, from 25 days into adulthood, the anterior and posterior cortical amygdaloid areas and the cortical amygdaloid nuclei also contained estrogen receptor-like immunoreactive cells. Fox *et al.* (1991b) point out that the appearance of estrogen receptor-like immunoreactivity in the POA and hypothalamus of *Monodelphis* pups between the ages of 10 and 15 days occurs just 1–6 days after neurogenesis is completed in these regions. This is similar to the situation in many

eutherians. Moreover, the time period coincides with the onset of high levels of aromatase activity in *Monodelphis,* as reported by Fadem *et al.* (1993). Fox *et al.* (1991b) suggest that the time course for estrogen expression, as in other mammals, may indicate the beginning of a critical period for sexual differentiation of the opossum brain. Although certain sexual behavior in *Monodelphis* differs between the sexes, suggesting there may be sexually dimorphic circuits in the brain, few morphological sex differences have been found. There are also no apparent sex differences in the density of estrogen receptor immunoreactive cells in the adult brain of *Monodelphis* or in nuclear or cytoplasmic staining patterns (Fox *et al.,* 1991c).

b. Androgen Receptors Butler *et al.* (1998) investigated the developmental expression of the androgen receptor in the tammar wallaby (*M. eugenii*). Androgen receptor gene transcript was detected in the brain of both sexes from Day 23 of the 26.5-day gestation and remained present until Day 4 postpartum (the oldest examined). In the tammar, sensitivity of the developing male reproductive tract to androgens appears in a relatively narrow window of time between Days 20 and 25 following birth (Shaw *et al.,* 1988; Lucas *et al.,* 1997). This is 3 weeks after the initiation of testosterone production on the day of birth. Presumably masculinization of brain structures (if they do take place) would, as in all other mammals studied, occur sometime later.

Iqbal *et al.* (1995) investigated the distribution of androgen receptor-like immunoreactivity in the CNS of adult male opossums (*Monodelphis*) and examined the effects of castration and testosterone replacement. As might be expected, androgen receptor-immunoreactive cells were found to be widely distributed throughout the brain and this broadly resembled the situation in the rat and other mammals. However, Iqbal *et al.* also reported the presence of androgen receptor-like receptivity in the SCN and lateral habenula; this has not been recorded in eutherians. Furthermore, no androgen receptor-like immunoreactivity was detected in the opossum cerebral cortex, although it is present in the rat. The highest numbers of cells staining for androgen receptor-like immunoreactivity were present in the dorsal and ventral nuclei of the lateral septum, medial region of the BNST, the MPOA, median preoptic nucleus, nucleus of the lateral olfactory tubercle, specific nuclei in the amygdala, the subiculum, VMHN, ARN-ME region, and ventral PMN as well as in the anterior pituitary and in the brain stem. Castration of opossums 4 days prior to mapping practically eliminated all androgen receptor-like immunoreactivity, but testosterone replacement 2h before mapping restored the distribution to that seen in intact males. Although the ontogeny of androgen receptors in the opossum brain has not been studied in detail, Sonea *et al.* (1997) reported that androgen receptor-like immunoreactivity is present in the male reproductive tract of *Monodelphis* from at least Postnatal Day 5. Preliminary studies from our laboratory indicate the definite presence of androgen receptors in the developing testis and scrotum of *Monodelphis* pups as early as Postnatal Day 2, and it appears that these are probably even there at birth. Certainly, the enzyme 3β-hydroxysteroid dehydrogenase (involved in steroid biosynthesis) is detectable in the

developing testis of *Monodelphis* on the day prior to birth; this has very recently been demonstrated in our laboratory.

We have also recently used double fluorescent labeling for CBT and for both androgen and estrogen receptor immunoreactivity to study the situation in the spinal cord of *Monodelphis* and can confirm that many (but not necessarily all) cremasteric motoneurons present there in the adult possess both androgen and estrogen receptors; no obvious sex differences are evident, however (Fig. 6). The situation in the neonate is unknown.

2. Aromatization Effects

Strong evidence exists, largely from work on rodents, that the organizational effects of androgens on the CNS follow their intracellular aromatization to estradiol in brain areas important for the control of sexual behavior. Male rodents show higher levels of neural aromatase activity than do females during the perinatal period when sex differentiation of the brain takes place (MacLusky *et al.,* 1985). Moreover, administration of aromatase inhibitors to male rats at this time increases the capacity for female sexual behavior in adulthood (McEwen *et al.,* 1977; Fadem and Barfield, 1981). Thus, both the suppression of feminine and the enhancement of masculine behavior are assumed to occur as a consequence of the effects of estrogen following its intracellular conversion from testosterone by aromatase activity (Hutchison, 1997). The P450 aromatase enzyme, responsible for the conversion of androgens to estrogens, appears to be a crucial factor in the development of neuronal systems and in determining brain sexual differentiation.

Callard *et al.* (1978) were unable to detect aromatase activity in any region of the brain of the adult American opossum (*D. virginiana*), leading them to speculate that it is unlikely that aromatization mediates androgen action in the brain of adult marsupials. Callard *et al.* (1982) investigated aromatase activity in the brains, ovaries, and adrenals of two Australian marsupials (the tammar wallaby *M. eugenii* and the brush-tailed possum *T. vulpecula*). They incubated tissue samples with [7^3-H]androstendione and an $NADH_2$–$NADPH_2$-generating system to isolate estrogen products but were unable to detect these in the adult brain. However, they did identify aromatase activity in the brains (principally in the POA, hypothalamus, septum, and amygdala) of male tammar wallabies between 20 and 25 days of age.

Fadem *et al.* (1993) examined aromatase activity in the brains of *M. domestica* pups and adults. Up to 8 days, whole brains from both sexes were pooled; at 16, 30, 60, and 84 days and in the adult, the hypothalamus and POA were separated, as was the cerebral cortex, from the remainder of the brain. Aromatase activity was detected in the brains of newborn opossums and showed a fourfold increase over the first 2 weeks of life. At 16 days aromatase activity in the hypothalamus–POA was significantly higher than at all other ages examined, including the adults, and there was a significant age by sex relationship as well. Overall, the tissue from males showed higher aromatase activity than did that from females, but this only reached significance in 16-day-old pups and in the adults. Moreover, it was only in

the adult that significant sex differences were seen in the aromatase activity of tissue removed from the cerebral cortex. Castration of adult males at least 7 days prior to measurement of aromatase activity caused its levels to decrease by almost 50% in the cerebral cortex, but this remained virtually unaltered in the hypothalamus–POA. Fadem *et al.* (1993) believe that the higher levels of aromatase activity observed in the hypothalamus–POA of male *Monodelphis* pups at approximately 16 days might indicate that the aromatization of androgen to estradiol, as in eutherians, is involved in masculinization of the brain at this time. Moreover, the presence of aromatase activity in the cerebral cortex over an extended period, along with high levels of circulating estradiol, led Fadem *et al.* to speculate that this estrogen is important not only in sexual differentiation but also for stimulating and supporting brain growth and development in both sexes.

IV. Factors Involved in Brain Sexual Differentiation

A. Brain Monoamines in Sexual Differentiation and the Control of Reproductive Behavior

The catecholaminergic and serotoninergic systems play important roles in the control and regulation of many brain functions. There is substantial evidence to indicate that the neurotransmitters of these pathways—norepinephrine, epinephrine, dopamine, and serotonin (5-HT)—exert a vital influence on the regulation of the hypothalamic stimulatory and inhibitory hormones both in fetal and in adult life, and that androgens acting on these systems during the critical period can have generalized and long-lasting effects. There are strong indications that 5-HT is an important developmental signal in the immature brain (Lauder, 1990). Moreover, there is considerable overlap in brain areas involved in the control of sexual behavior between steroid binding sites and the monoaminergic systems. The monoamines are also known to have direct behavioral effects. As reported by Fabre-Nys (1998), dopamine modulates the expression of sexual behavior mainly through an action on the D_2 receptor. It stimulates sexual behavior in males, but both facilitatory and inhibitory effects of dopamine on female sexual behavior have been reported (Melis and Argiolas, 1995). Norepinephrine exerts a predominantly stimulatory effect on sexual behavior acting through α_1 receptors involved in attention mechanisms and sensory processing (Crowley *et al.,* 1989). In contrast, α_2 receptor activation inhibits male sexual behavior (Pfaus and Everitt, 1995). Increased levels of 5-HT have been shown to reduce sexual behavior in both males and females (Fabre-Nys, 1998).

Research that we carried out on the rat demonstrated that androgens, acting perinatally, affect both the catecholaminergic and the serotoninergic systems within the various brain regions (Siddiqui and Gilmore, 1988; Siddiqui *et al.,* 1989). Some of the changes observed to take place are of late onset, occurring long after puberty.

Furthermore, not only those regions of the brain directly involved with the regulation of gonadotropin release or sex-specific behavior are altered by exposure to androgens, but many other areas as well. Other research in our laboratory has indicated that a reduction in 5-HT levels in male hamsters shortly after the period for brain sexual differentiation increases the amount of female sexual behavior in adulthood (Johnston *et al.,* 1990). This indicates that 5-HT may be modulating a neural substrate already differentiated by androgens during the perinatal period. In male hamsters, we have shown that perinatal exposure to a long-acting form of morphine increases the amount of both male and female reproductive behavior in adulthood (Johnston *et al.* 1990, 1992). This suggests that the defeminization process had been affected, possibly by the opiate administration delaying and inhibiting neuronal growth and synaptogenesis. Djavadian *et al.* (1999) examined the distribution of the 5-HT_{1A} receptor subtype in the brain of four adult opossums (*M. domestica*). In general, the distribution and density of this frequently expressed and functionally important receptor were found to be very similar to those already described for the rat and monkey. Observations by Stevenson *et al.* (1991) on tissue removed from the hypothalamus and cerebral cortex of *M. domestica* indicated that in juveniles 5-HT levels are higher in the hypothalamus of males aged 60–80 days than in females of the same age. No sex differences were recorded in the levels of epinephrine and norepinephrine at these times.

B. Roles of Neuropeptides in Sexual Differentiation

Sex differences have been identified in the pattern of immunoreactivity of several neuropeptides in different regions of the brain in rodents (Cooke *et al.,* 1998). These neuropeptides, which are involved in the control of social and sexual behavior, include substance P, cholecystokinin (CCK), and arginine vasopressin (AVP). It is likely that the gonadal steroids play a role in the regulation of the expression of these neuropeptides (Iqbal *et al.,* 1995). Substance P staining in the posterodorsal portion of the rat medial amygdala is twice as great in males as in females, with this difference dependent on circulating androgen levels (Malsbury and McKay, 1994). More CCK immunoreactivity is also present in the posterodorsal portion of the medial amygdala of male rats than in their female counterparts (Micevych *et al.,* 1987). Likewise, AVP immunoreactivity is greater in the posterodorsal portion of the medial amygdala and medial posterior portion of the BNST in male than in female rats (De Vries *et al.,* 1984).

1. Cholecystokinin

The neuropeptide CCK is widely distributed throughout the CNS in mammals from early in development. Although in the adult it is known to stimulate gonadotropin secretion and increase sexual behavior, its role in the developing brain is unclear. Like the situation in rodents, the opossum, *M. domestica,* exhibits a sex difference

in the localization of CCK immunoreactive structures in the MPOA, with more CCK immunoreactive fibers being present in the periventricular POA in the male than in the female (Fox *et al.,* 1990).

Fox *et al.,* (1991a) investigated the ontogeny of CCK-like immunoreactivity in *M. domestica* and identified CCK immunoreactive cell bodies in the cortex, hippocampus, hypothalamus, thalamus, midbrain, and brain stem of the adult. They found that the general distribution of CCK immunoreactive cells and fibers was similar to that reported for the rat, with the cerebellum being the only major brain region not to contain CCK immunoreactive fibers. Within the hypothalamus intensely staining cell bodies were seen in the periventricular POA and in the SCN, but not in the MPOA or in any other nucleus. The MPOA of male opossums was found to contain a dense collection of CCK immunoreactive fibers close to the third ventricle, whereas the density was lower in females. In the anterior hypothalamus high densities of CCK immunoreactive fibers were present in the SCN and periventricular nucleus. In the posterior hypothalamus these were seen in the ventral parts of the DMN and the dorsal half of the ARN. CCK immunoreactive fibers were also observed in the median MPOA, the lateral hypothalamus, the zona incerta (ZI), and the dorsal and ventral PMN.

Interestingly, Fox *et al.,* (1991a) found that CCK immunoreactive perikarya were present in the MPOA of male opossums, although only from 25 to 35 days of age; at no time were they observed in the MPOA of females. Many cells in the SCN expressed CCK immunoreactivity in animals aged 2 and 6 months, respectively, and in the dorsal PMN (situated in the caudal hypothalamus), but just at 35 days of age. No other regions of the developing opossum hypothalamus contained CCK immunoreactive cells. The first appearance of CCK immunoreactive fibers in many areas of the hypothalamus was at 35 days of age, and these were maintained to adulthood. However, CCK immunoreactive fibers were identified in both the ZI and MPOA of male opossums aged 15 and 25 days, respectively, but only at 35 days were such cells seen in the SCN; CCK immunoreactive fibers were not found in other areas of the CNS until the animals were 2 months old. The developmental pattern of CCK immunoreactive fibers in the MPOA of young male and female opossums also differed. It was not until female opossums were 35 days of age that such fibers were identifiable. Although the fibers increased in both sexes to adulthood, there were always more present in males.

Fox *et al.* (1991a) argue that the ontogeny of CCK immunoreactivity in the MPOA of *Monodelphis* means that it is unlikely that the sex difference in CCK immunoreactive fibers results from a disparity in the number of cells capable of synthesizing CCK. If this were so, one would anticipate the identification of CCK immunoreactive fibers at the same time points during development with simply fewer fibers being present in the female. The fact that CCK immunoreactive cells and fibers appear at different ages in male and female opossums indicates that the expression of CCK is differentially regulated in each sex. Fox *et al.* (1991b) and Handa *et al.* (1991) demonstrated that estrogen receptors are in the right place

at the right time during brain development in *Monodelphis* to enable them to regulate CCK expression. Fox *et al.* (1991b) detected the presence of estrogen receptor immunoreactivity within the MPOA of *Monodelphis* pups by 15 days of age, indicating that the receptive machinery is already in place from that time for CCK to respond to any sex differences in estrogen levels. As it is another 10 days before CCK-immunoreactive cells and fibers are identifiable in the male MPOA, Fox *et al.* (1991a) speculate that estrogen may regulate CCK expression from its onset, although they do not see a role for CCK in early developmental events in the MPOA or in the SCN.

2. Arginine Vasopressin and Oxytocin

Both AVP and the closely related oxytocin are nonopeptides principally synthesized in neurosecretory cells in the paraventricular nucleus (PVN) and SON. Axons from these neurons project to the limbic structures, the ME, the posterior pituitary, the midbrain, the brain stem, and the spinal cord; cells and fibers with AVP-like and oxytocin-like immunoreactivity have also been demonstrated in other parts of the brain, including the SCN of the hypothalamus (Iqbal and Jacobson, 1995a,b). Oxytocin and AVP are transported via modified axons to the posterior pituitary, from which they are released into the bloodstream. Both neuropeptides have major roles in many centrally regulated functions, including sexual and social behavior (Bluth and Dentzer, 1993; Wang *et al.,* 1993; Argiolas and Gessa, 1991).

Iqbal and Jacobson (1995a) investigated the ontogeny of AVP-like immunoreactivity and of AVP binding sites in the brain of the opossum (*M. domestica*). AVP-like immunoreactive fibers are widely distributed throughout the brain, extending from the olfactory bulbs to the spinomedullary junction. In the adult the distribution of AVP-like immunoreactivity is generally similar to that reported for many other mammalian species including the rat. Cells containing ADH-like immunoreactivity are located in the lateral and medial POA, the PVN, the SON and the lateral hypothalamic area. Interestingly, AVP-like immunoreactive cell bodies have also been detected in an area around the substantia nigra and dorsal raphe nucleus, where they have not been identified in other mammals. The medial amygdala and locus coeruleus of the adult opossum brain are devoid of AVP-like immunoreactive cell bodies, whereas in the rat they are present in these regions. A sex difference in the density of AVP-like immunoreactive fibers in the lateral septum and lateral habenula of the adult rat has been reported by De Vries and Buijs (1983). AVP-expressing neurons in the BNST of the rat have been found to provide a greater density of immunoreactive fibers to the lateral septum of the male than to that of the female (De Vries and Buijs, 1983; De Vries *et al.,* 1984; Wang *et al.,* 1993). Iqbal and Jacobson (1995a) found a similar situation regarding the density of AVP-like immunoreactive fibers in the lateral septum of the opossum, although not in the lateral habenula. These differences appeared at Day 60, well before puberty but after the appearance of androgen receptors.

When Iqbal *et al.,* (1995) examined the condition existing in opossum pups ranging from Embryonic Day 12 to Day 60 of life, they found that neurogenesis in the SON and PVN was completed by Postnatal Days 5 and 7 respectively, and only slightly later in other regions. However, this was preceded by the appearance of AVP-like immunoreactivity in the forming mesencephalon and diencephalon as early as Embryonic Days 12 and 13. Moreover, even before neurogenesis was completed in the SON and PVN, cell bodies containing AVP-like immunoreactivity were located in these regions on Postnatal Days 1 and 3, respectively. This occurs in a developmental period earlier than that in the rat. The distribution pattern of AVP-like immunoreactivity resembled that in the adult by Postnatal Day 60. The findings led the authors to suggest that the very early appearance of AVP-like immunoreactivity in the *Monodelphis* brain, before neurogenesis is complete, may indicate that this neuropetide is involved in morphogenesis of the CNS.

Kuehl-Kovarik *et al.* (1997), who utilized autoradiography to examine [^{3}H]AVP binding sites in the CNS of young and adult opossums (*M. domestica*), found that these resembled those seen in other mammals but were few in number in the developing brain. Some of the binding sites, including those in the cerebellum, appear to be unique to opossums. At birth only the ventral tegmental area, along with the solitary tract, was labeled and [^{3}H]AVP binding sites in the forebrain did not appear until the animals reached 25 days of age. Binding remained at low levels in other parts of the brain until the opossums were 35 days old. Interestingly, and contrary to expectations, [^{3}H]AVP binding sites appeared much later than AVP immunoreactivity, often after neurogenesis was complete. Moreover, at birth only the ventral tegmental area, along with the solitary tract, was labeled. No sex differences in [^{3}H]AVP binding were detected in the brains at any age. Dubois-Dauphin *et al.* (1990) also reported that [^{3}H]AVP binding in adult golden hamsters is not sexually dimorphic. Kuehl-Kovarik *et al.* (1997) concluded that although AVP appears early in life, the binding sites do not play a developmental role in these animals.

Iqbal and Jacobson (1995b) examined the ontogeny of oxytocin-like immunoreactivity in *M. domestica*. Its appearance in the hypothalamic SON and PVN occurred slightly later than did immunoreactivity for AVP. These authors failed to find sex differences for oxytocin-like immunoreactivity in any brain region of either developing or adult opossums, but they reported a general similarity in the distribution of this immunoreactivity in the opossum brain to that found in other mammals.

V. Sex Differences in the CNS Affecting Reproductive Behavior

Adult vertebrates tend to exhibit sex differences in the degree of certain types of behavior, including that of courtship, copulation, aggression, and parenting. As

mentioned earlier, in eutherians sex-specific behavior can frequently be linked to circulating gonadal steroid hormones acting on specific areas of the brain sexually differentiated prenatally or shortly following birth.

More than 30 years ago, Roberts *et al.* (1967) investigated hypothalamic mechanisms regulating sexual and aggressive behavior in the opossum *D. virginiana.* They reported dramatically complete sequences of male mating behavior in both sexes following electrical stimulation of the hypothalamus and POA. This behavior included mounting, rubbing, biting, clicking, and, in the males, penile erection. However, it only occurred in the presence of another animal and was thus interpreted as an indicator of a reduction of tonic inhibition in specialized sensory motor mechanisms located outside the hypothalamus and POA. These findings led Roberts *et al.* to suggest that a relatively complete male response mechanism exists in the brains of both sexes in this marsupial but is more easily elicited in the male. They further concluded that androgens, acting early in the life of *Didelphis,* rather than being essential for the development of most of the complex aspects of sexual behavior that take place, must exert their critical influence on more subtle aspects, such as the sensitivity of the mechanisms to hormones or on the development of neural input. It has also been argued by Rudd *et al.* (1996) from work on the tammar wallaby that, unlike the situation in eutherians, androgens acting early in life have no long-term organizational effects on the neural pathways controlling male-type sexual behavior in marsupials. These authors believe that such behavior is completely dependent on the adult steroid hormone environment because of the similarity in the behavioral responses of both sexes in the presence or absence of testosterone. However, this viewpoint is not universally accepted.

Fadem (1995a) examined sex differences in aggressive behavior in *M. domestica* and found that the types of aggression seen were similar to those recorded in other species of marsupials. Clear sex differences were present and both hormonal and pheromonal factors appeared to be involved in mediating such behavior. In the same species, Fadem (1990) also explored the effects of the gonadal hormones on scent marking in which clear sex differences are normally apparent; intact females rarely scent mark. In ovariectomized females administration of testosterone stimulated chest marking and both exogenous testosterone and estradiol administration induced head marking. The presence of males inhibited scent marking of either type. It was not apparent, however, whether the sex differences in behavior involved sexually dimorphic pathways in the CNS.

Fadem (1995b, 2000, 2001) investigated the effects of treatment with the antiestrogen tamoxifen on the organization of sexually dimorphic behavior in *Monodelphis*. In the earliest study neonatal opossums were injected with tamoxifen, gonadectomized, and as adults, after receiving silastic implants of estradiol and then testosterone, tested for scent-marking, aggressive, and precopulatory behavior. The findings suggest that in adults of this species early exposure to tamoxifen defeminizes aggressive behavior in females and reduces masculine-typical behavior in males but does not affect the display of testosterone-activated behavior. Furthermore, such treatment was observed to increase the activity level

following administration of estradiol in adulthood and to increase body weight. Because the exposure to tamoxifen took place during a time when high levels of aromatase activity had been recorded in the hypothalamus–POA (Fadem *et al.,* 1993), the authors speculated that the decreased levels of estradiol resulting from tamoxifen administration could have accounted for the effects observed. In a recent study (Fadem, 2000), opossum pups of both sexes were treated with estradiol or the estrogen receptor antagonist tamoxifen on Days 4 and 8 postnatally. Following gonadectomy at 10 weeks and the administration of testosterone in adulthood, intact males showed more scent-marking behavior than did females, and the estrogen-treated animals exhibited even more scent-marking behavior than either the controls or the tamoxifen-treated opossums. Estrogen-treated animals of both sexes also showed less female-typical screeching threat behavior than did the controls or those treated with estradiol. Findings from both these studies led Fadem to speculate that, as in eutherians, exposure of marsupials to estrogens during a critical period in development may have behavioral masculinizing effects. The research lends further support to the notion that at least in some marsupials as in eutherians, the brain does become sexually differentiated during a critical period early in development following exposure to androgens and their subsequent intraneuronal conversion to estrogens. Fadem (2001) concluded that gonadal hormones probably act on the brain of *Monodelphis* over an extended period to organize sexually dimorphic behavior.

The findings of another study by Fadem and Erianne (1997), however, seem to play down the early organization of sexual behavior, Such behavior was examined in opossums (*M. domestica*) gonadectomized prepubertally and treated with estradiol and progesterone when adults. Experimental animals of both sexes permitted penile intromission by intact males. These results may indicate that male opossums do not undergo receptive defeminization early in life as do the males of most nonprimate mammals. Fadem and Erianne postulate that one explanation for the behavioral similarities between the sexes could be that the males receive little prenatal exposure to the defeminizing effects of either testicular androgens or maternal hormones.

The original claim by Rudd *et al.* (1996) that androgens are not involved in brain sexual differentiation in marsupials has been thrown into question by recent findings from the same group. Rudd *et al.* (1999) demonstrated a clear dimorphism in the ability of the male and female tammar wallaby to produce an LH surge in response to estradiol. They found that a sharp increase in plasma LH occurred between 15 and 28 h after an injection of estradiol benzoate in 13 of 16 intact females but in none of 11 intact males. When males were castrated early in pouch life (24–26 days of age), as adults they showed pronounced surges of LH in response to estradiol. Males castrated at 14 months showed similar responses to intact males. These results indicate that testicular androgens, acting early during life, exert an irreversible inhibitory effect on the estradiol–LH positive feedback system in the tammar, as they do in most nonprimate eutherians studied.

Research by Hinds *et al.* (1990), Shaw (1990), Gemmel *et al.* (1991), Rose and MacFadyen (1997), and Smith *et al.* (2001) indicates that the behavioral mechanisms controlling parturition in several Australian marsupials are not sexually differentiated. This is clear because the behavior associated with parturition can easily be induced in adult males, nulliparous females, and even pouch young by administering prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or, in certain instances, oxytocin, which appears to act by stimulating prostaglandin release. The species studied include *M. eugenii, T. vulpecula,* the bettong (*Bettongia gaimardi*), and the bandicoots (*Isoodon obesulus* and *Perameles gunnii*). Why do male marsupials respond to prostaglandin and oxytocin by exhibiting birth behavior (associated with the female)? Smith *et al.* (2001) suggest that such behavior may be "hard-wired" in the brain and not easily altered by natural selection. Alternatively, they postulate that the gene(s) responsible is perhaps closely associated with other genes essential for both sexes. Interestingly, Rose and Fadem (2000) reported that in *Monodelphis* birth behavior cannot be elicited in the male by oxytocin, although it can be induced in the female by this hormone and in both sexes by an injection of $PGF_{2\alpha}$. Could this sex difference in the response of *Monodelphis* to oxytocin possibly be due to long-term organizational effects of the gonadal hormones on areas of the brain regulating behavior?

VI. Concluding Remarks

Few sexually dimorphic structures have been identified in the marsupial CNS; this is at least partly due to the relative paucity of research on this topic. However, marsupials are eminently suitable animal models for research into organogenesis because of their immaturity at birth and the accessibility of the newborn to experimental manipulation. This is especially true for the opossum, *M. domestica,* which lacks a pouch, breeds readily in captivity, and gives birth to several young after a gestation of only 14 days. Because the marsupial CNS is very underdeveloped in the neonate, it readily lends itself to detailed investigations of the morphological and physiological changes that occur as it grows and matures (Saunders, 1997; Saunders *et al.,* 1989).

Is the male marsupial CNS, like that in eutherians, irreversibly altered by androgens acting on it during a limited critical period during development? It appears that the jury is still out considering the evidence, with no final verdict having yet been reached. However, the availability and application of new techniques, particularly in the field of molecular biology, will no doubt reveal further structural sex differences in the marsupial CNS. It would indeed be most surprising if the situation in marsupials regarding masculinization of the CNS was fundamentally different from that in eutherians. Certainly, recent papers, including those by Rudd *et al.* (1999) and Fadem and colleagues, suggest that gonadal steroids do act well

prior to puberty to irreversibly alter the pattern of gonadotropin release and at least certain aspects of reproductive behavior in marsupials.

The presence of binding sites for both androgens and estrogens on neurons in the marsupial CNS from early in development undoubtedly indicates that targets exist for possible action by the gonadal (or possibly even the adrenal) steroids. Finally, it is extremely important to emphasize that a marsupial such as *M. domestica* may eventually prove an excellent model for studying how environmental contaminants that mimic the action of the gonadal steroids affect neurogenesis, functional neuronal development, and adult sexual behavior. It is hoped that some exciting advances in this field will occur during the next decade, taking advantage of the unique opportunities offered by the peculiarities of marsupial reproduction.

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Signaling to Gene Activation and Cell Death by Tumor Necrosis Factor Receptors and Fas

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Tumor necrosis factor (TNF) receptors and Fas elicit a wide range of biological responses, including cell death, cell proliferation, inflammation, and differentiation. The pleiotropic character of these receptors is reflected at the level of signal transduction. The cytotoxic effects of TNF and Fas result from the activation of an apoptotic/necrotic program. On the other hand, TNF receptors, and under certain conditions also Fas, exert a proinflammatory function that results from the induction of several genes. In this context, the transcription factor nuclear factor-kappa B (NF-κB) plays an important role. NF-κB is also important for the induction of several antiapoptotic genes, which explains at least partially why several cell types can only be killed by TNF in the presence of transcription or translation inhibitors. It is the balance between proapoptotic and antiapoptotic pathways that determines whether a cell will finally die or proliferate. A third signal transduction pathway that is activated in response to TNF is the mitogen-activated protein kinase cascade, which plays an important role in the modulation of transcriptional gene activation.

KEY WORDS: TNF, Fas, NF-κB, MAP kinases, Caspases, Apoptosis, Necrosis, Inflammation, Death receptors

I. Introduction

A. Tumor Necrosis Factor

Tumor Necrosis Factor (TNF) was identified as a serum factor that is responsible for the hemorrhagic necrosis of tumors that could be observed in patients following

acute bacterial infections (Carswell *et al.,* 1975). This serum factor was found to be cytotoxic to tumor cells in culture, but its molecular identity was not clarified until a decade later (Aggarwal *et al.,* 1985). Although originally identified because of its antitumor activity, the pluripotent activity of TNF led to its independent discovery as a protein that was responsible for endotoxin-induced cachexia in mice (Beutler *et al.,* 1985). Since its initial isolation, TNF has been determined to be a cytokine with very diverse biological activities, including direct cytotoxicity for cancer cells, differentiation, growth stimulation, and antiviral and proinflammatory effects. This might explain its role in various physiological and pathological phenomena, such as infection, inflammation, cancer, cachexia, lethal septic shock, and autoimmune diseases including Crohn's disease and rheumatoid arthritis (Kollias *et al.,* 1999). The main physiological role of TNF is undoubtedly activation of the first-line reaction of an organism to microbial, parasitic, viral, or mechanical stress. The activity of TNF is tightly regulated at the levels of secretion and receptor expression. Additional regulatory mechanisms are provided by concomitant actions of different cytokines and the presence in biological fluids of specific inhibitory proteins, especially soluble cytokine receptors. Abnormalities in the production of these substances and/or overreaction of the host might contribute to the pathophysiology of immune and neoplastic diseases.

Originally, it was believed that TNF was produced by monocytes and macrophages only. Now, it seems that, at least *in vitro,* many cell types can produce TNF after an appropriate stimulus (Sidhu and Bollon, 1993). In addition to lipopolysaccharide (LPS), which represents the main stimulus, virus, fungal, and parasital antigens, enterotoxin, mycobacterial cord factor, C5a anaphylatoxin, immune complexes, interleukin (IL)-1, IL-2, and, in an autocrine manner, TNF may trigger the synthesis of TNF. In macrophages, TNF transcription has been shown to be dependent on nuclear factor-kappa B(NF-κB) activation (Foxwell *et al.,* 1998). The ability of most cells to produce TNF is further regulated by the 3′ AU-rich elements (ARE) region of the gene. The ARE is common to many cytokine mRNAs and is bound by tristetraprolin (TTP), a zinc finger-containing protein that accelerates the turnover of ARE-containing mRNAs (Carballo *et al.,* 1998). TTP is induced by TNF as a negative feedback loop that limits TNF activity. Mice lacking TTP develop spontaneous, chronic, TNF-induced inflammation. Transgenic mice containing TNF genes with a deleted ARE sequence produce increased levels of TNF and also develop an inflammatory phenotype (Kontoyiannis *et al.,* 1999).

TNF is expressed as a 26-kDa integral transmembrane precursor protein from which a 17-kDa mature TNF protein is released into the medium by proteolytic cleavage of the 76-amino acid signal peptide by the membrane-bound matrix metalloproteinase TNF-α converting enzyme (TACE), whose regulation is obscure (Pennica *et al.,* 1984; Kriegler *et al.,* 1988; Blobel, 1997; Black and White, 1998). Not only secreted TNF but also the membrane-bound form are biologically active (Perez *et al.,* 1990). Membrane insertion may be an effective way to keep the action of TNF restricted to specific locations. There are reports that the transmembrane

form of TNF binds preferentially to the TNF receptor (TNF-R) 2 and is able to kill target cells by cell-to-cell contact (Grell *et al.,* 1995). However, we have found that membrane-bound TNF also efficiently triggers TNF-R1 (Decoster *et al.,* 1995). Therefore, we think that the apparent specificity of membrane-bound TNF for TNF-R2 reflects the relative abundance of this receptor on the target cells. Interestingly, evidence is accumulating that the intracellular part of TNF also has a signaling function. In this context, reverse signaling by TNF has been shown to generate a negative signal by which cells become resistant to LPS (Eissner *et al.,* 2000).

Originally, the unique ability of TNF to kill specifically tumor cells held promise in the treatment of cancer. However, the use of systemic TNF treatment has been hampered by severe toxicity (fever, hypertension, weight loss, and septic shock), as well as the resistance of various tumor cells to TNF. This is mainly due to the gene-activating properties of TNF, which include several proinflammatory proteins and antiapoptotic proteins, respectively. Perhaps the most encouraging studies to date show that high doses of TNF along with chemotherapy and interferon-γ can be safely administered regionally through isolated limb perfusion. This procedure produced up to 80% complete remission in cases of melanoma and soft tissue sarcomas (Lejeune *et al.,* 1998).

Since TNF is also an important inflammatory disease mediator, there has been much interest in strategies that interfere with this activity of TNF. Recently, TNF inhibitors have emerged as a new treatment option for rheumatoid arthritis and inflammatory bowel disease. Currently, two TNF inhibitors are available for clinical use—etanercept and infliximab. Etanercept is a soluble TNF-R:Fc fusion protein that competes with the endogenous TNF receptors for TNF binding. Infliximab is a chimeric anti-TNF monoclonal antibody, which also binds with high affinity to soluble TNF (Feldmann and Maini, 2001).

B. Fas Ligand

Fas ligand (FasL) was discovered on the cell surface of cytotoxic T lymphocytes (CTLs) as a protein that kills Fas-bearing cells (Rouvier *et al.,* 1993), but evidence for the biological role of FasL derived from the finding that the coding gene is mutated in mice suffering from a severe autoimmune disease, generalized lymphoproliferative disease (*gld*). These mice develop lymphadenopathy and splenomegaly by accumulating $CD4^{-}CD8^{-}$ T cells. The defect arises from a point mutation in the FasL gene preventing its ability to bind to the Fas receptor (Takahashi *et al.,* 1994). A similar defect is seen in *lpr* (lymphoproliferation) mice which lack a functional Fas receptor caused by an aberrant transcription of the gene (Watanabe-Fukunaga *et al.,* 1992a; Adachi *et al.,* 1993). Mature T cells from *lpr* and *gld* mice do not die after activation by Fas, and they accumulate in the lymph nodes and the spleen, indicating that the FasL/Fas system is involved in downregulation of the immune response (Nagata and Golstein, 1995; Nagata, 1997). Defects

in the FasL/Fas system have been described in children with autoimmune lymphoproliferative syndrome or Canale–Smith syndrome and exhibit lymphadenopathy, hepatosplenomegaly, and other manifestations of systemic autoimmunity (Fisher *et al.,* 1995; Rieux-Laucat *et al.,* 1995; Nagata, 1998, 1999).

FasL is predominantly expressed in natural killer (NK) cells and activated T lymphocytes, in which it is involved in activation-induced suicide of T cells to downregulate the immune reaction. Mature T lymphocytes are activated by T cell receptor interaction with antigen-presenting cells. The activated T cells express FasL, which binds to the Fas-expressing activated T cells to induce apoptosis (Nagata and Golstein, 1995; Nagata, 1997). Together with perforins and granzymes, FasL is the major actor in CTL-mediated cytotoxicity (Nagata and Golstein, 1995; Nagata, 1997) by binding to Fas on the target cells, such as virus-infected cells or cancerous cells, and inducing apoptosis by activating caspases. FasL is also constitutively expressed in immune privileged tissues such as testis and eye, which do not tolerate an inflammatory response upon infection. The mechanism by which these organs protect themselves against harmful immune reaction consists of immediate killing of inflammatory cells by FasL (Bellgrau *et al.,* 1995; Griffith *et al.,* 1995). This suggests the therapeutic use of FasL as an immunosuppressive agent to inhibit graft rejection. Interestingly, some tumor cells have usurped the FasL-mediated killing to escape the immune surveillance system by inducing Fas-mediated apoptosis in attacking CTL and NK cells (Hahne *et al.,* 1996; Strand *et al.,* 1996).

FasL is synthesized as a type II transmembrane protein consisting of a C-terminal extracellular region of 179 amino acids and an N-terminal cytoplasmic part of 80 amino acids (Suda *et al.,* 1993; Takahashi *et al.,* 1994). N-glycosylation seems to be essential for expression and secretion of FasL on the cell surface (Schneider *et al.,* 1997; Tanaka *et al.,* 1997). Membrane-bound FasL can be processed by a metalloproteinase to generate a soluble cytokine (Tanaka *et al.,* 1996; Schneider *et al.,* 1998). Soluble FasL exist as trimers (Tanaka *et al.,* 1997), similar as TNF molecules. Whereas soluble human FasL is functional (Tanaka *et al.,* 1995), murine FasL rapidly loses its apoptotic activity when processed from the membrane-bound form (Suda *et al.,* 1997; Schneider *et al.,* 1998). However, active soluble FasL can be obtained by cross-linking monomers with an immobilized monoclonal antibody (Schneider *et al.,* 1998) or by fusing the ligand with a leucine zipper motif which facilitates and stabilizes oligomerization (Walczak *et al.,* 1999). The results in mice may indicate that FasL preferentially acts in a juxtracrine context, and that shedded FasL may attenuate the response and therefore downregulate the potential harmful apoptotic activity. Recently, extracellular matrix proteins have been shown to interact with soluble FasL and potentiate its proapoptotic activity (Aoki *et al.,* 2001). Extracellular matrix likely enhances the biological activity of soluble FasL through its ability to retain and increase its local concentration.

Administration of an agonistic anti-Fas antibody kills lymphomas growing in nude mice (Trauth *et al.,* 1989), indicating a possible use in cancer therapy. However, as for TNF, application of Fas-induced apoptosis in cancer therapy was

not successful due to its high cytotoxic effect, especially on the liver. This suggests that a method of delivering FasL specifically at the tumor site should be developed. No clinical trials with FasL or the antibody have been reported (Nagata, 2000).

II. TNF Receptors, Fas, and Associated Proteins

A. TNF Receptors

Receptors for TNF are present on nearly all cell types, except for erythrocytes and unstimulated lymphocytes. Two receptors have been cloned which differ in size and in binding affinity. Based on the molecular weight of the proteins, they are referred to as p55 (TNF-R1, TNFRSF1A, or CD120a) and p75 (TNF-R2, TNFRSF1B, or CD120b) (Vandenabeele *et al.,* 1995; Locksley *et al.,* 2001). The binding constant is about 2×10^{-10} M and the number of receptors varies from about 200 to 10,000 per cell. Of considerable interest is the fact that human TNF does not bind to murine p75, although murine TNF binds to both human receptors (Lewis *et al.,* 1991). The two TNF-R types consist of an extracellular domain, which binds TNF and is homologous for 28%; a transmembrane region; and an intracellular part, which is totally different in both receptors. Each extracellular domain contains four conserved, cysteine-rich repeats about 38 to 42 amino acids in length. Based on similarities in their extracellular domains, these receptors belong to a receptor superfamily including the low-affinity nerve growth factor receptor, the Fas antigen, death receptor (DR)-3, DR4, DR5, DR6, the human B-lymphocyte activation molecule CD40, and the OX40 antigen found on activated T cells (Locksley *et al.,* 2001). The intracellular part of p55 can be divided into two domains: a membrane-proximal and a membrane-distal (i.e., C-terminal) domain. The latter is largely homologous to the intracellular domain of Fas, DR3, DR4, DR5, and DR6, all of which are implicated in cell death. Therefore, this domain has been referred to as the "death domain" (DD) (Ashkenazi and Dixit, 1998; Tartaglia *et al.,* 1993b). The membrane-proximal domain as well as the DD of p55 are important for nitric oxide (NO) synthase induction and induction of mitochondrial motility (De Vos *et al.,* 1998), but the DD is sufficient for cytotoxicity and NF-κB activation. Furthermore, TNF-induced activation of two distinct types of sphingomyelinase (SMase), a membrane-associated neutral SMase and an endosomal acidic SMase, is mediated by the membrane-proximal domain and the membrane-distal (death) domain of p55, respectively (Wiegmann *et al.,* 1994). The p75 receptor has no specific domains but is characterized by a high content of Ser residues. Its C-terminal part is involved in binding of specific TNF receptor associated factors (TRAFs), which also mediate signaling by CD30, CD40, OX40, RANK, and a few other receptors (Rothe *et al.,* 1994; Arch *et al.,* 1998). The remarkable absence of homology between the intracellular regions of both TNF receptors suggests that they

are involved in different functions or signal-transducing pathways. Most of the TNF responses known (NF-κB activation, cytotoxicity, IL-6 induction, and fibroblast proliferation) occur by activation of p55 (Engelmann *et al.,* 1990; Thoma *et al.,* 1990). However, TNF activities on thymocytes and circulating T lymphocytes seem to be p75 mediated (Tartaglia *et al.,* 1991; Vandenabeele *et al.,* 1992). Interestingly, p75 signaling in T cells is dramatically affected by the intracellular mediator receptor interacting protein (RIP), which is required for NF-κB activation by p55. In the presence of RIP, p75 triggers cell death, whereas in the absence of RIP p75 activates NF-κB (Pimentel-Muinos and Seed, 1999). These results demonstrate that the signaling output of p75 triggering can be shaped by specific intracellular constraints. Apart from its role in T cells, a contribution of p75 was also demonstrated in TNF-induced killing of tumor cells (Grell *et al.,* 1993; Bigda *et al.,* 1994), in endothelial and neutrophil functions (Barbara *et al.,* 1994; Dri *et al.,* 1999), in the expression of NO and inducible NO synthase by macrophages (Riches *et al.,* 1998), in inhibition of early hematopoiesis (Jacobsen *et al.,* 1994), and in transmembrane TNF-mediated arthritis (Alexopoulou *et al.,* 1997). In all these cases, simultaneous triggering of p55 and p75 TNF receptors leads to a cooperative effect. Several mechanisms of cooperation have been proposed: (i) ligand passing, in which the fivefold higher affinity and fast dissociation rate of p75 allow the presentation of TNF to neighboring p55 molecules (Tartaglia *et al.,* 1993a; Dri *et al.,* 1999); (ii) p75-mediated signaling events (Declercq *et al.,* 1998; Riches *et al.,* 1998; Chan and Lenardo, 2000); (iii) p75-induced production of endogenous TNF and autotropic or paratropic activation of p55 (Grell *et al.,* 1999; Pelagi *et al.,* 2000); and (iv) TNF-induced formation of p55/p75 heteroduplexes (Pinckard *et al.,* 1997). It has also been suggested that p75 is unique in mediating signaling initiated by the transmembrane form of TNF (Grell *et al.,* 1995).

Mice in which the p55 or the p75 TNF receptor is deleted have been described (Rothe *et al.,* 1993; Pfeffer *et al.,* 1993; Erickson *et al.,* 1994). Deletion of the p55 receptor gene causes pronounced immunodeficiency, in which animals show enhanced susceptibility to *Listeria monocytogenes,* indicating that the p55 TNF receptor plays an important role in defense against microorganisms. However, "knockout" of p55 does not confer resistance to the lethal effect of LPS; only in galactosamine-treated animals does p55 gene deletion abrogate the sensitivity to LPS. p55 was also found to control early graft-versus-host disease (Speiser *et al.,* 1997). Deletion of the p75 gene causes a minimal phenotype, in which scab formation fails to occur in response to repeated intradermal injection of TNF, and there is modest resistance to the lethal effect of TNF. In addition, p75-deficient mice exhibit depressed Langerhans cell migration and reduced contact hypersensitivity (Wang *et al.,* 1997). It was found that production of human p75 in transgenic mice results in a severe inflammatory syndrome involving mainly the pancreas, liver, kidney, and lung, and characterized by constitutively increased NF-κB activity in the peripheral blood mononuclear cell compartment (Douni and Kollias, 1998). Studies with p55- and p75-deficient mice also revealed that both receptors differentially

regulate osteoclastogenesis (Abu-Amer *et al.,* 2000), with p55 enhancing and p75 suppressing osteoclastogenesis.

Soluble TNF-binding proteins, which were identified as extracellular domains of the TNF receptors, have been found in urine, serum, synovial fluids, and cerebral spinal fluids of patients with various diseases (Nophar *et al.,* 1990). They arise by proteolytic cleavage from surface-bound receptors (Porteu *et al.,* 1991). How these receptors are released is not fully understood. TACE, the enzyme that causes the release of TNF, is also involved in the release of the receptor (Peschon *et al.,* 1998). Receptor shedding from cell lines can be induced by protein kinase C activators, *N*-formyl-methionyl-leucyl-phenylalanine, complement fragment 5A, the calcium ionophore A23187, or by TNF (Porteu and Nathan, 1990; Porteu and Hieblot, 1994). It has been found that in a wide spectrum of diseases, such as systemic lupus erythematosus (Aderka *et al.,* 1993) and rheumatoid arthritis (Cope *et al.,* 1992), the levels of soluble TNF receptors are augmented as disease gets worse; therefore, it is a useful marker of disease activity. Soluble TNF receptors retain the ability to bind TNF and thus can limit acute TNF effects (Engelmann *et al.,* 1990). In addition, in certain conditions, these naturally occurring inhibitors might function as TNF carriers (Aderka *et al.,* 1992; Klein and Brailly, 1995). Although regulation through cleavage of TNF receptors remains a plausible but unproven regulatory mechanism, constitutional inflammation and fever in some cases of TNF receptor-associated periodic syndrome may be due to defective shedding of p55 caused by amino acid changes in its extracellular domain (Galon *et al.,* 2000). The soluble form of p75 has proven very successful for the treatment of inflammatory conditions such as arthritis and inflammatory bowel disease. Typically, fusions with immunoglobulin or leucine zipper oligomerization domains are used to achieve high-level activity of the soluble receptor. A p75 dimeric Fc fusion protein (known as Etanercept or Enbrel) has been approved for human use (Feldmann and Maini, 2001).

The TNF receptors have a half-life between 30 min and 2 h, and it has been suggested that this rapid turnover is related to the high content of so-called PEST sequences (proline, glutamic acid, serine, and threonine) in the intracellular domains. Ubiquitination may be involved in this degradation process, at least as far as p75 is concerned (Loetscher *et al.,* 1990). TNF receptors can be up- or downmodulated by several agents, including interferons, cAMP, protein kinase C modulators, and TNF (Aggarwal and Natarajan, 1996). TNF receptors are rapidly internalized and there is no recycling.

B. Fas

Fas (CD95 and APO-1) was discovered as a cell surface antigen recognized by a cytotoxic monoclonal antibody against human cell surface protein (Trauth *et al.,* 1989; Yonehara *et al.,* 1989). As already mentioned, the function of Fas was

mainly elucidated by genetic and molecular analyses of mice showing an *lpr* phenotype (Watanabe-Fukunaga *et al.,* 1992a). The Fas-null mice, established by gene targeting, also show lymphadenopathy and splenomegaly, which is even more pronounced than in mice carrying the leaky *lpr* mutation (Adachi *et al.,* 1995). This lymphoproliferative phenotype was rescued by expressing Fas as a transgene in the lymphocytes of *lpr* mice (Wu *et al.,* 1994). These findings confirm the essential role of Fas in the cell death program of T lymphocytes. Fas is ubiquitously expressed in various tissues and cells with abundant expression in the thymus, liver, kidney, and heart (Watanabe-Fukunaga *et al.,* 1992b).

Human Fas is a type I transmembrane receptor with a molecular mass of 48 kDa (Itoh *et al.,* 1991; Oehm *et al.,* 1992) that also exists in a soluble form as a result of alternative splicing (Cascino *et al.,* 1995). FasL binds to Fas with a binding constant of approximately 1×10^{-9} M. DcR3, a soluble decoy receptor for FasL, binds with the same K_d and antagonizes the function of FasL (Pitti *et al.,* 1998). Binding of FasL to Fas or cross-linking the receptor with agonistic antibodies induces apoptosis (Trauth *et al.,* 1989; Yonehara *et al.,* 1989; Itoh *et al.,* 1991). The Fas receptor also contains the extracellular cysteine-rich motif characteristic of the TNF receptor superfamily. The cytoplasmic region of the Fas receptor has little sequence similarity with other members of the receptor family, except for a typical 80-amino acid stretch that has been designated as the DD and is responsible for transducing the death signal (Itoh and Nagata, 1993; Tartaglia *et al.,* 1993b). The tertiary structure of the Fas DD revealed the presence of six antiparallel, amphipathic α helices, with many exposed charged residues probably responsible for mediating the interactions between DDs (Huang *et al.,* 1996).

C. TNF Receptor-Associated and Fas-Associated Proteins

The primary trigger for signaling is clustering of the TNF receptors, which is brought about by binding to the trimeric TNF. The main argument in support of a clustering mechanism comes from the observation that monoclonal antibodies can mimic TNF action (Engelmann *et al.,* 1990; Espevik *et al.,* 1990). The fact that a pentameric immunoglobulin M monoclonal antibody is considerably more active in mimicking TNF action compared to the bivalent immunoglobulin G is further support for a clustering mechanism. Moreover, stoichiometric binding studies (Pennica *et al.,* 1992) and three-dimensional resolution of ligand/receptor complexes which employed genetically engineered TNF receptor ectodomains (Banner *et al.,* 1993) have confirmed that trimeric TNF is indeed able to bind up to three TNF receptors. Recently, TNF receptors as well as Fas have been shown to exist as preassembled receptor complexes at the cell membrane, which is mediated by a pre-ligand activation domain that is located in the first cysteine-rich domain in the extracellular part. Triggering of these preassembled oligomers by trimeric TNF leads to a conformational change in the cytoplasmic regions of the receptor and subsequent signaling (Chan *et al.,* 2000).

Spontaneous signaling of clustered p55 receptors is blocked by the binding of silencer of death domain (SODD; BAG-4) to the DD of p55 (Jiang *et al.,* 1999; Takayama *et al.,* 1999). SODD binding prevents the recruitment of other signaling proteins to the p55 DD. TNF treatment has been shown to result in the release of SODD from the p55 and the subsequent recruitment of TNF-R associated DD (TRADD) (Fig. 1). The latter was originally identified in a yeast two-hybrid screening with the intracellular domain of p55 as a 34-kDa cytoplasmic protein containing a C-terminal DD (Hsu *et al.,* 1995, 1996a). As observed for p55, overexpression of TRADD causes apoptosis and activation of NF-κB and MAP kinases, suggesting that TRADD is critically involved in p55 signal transduction. The further discovery that the N-terminal domain of TRADD interacts with TRAF2, whereas its C-terminal DD binds to Fas associated DD (FADD) and RIP, demonstrated that TRADD functions as an adaptor to recruit other signaling proteins to p55 after TNF stimulation (Hsu *et al.,* 1996a,b).

TRAF2 was originally identified as a protein that associates with a C-terminal region in the cytoplasmic domain of p75, which is indispensable for p75-mediated signal transduction (Rothe *et al.,* 1994). TRAF2 is characterized by the presence of an N-terminal ring and zinc finger-containing domain and a C-terminal TRAF domain (Rothe *et al.,* 1994), which mediates its interaction with p75 and TRADD. Since then, six related proteins have been identified (Arch, 1998), but only TRAF1 and TRAF2 have been shown to be involved in TNF signaling. Also, two other proteins, known as cellular inhibitors of apoptosis (c-IAP1 and c-IAP2), are recruited to the TNF receptors by binding to TRAF2 (Rothe *et al.,* 1995b); however, the physiological function of their recruitment is not clear. TRAF2 has been shown to be essential for TNF-induced activation of stress-activated protein kinases (JNK and p38 kinase) and to at least partially mediate TNF-induced NF-κB activation. This was originally proposed based on the observation that transient overexpression of TRAF2 in human embroyonic kidney HEK293 cells induces activation of NF-κB and stress-activated protein kinases, whereas a dominant-negative mutant of TRAF2 lacking the ring finger domain (TRAF2-DN) was able to block TNF-induced NF-κB activation (Rothe *et al.,* 1995a). However, the role of TRAF2 in NF-κB activation seems to be quite complex since the TNF-induced nuclear translocation of NF-κB was recently shown to be only delayed and slightly reduced in murine embryonic fibroblasts (MEFs) and thymocytes derived from TRAF2-deficient mice (Table I), as well as in lymphocytes from TRAF2-DN transgenic mice. Nevertheless, the activation of the IκB kinase (IKK) complex that is directly responsible for phosphorylation of the NF-κB inhibitor IκB and subsequent nuclear translocation of NF-κB (Karin, 1999) is dramatically reduced in TRAF2-deficient MEF cells (Lee *et al.,* 1997; Yeh *et al.,* 1997; Devin *et al.,* 2000). TNF-induced JNK and p38 activation is completely disrupted in TRAF2-deficient cells.

TNF triggering of the p55 also leads to the recruitment of RIP to the p55 complex via binding of its C-terminal DD with the DD of TRADD (Fig. 1). Overexpression of RIP induces cell death and NF-κB activation. However, by generating RIP-deficient human T cell leukemia Jurkat cells, as well as by the isolation of

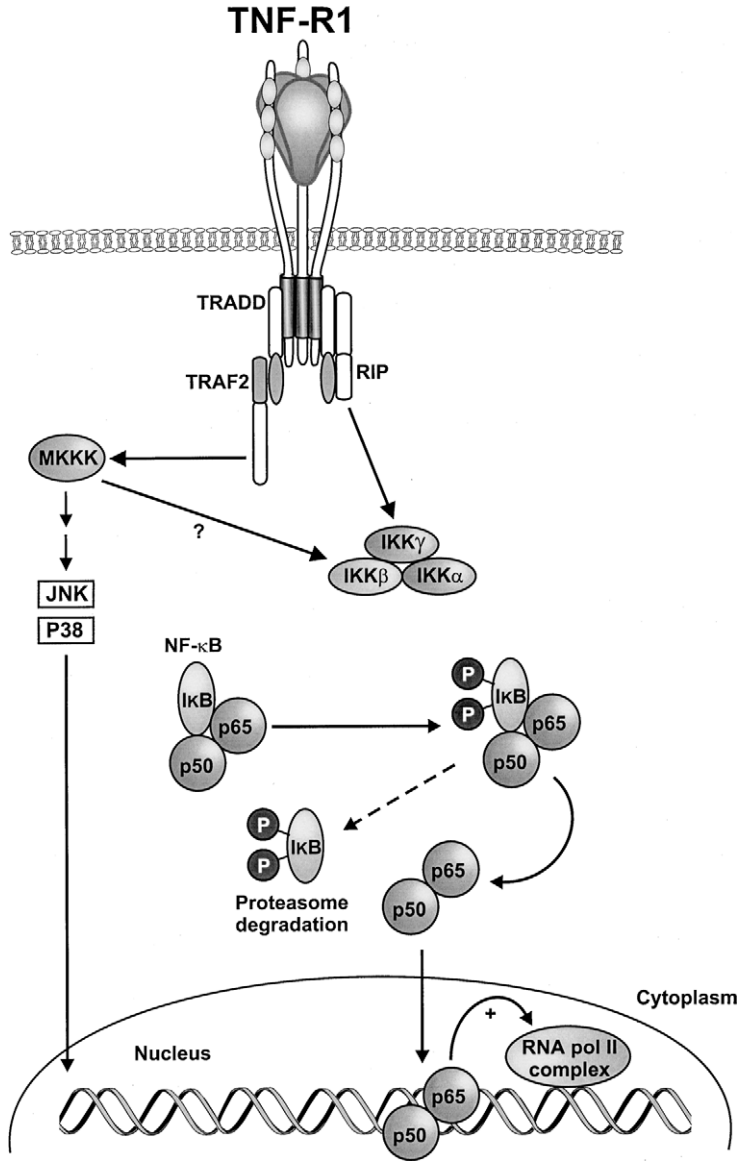

FIG. 1 Overview of TNF-R1-induced signaling pathways leading to NF-κB-dependent gene expression.

TABLE I

Phenotype of Mice or Cells Deficient for Specific Proteins That Are Involved in the Activation and Regulation of NF-κB in Response to TNF

Knockout	Developmental defects	Cellular and molecular phenotype	Reference
RIP	Perinatally lethal death at 1–3 days of age	Extensive apoptosis in lymphoid and adipose tissue Failure to activate NF-κB	Kelliher *et al.* (1998)
TRAF2	Normal at birth, die prematurely	Atrophy of the thymus and spleen and depletion of B cell precursors Reduced IKK activation Mild effect on NF-κB translocation Defective JNK activation	Yeh *et al.* (1997) Devin *et al.* (2000)
NIK	Normal	Abnormalities in lymphoid tissue development and antibody responses Normal NF-κB DNA binding activity Deficiency of NF-κB transactivation upon LTβ stimulation	Yin *et al.* (2001)
MEKK1	Normal	Failure of eyelid closure Normal NF-κB activation but defective JNK activation	Yujiri *et al.* (2000)
IKK-α	Perinatally lethal	Thicker, undifferentiated epidermis Altered limb and skeletal patterning Diminished NF-κB activation by TNF and IL-1	Li *et al.* (1999b) Hu *et al.* (1999)
IKK-β	Embryonic lethal, death around E13.5	Massive liver apoptosis Defective in NF-κB activation by TNF and IL-1	Li *et al.* (1999a) Tanaka *et al.* (1999) Li *et al.* (1999)
IKK-γ	Male lethality, die at E12.5–E13.0	Massive liver apoptosis Skin lesions in heterozygous females Complete inhibition of NF-κB activation by proinflammatory cytokines	Schmidt-Supprian *et al.* (2000) Makris *et al.* (2000) Rudolph *et al.* (2000)
IκBα	Normal, die at 7–10 days of age	Severe widespread inflammatory dermitis and granulocytosis Cell-type-dependent constitutive nuclear localization of NF-κB Prolonged nuclear localization of NF-κB after activation signals	Beg *et al.* (1995a) Klement *et al.* (1996)

(*continued*)

TABLE I (*continued*)

Knockout	Developmental defects	Cellular and molecular phenotype	Reference
p50	Normal	Massive liver apoptosis Defective B cell proliferation and defective in basal and specific antibody production	Sha *et al.* (1995)
p65	Embryonic lethality, die at E15–E16	Massive liver apoptosis Enhanced sensitivity to TNF-induced apoptosis Defective in TNF-induced activation of NF-κB	Beg *et al.* (1995b)
A20	Normal at birth, die prematurely at approximately 1 week of age	Severe inflammation and cachexia Fail to terminate TNF but not IL-1-induced NF-κB responses	Lee *et al.* (2000)

liver-derived Abelson transformed pre-B cells or MEF cells from RIP knockout mice, RIP has been shown to have an indispensable role in TNF-induced NF-κB activation, whereas TNF-induced apoptosis was not affected (Tables I and II) (Ting *et al.,* 1996; Kelliher *et al.,* 1998). The DD of RIP (Fig. 2) also interacts with the DD of RAIDD, which is a caspase-binding protein with unknown function (Duan and Dixit, 1997). RIP also consists of an N-terminal kinase domain which is not required for NF-κB activation in response to TNF. In contrast, the intermediate domain of RIP contributes to the activation of NF-κB and directly interacts with a nonkinase regulatory subunit of the IKK complex, IKKγ or NEMO (Zhang *et al.,* 2000). Recently, two proteins that are homologous to RIP have been identified, named RIP2 (also known as CARDIAK or RICK) and RIP3. Both proteins contain the conserved kinase domain but have variable C-terminal domains. Although overexpression of RIP2 and RIP3 induces NF-κB activation, it is unclear if these proteins are also involved in TNF-mediated signaling (McCarthy *et al.,* 1998; Thome *et al.,* 1998; Yu *et al.,* 1999).

Another protein that interacts with the DD of TRADD is FADD (Hsu *et al.,* 1996a). This protein also contains two functionally and structurally distinct domains: a C-terminal DD that is necessary for its recruitment to the p55 TNF receptor and an N-terminal death effector domain (DED) that promotes self-association and activation of a downstream proteolytic cascade through binding of the homologous DEDs of procaspase-8 (previously called FLICE or Mach; Boldin *et al.,* 1996; Muzio *et al.,* 1996) (Fig. 2). Binding of procaspase-8 to FADD leads to its recruitment to the p55 and oligomerization-catalyzed autoproteolytic activation (Muzio *et al.,* 1996). Active caspase-8 can then initiate a proteolytic cascade that will eventually lead to cell death.

In addition to TRADD, TRAF2, RIP, and FADD, several other proteins whose functions are largely unclear have been shown to bind to the p55. Yeast two-hybrid

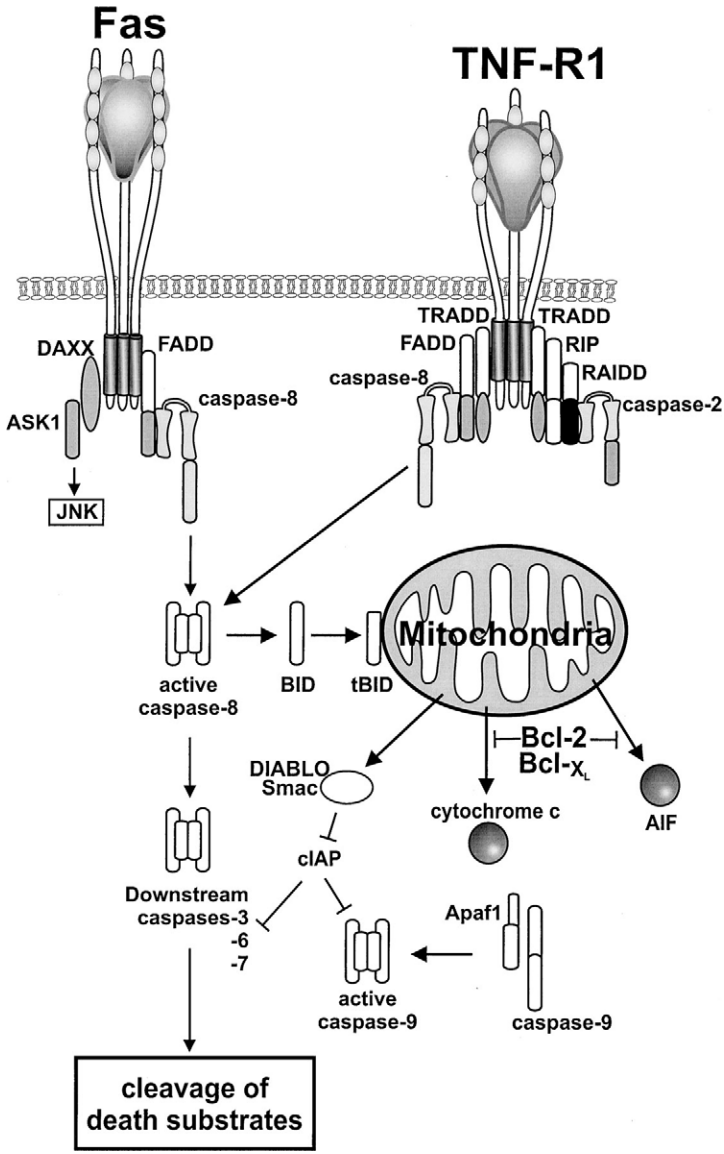

FIG. 2 Overview of TNF-R1 and Fas-induced signaling pathways leading to apoptosis.

screening led to the characterization of 55.11, which binds to the membrane-proximal region of the p55 and is almost identical to the p97 subunit of the 26S proteasome (Boldin *et al.,* 1995a; Tsurumi *et al.,* 1996). The group of Donner cloned two TNF receptor-associated proteins (TRAPs) (Song *et al.,* 1995; Dunbar *et al.,* 1997), which also interact with the membrane-proximal part. TRAP-2 turned

out to be identical to 55.11, whereas TRAP-1 shows strong homology with members of the hsp90 family. Evidence has also been obtained that both receptors can be phosphorylated by an associated kinase (Darnay *et al.,* 1994, 1995; Beyaert *et al.,* 1995), but its biological function remains largely unknown. Recently, a role for p55 phosphorylation by MAP kinases in the cellular redistribution of the receptor has been described (Cottin *et al.,* 1999; Van Linden *et al.,* 2000).

The membrane-proximal part of p55 has been shown to associate with factor-associated with neutral Smase (FAN) (Adam-Klages *et al.,* 1996), which was shown to be involved in the TNF-induced activation of neutral Smase and ceramide production in some cells. The adaptor protein Grb2 can also associate with the membrane-proximal region of p55, and it has been proposed to be involved in TNF-induced activation of Raf that occurs in some cell lines (Hildt and Oess, 1999).

The number of Fas receptor associated proteins that have been identified is far more limited compared to that of TNF-R1. The main adaptor protein that is recruited to the Fas receptor upon stimulation is FADD (Boldin *et al.,* 1995b; Chinnaiyan *et al.,* 1995) (Fig. 2). Although most of the Fas receptor interacting proteins were obtained using yeast two-hybrid screening, Fas signaling complexes have also been identified *in vivo* using biochemical methods on receptor complexes (Kischkel *et al.,* 1995). Activation of the receptor with agonistic anti-Fas antibodies followed by coimmunoprecipitation of Fas resulted in the identification of four Fas associated proteins (CAP1–4 for APO-1–associated proteins) on two-dimensional gels. Two associated proteins were identified as different phosphorylated FADD species, and the two other proteins revealed the existence of a FADD interacting caspase, viz. caspase-8 (Muzio *et al.,* 1996). These proteins, together with the receptor, were named the death-inducing signaling complex (DISC). FADD interacts directly with the DD of Fas and recruits and activates procaspase-8 through its DED (Boldin *et al.,* 1996; Muzio *et al.,* 1996). The N-terminal DED domain of FADD is essential for Fas-mediated apoptosis since overexpression of a FADD mutant lacking the DED acts as a dominant-negative mutant (FADD-DN) and protects cells from Fas and TNF-induced apoptosis (Chinnaiyan *et al.,* 1995; Hsu *et al.,* 1996a). Moreover, FADD-deficient mice exhibited reduced Fas and TNF-induced cell death (Zhang *et al.,* 1998a) (Table II). Also, UV radiation- and anticancer drug-induced apoptosis are prevented by FADD-DN (Rehemtulla *et al.,* 1997; Micheau *et al.,* 1999; Fulda *et al.,* 2000). Surprisingly, FADD not only participates in the transduction of an apoptotic signal but also is essential for transmitting growth and/or survival signals because the FADD-null or FADD-DN transgenic mice are resistant to mitogen-induced T cell proliferation (Newton *et al.,* 1998, 2000, 2001; Zhang *et al.,* 1998a). The failure of FADD-DN T cells to proliferate *in vivo* offers the simplest explanation for why FADD-DN mice do not develop the lymphadenopathy seen in Fas-deficient *lpr* mutant mice (Newton *et al.,* 1998).

TABLE II
Phenotype of Mice and Cells Deficient in Specific Proteins That Are Involved in Fas or TNF-R-Induced Apoptosis

Knockout	Developmental defects	Cellular and molecular phenotype	Reference
Caspase-1	Normal	Resistant to LPS-induced septic shock No IL-1β and IL-18 processing	Kuida *et al.* (1995) Li *et al.* (1995)
Caspase-2	Normal	Excessive oocytes resistant to apoptosis Accelerated apoptosis of facial motor neurons	Bergeron *et al.* (1998)
Caspase-3	Perinatally lethal, die at 1–3 weeks of age	Thymocytes, MEFs, and hepatocytes show no bleb formation, DNA fragmentation, nuclear breakdown Delayed or absent cleavage of cellular substrates (PARP, ICAD, lamins, etc)	Kuida *et al.* (1996)
Caspase-6	Normal		Zheng and Flavell (2000)
Caspase-7	Embryonic lethal during gestation		Zheng and Flavell (2000)
Caspase-8	Embryonic lethal, die around E11.5	MEFs resistant to TNF-R1, Fas, and DR3 Sensitive to drug- and UV-induced apoptosis	Varfolomeev *et al.* (1998)
Caspase-9	Perinatally lethal	Sensitive to UV radiation and Fas	Kuida *et al.* (1998)
		Thymocytes resistant to γ-irradiation and drugs	Hakem *et al.* (1998)
Caspase-11	Normal	Resistant to endotoxic shock Lack of IL-1α and IL-1β production Cells resistant to ischemia-induced cell death	Wang *et al.* (1998)
Caspase-12	Normal	Resistant to ER stress-inducing agents	Nakagawa *et al.* (2000)
FADD	Embryonic lethal	MEFs resistant to death receptor-induced death Sensitive to drug-, E1A-, cMyc-induced death Thymocytes show impaired survival and proliferation	Yeh *et al.* (1998) Zhang *et al.* (1998a)
RIP	Perinatally lethal, die at 1–3 days of age	Extensive apoptosis in lymphoid and adipose tissue	Kelliher *et al.* (1998)
		Normal anti-Fas response, highly sensitive to TNF-R1-induced apoptosis Failure to activate NF-κB	

(*continued*)

TABLE II (*continued*)

Knockout	Developmental defects	Cellular and molecular phenotype	Reference
DAXX	Embryonic lethal	Extensive apoptosis	Michaelson *et al.* (1999)
FLIP	Embryonic lethal, die at E10.5	Highly sensitive to TNF and FasL-induced apoptosis	Yeh *et al.* (2000)
XIAP	Normal	No clear phenotype	Harlin *et al.* (2001)
		Levels of cIAP-1 and cIAP-2 were increased	
BID	Normal	Resistant to Fas-induced apoptosis	Yin *et al.* (1999)
Cytochrome c	Embryonic lethal, die at E10.5	Resistant to death induced by UV, serum withdrawal, staurosporin	Li *et al.* (2000)
		Increased sensitivity to TNF	
AIF	Embryonic lethal	Defective activation of embryoid bodies, essential for the initiation of gastrulation	Joza *et al.* (2001)
Apaf-1	Embryonic lethal, die at E16.5	Resistant to death induced by drugs and irradiation	Cecconi *et al.* (1998)
		Increased sensitivity to Fas	Yoshida *et al.* (1998)

Besides its function in signaling to gene induction, RIP was also identified in the Fas DISC complex and turned out to be a crucial factor in Fas- and TNF-mediated necrotic cell death independent of caspases (Holler *et al.,* 2000). Recently, a new protein, SADS (small accelerator for death signaling), was identified that is recruited to the DISC complex upon Fas ligation (Suzuki *et al.,* 2001). SADS is not essential for Fas-mediated apoptosis signaling but rather enhances it. It interacts with the DED of FADD and procaspase-8 and is not involved in TNF-induced cell death. Moreover, SADS expression seems to be lost in some human colon cancer cells, suggesting that downregulation or loss may contribute to tumor cell survival (Suzuki *et al.,* 2001).

An alternative Fas pathway, based on yeast two-hybrid screening and transient overexpression data, may involve activation of caspase-2 through RIP and the RIP-interacting protein RAIDD (Ahmad *et al.,* 1997; Duan and Dixit, 1997). The death adaptor protein RAIDD contains a DD and a caspase recruitment domain (CARD) by which procaspase-2 is recruited. A third pathway may implicate the protein DAXX that specifically associates with the DD of Fas (Yang *et al.,* 1997), leading to the downstream activation of ASK1 and JNK (Chang *et al.,* 1998). These kinases can activate several transcription factors, although conflicting results have been published regarding the role of JNK signaling in the control of apoptotic cell death (Villunger *et al.,* 2000). Finally, there has been a report on the association

of FLASH with the Fas receptor (Imai *et al.,* 1999). However, the contributions of the former two pathways to TNF and Fas cytotoxicity in physiological conditions remain unclear; the relevance of FLASH is highly controversial (Koonin *et al.,* 1999).

III. Gene Activation in Response to TNF

TNF induces many gene products involved in inflammation, tissue repair, hematopoiesis, immune response, and antitumor effects. An exhaustive list of such TNF-responsive genes has been compiled (Kyriakis, 1999) that includes genes coding for transcription factors, growth factors, cytokines, acute phase response proteins, cell adhesion molecules, enzymes, and cell surface antigens. Some of the TNF-responsive genes code for so-called TNF resistance proteins, which can inhibit TNF cytotoxicity. Indeed, the cytotoxic activity of TNF is enhanced up to 100-fold in the presence of transcription of translation inhibitors, such as actinomycin D or cycloheximide, respectively (Ruff and Gifford, 1981). Many cell lines are quite resistant to TNF but become sensitive when they are also treated with actinomycin D (Fransen *et al.,* 1986). Examples of TNF resistance proteins are manganese superoxide dismutase (Wong and Goeddel, 1988), the zinc finger protein A20 (Opipari *et al.,* 1992), and the heat shock protein hsp70 (Jaattela *et al.,* 1992).

A. NF-κB as a Key Player

Changes in gene expression result from the activation of transcription factors by TNF. The most important transcription factor involved in TNF-induced gene activation is undoubtedly NF-κB, which is activated within 5 min after TNF stimulation. NF-κB has been shown to be essential for TNF-mediated induction of various genes, including cytokines (TNF, IL-1, and IL-6) and chemokines (RANTES, MCP-3, and eotaxin), inducible NO synthase, and adhesion molecules (ICAM-1 and VCAM-1). Moreover, the observation that some proinflammatory cytokines regulated by NF-κB can also activate NF-κB indicates that this may represent a feed-forward amplifying loop that could form the basis for the persistence of chronic inflammatory diseases.

NF-κB is made up of two subunits which belong to the Rel family, whose members share a $\sim$300-amino acid region known as the Rel homology domain that contains the DNA binding elements (Chen and Ghosh, 1999). The most usual form of NF-κB is a heterodimer of a 65-kDa polypeptide subunit (p65, also referred to as Rel-A) and a 50-kDa subunit (p50). Other subunits, such as p52, p105, p100, c-Rel, and Rel-B, may also occur. These subunits form various homodimers

and heterodimers with different regulatory activities. The p50 subunit within the p50/p65 heterodimer facilitates DNA binding, whereas the p65 subunit is required for adequate transactivation (Chen and Ghosh, 1999). Targeted disruption of the genes coding for p65 or p50 in mice results in severe immune deficiency which is lethal in the case of p65 (Beg *et al.,* 1995b; Sha *et al.,* 1995). In unstimulated cells, NF-κB is mostly localized to the cytoplasm in a dormant form bound to an inhibitory protein, lκB (Karin and Ben-Neriah, 2000) (Fig. 1). IκB has several isoforms (IκB-α, -β, -γ/p105, -δ/p100, -ε, and Bcl-3), the most predominant being the α and β subunits. IκB-γ and IκB-δ also function as precursor proteins for p50 and p52, respectively. IκB family proteins have five to seven conserved domains known as ankyrin repeats, each consisting of 33 amino acids, which are involved in the interaction with NF-κB. Binding of IκB masks the nuclear localization signal of the Rel proteins and thus prevents NF-κB from entering the nucleus. Upon appropriate cell stimulation, specific kinases phosphorylate IκB, leading to its ubiquitination which makes it a substrate for rapid degradation by the proteasome, a multifunctional cellular protease (Karin and Ben-Neriah, 2000). This releases NF-κB from IκB, resulting in the translocation of active NF-κB into the nucleus, where it binds to specific IκB recognition elements—5′-GGGPuNNPyPyCC-3′ (p65/p50) or 5′-GGGPuNPyPyCC-3′ (p65/c-Rel)—in the promoter region of inducible genes.

In addition to NF-κB, TNF has been reported to also activate or induce the transcription factors activation protein 1 (AP-1), nuclear factor IL-6, cAMP-responsive element-binding protein, and possibly others (Zhang *et al.,* 1988; Brenner *et al.,* 1989; Kyriakis, 1999).

B. Role of Mitogen-Activated Protein Kinases

In addition to activation of NF-κB and cell death, TNF receptors can also activate several MAP kinases (ERK, JNK, and p38 MAP kinase). Among many substrates, these kinases have also been shown to phosphorylate several transcription factors or other proteins that are involved in the regulation of gene expression. For example, the activation of AP-1 by the p55 receptor is mediated through JNK (Brenner *et al.,* 1989). Inhibition of p38 MAP kinase and ERK kinase activation have also been shown to prevent NF-κB-dependent gene expression in response to TNF, without affecting the nuclear translocation and DNA binding of NF-κB (Beyaert *et al.,* 1996; Vanden Berghe *et al.,* 1998). This is most likely due to the phosphorylation of a cofactor or of a cooperative transcription factor by p38 MAP kinase or a downstream activated kinase. Alternatively, NF-κB might be a target for specific protein kinases. In this context, phosphorylation of the p65 subunit in response to TNF has been shown to contribute to NF-κB transactivation (Sakurai *et al.,* 1999). However, p38 and ERK signaling have not been found to be responsible for TNF-induced p65 phosphorylation.

C. Cytoplasmic Signaling Cascades

1. Activation of NF-κB

Recently, a specific IKK complex has been identified (Karin, 1999) consisting of at least two inducible IκB kinases (IKK-α and IKK-β), which phosphorylate IκB-α at serines 32 and 36 and IκB-β at serines 19 and 23. The IKKs can form homo- and heterodimers via their leucine zipper domains, although heterodimers appear to be favored *in vivo*. MEF cells that were isolated from IKK-β-deficient mouse embryos showed a marked reduction in TNF-induced NF-κB activity (Tanaka *et al.,* 1999), whereas NF-κB activation was not impaired in IKK-α-deficient fibroblasts (Hu *et al.,* 1999) (Table I). These results show that IKK-β is essential for cytokine-induced NF-κB activation, and that it cannot be substituted with IKK-α. Nevertheless, IKK-α was found to be essential for NF-κB activation in the limb and skin during embryogenesis (Hu *et al.,* 1999). Apart from IKK-α and IKK-β, IKK-γ (also called NEMO/IKKAP-1/FIP-3) has been identified as an essential regulatory subunit of the IKK complex (Rothwarf *et al.,* 1998). The upstream signaling pathways which are involved in activation of the IKK complex in response to TNF are unclear. Activation of IKK-α and -β is dramatically reduced in TRAF2-deficient MEF cells, indicating an important role for TRAF2 in TNF-induced activation of the IKK complex (Devin *et al.,* 2000). This might involve the ubiquitin protein ligase function that has been assigned to the ring finger domain of TRAF2. In fact, IKK activation by TRAF6, which is a TRAF2-related protein that is involved in NF-κB activation in response to IL-1 and LPS, was recently shown to require a ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain (Deng *et al.,* 2000). In addition, TRAF2 was also shown to be required for the recruitment of the IKK complex to the p55 TNF receptor (Devin *et al.,* 2000). In addition to ubiquitination, phosphorylation of IKK has also been proposed to be involved in IKK activation. In this context, a role for the TRAF2 interacting kinases NIK and MEKK1, which are members of the MAP kinase kinase kinase (MKKK) family, has been proposed (Malinin *et al.,* 1997; Nakano *et al.,* 1998; Baud *et al.,* 1999). However, gene targeting studies recently demonstrated that disruption of MEKK1 or NIK does not impair TNF-induced IKK activation (Yujiri *et al.,* 2000; Yin *et al.,* 2001). Also, protein kinase C ζ activation has been shown to contribute to IKK activation in response to TNF (Lallena *et al.,* 1999). Protein kinase C ζ can be recruited to TNF-R1 via an indirect interaction with RIP that is mediated by the adaptor protein p62 (Sanz *et al.,* 1999). Another regulatory step in IKK activation may be its dimerization, which is again mediated by RIP (Poyet *et al.,* 2000).

Phosphorylation of IκB by the IKK complex leads to its subsequent ubiquitination and degradation by the proteasome. Recently, the component of the ubiquitin ligase which specifically recognizes the IκB-α degradation motif was identified (Yaron *et al.,* 1998; Spencer *et al.,* 1999). It has been reported that IκB-α can also be modified by SUMO-1 (small ubiquitin-like modifier, also known as sentrin)

(Desterro *et al.,* 1998). This occurs at the same lysine residue which is conjugated by ubiquitin, making it resistant to proteasomal degradation and thus inhibiting NF-κB-dependent gene expression. Interestingly and in contrast to ubiquitin, SUMO-1 specifically modifies the unphosphorylated form of IκB.

The IκB-α, IκB-γ, IκB-ε, and Bcl-3 genes have several κB sequences in their promoter region so that NF-κB induces their synthesis. IκB-α molecules enter the nucleus to bind to activated NF-κB and cause the return of NF-κB to the cytoplasm, thus terminating activation (Tam and Sen, 2001). Targeted disruption of the IκB-α gene results in prolonged NF-κB activation, and the animals die of severe inflammation, including widespread dermatitis (Klement *et al.,* 1996). Replacing the IκB-α gene with the IκB-β gene by generating knockin mice restored the normal phenotype, demonstrating the functional redundancy between both inhibitors (Cheng *et al.,* 1998). In contrast to IκB-α and IκB-β, the function of the other members of the IκB family is not clear, but it is likely that they are important in balancing the NF-κB activation and specific gene regulation.

Although IκB is generally believed to be the main inhibitor of NF-κB activity, other cellular inhibitors of NF-κB exist. However, their mechanism of action is largely unclear. The best known example is A20, a TNF-inducible zinc finger protein which inhibits NF-κB activation by a variety of agents upon overexpression (Beyaert *et al.,* 2000). Because A20 interacts with TRAF2 (Song *et al.,* 1996), it is assumed that the latter interaction might be responsible for its inhibitory effect on TNF-induced NF-κB activation. Recently, many novel A20-binding proteins that are likely to mediate the inhibitory effect of A20 have been identified (De Valck *et al.,* 1999; Heyninck *et al.,* 1999; Beyaert *et al.,* 2000). Other proteins that have been shown to inhibit TNF-induced NF-κB activation are TRIP and I-TRAF (Rothe *et al.,* 1996; Lee and Choi, 1997).

Recent studies have demonstrated that RIP can be proteolytically cleaved by caspase-8 in its intermediate domain (Lin *et al.,* 1999; Kim *et al.,* 2000; Lewis *et al.,* 2000; Martinon *et al.,* 2000). This cleavage of RIP abrogates TNF-induced NF-κB activation and further enhances TNF-mediated apoptotic events, most likely by switching off the NF-κB-mediated expression of survival proteins. Moreover, the C-terminal fragment of RIP that is produced upon cleavage enhances the association between TNF-R1, TRADD, and FADD, thereby amplifying the activation of caspase-8. Similarly, caspase-induced inactivation of the antiapoptotic protein TRAF1 might be part of the proapoptotic amplification system to ensure rapid cell death (Leo *et al.,* 2000; Jang *et al.,* 2001). Finally, caspase-independent degradation of TRAF2 has been observed in response to TNF-R and CD30 stimulation (Duckett and Thompson, 1997), again providing a potential mechanism to shift the balance to cell death.

2. Activation of Mitogen-Activated Protein Kinases

Both TNF receptors can activate ERK as well as JNK and p38 kinases within minutes. In contrast, activation of MAP kinases in response to Fas triggering

can only be observed after several hours. MAP kinase signaling pathways consist of a strongly conserved cascade of three kinases. MAP kinases are activated upon phosphorylation on threonine and tyrosine by dual-specificity MAP kinase kinases (MKKs). These MKKs are then phosphorylated by several serine/threonine MKKKs. The mechanisms that connect the MKKKs to the TNF receptor or Fas are less well characterized. ERK activation in response to TNF presumably involves the association of MAP kinase activating DD protein (MADD) with the DD of p55 (Schievella *et al.,* 1997). The signaling pathways downstream of MADD are unknown but are likely to involve a MKKK. A role for MEKK1 in TNF-induced ERK activation is unlikely because ERK activation is unaffected in MEKK1-deficient macrophages (Yujiri *et al.,* 2000). Interestingly, MADD is almost identical to the GDP/GTP exchange protein Rab3-GEP (Brown and Howe, 1998). This suggests a potential role for GTP-binding proteins in the regulation of ERK activation in response to TNF. JNK and p38 kinase activation is mediated by TRAF2, as demonstrated by the absence of their activation in TRAF2-deficient cells (Yeh *et al.,* 1997). How TRAF2 induces JNK and p38 kinase activation is not clear. Recent evidence indicates that apoptosis signal-regulating kinase 1 (ASK1) interacts with TRAF2 in a TNF-dependent manner (Nishitoh *et al.,* 1998; Liu *et al.,* 2000). ASK1 is a MKKK that activates MKK4/JNK and MKK6/p38 signaling cascades. Moreover, its activation by TRAF2 requires prior dissociation from thioredoxin, which is associated with ASK1, and most likely involves the TNF-induced oxidation of thioredoxin. Another study indicates that interaction of TRAF2 with members of the germinal center kinase (GCK) family leads to activation of JNK (Yuasa *et al.,* 1998). GCKs can directly bind to MEKK1 and may form an extra link between TRAF2 and MEKK1, which are already involved in a direct interaction (Baud *et al.,* 1999).

D. Gene Activation in Response to Fas

In contrast to the strong potency of TNF to activate several genes, gene activation in response to Fas triggering occurs only under specific conditions. FasL has been shown to upregulate the expression of various chemokines and cytokines (including IL-8) in different cell types (Schaub *et al.,* 2000). Fas engagement also induces the release of IL-1β and the production of interferon-γ in dendritic cells (Rescigno *et al.,* 2000). However, it is believed that only locally excessive levels of FasL may activate the immune system and exert a proinflammatory effect by activating the expression of a limited number of proinflammatory genes (O'Connell *et al.,* 2001). Although little is known about the activation of transcription factors by Fas engagement, Fas-dependent NF-κB activation has been reported in some cell lines (Rensing-Ehl *et al.,* 1995; Ponton *et al.,* 1996). A recent study demonstrates the role of the FADD-interacting protein and inhibitor of caspase-8 FLIP (FLICE inhibitory protein) in the proliferation of CD3-activated T lymphocytes by recombinant FasL through the activation of the transcription factors NF-κB and

AP-1 (Kataoka *et al.,* 2000). FLIP can be considered as a multifunctional protein capable of switching Fas from apoptotic pathways to proliferation and differentiation pathways by blocking progression of caspase-8 activation and initiating the recruitment of adaptor proteins and kinases, such as TRAF1, TRAF2, RIP, and Raf-1. This is in agreement with the high expression levels of FLIP in many tumors (Tschopp *et al.,* 1998), suggesting that on attacking CTLs FasL may under some conditions even stimulate tumor cell growth through Fas interaction. Moreover, FLIP-transfected tumor cell lines escape immune surveillance (Djerbi *et al.,* 1999; Medema *et al.,* 1999).

IV. Cell Death

In addition to its function in gene regulation, TNF family members play a crucial role in the induction of cell death. It has become clear that death is as important for a multicellular organism as other cellular processes, and that it can be considered as an essential part of life. Here, we review the main cell death pathways and basic components of the death machinery induced by TNF- and Fas-receptor activation.

A. Apoptosis versus Necrosis

Apoptosis and necrosis are two distinct forms of cell death (Fiers *et al.,* 1999; Denecker *et al.,* 2001a,b). There is controversy about the term necrosis because it is also used in a histological sense. Therefore, necrosis as a cell death process is also indicated by oncosis, reflecting the swollen appearance of cells at the initiation of this particular way of cell death. Apoptosis and necrosis represent two extremes of a wide range of possible morphological and biochemical ways of programmed cell death. Both types of cell death can occur simultaneously in cells or tissues exposed to the same stimulus. Often, the final result depends on the intensity of the signal or the concentration of the agent (Nicotera *et al.,* 1999). Notably, in several pathological conditions, such as liver damage or brain ischemia, cell death can occur by both necrosis and apoptosis (Leist *et al.,* 1995, 1996, 1997; Charriaut-Marlangue *et al.,* 1996). The term programmed cell death is used to refer to any kind of cell death—apoptosis, necrosis, or a mixed phenotype—mediated by an intracellular death program, irrespective of the trigger.

Apoptosis is considered the most common physiological way of cell killing that occurs during normal development and tissue homeostasis in multicellular organisms. Cells undergoing apoptosis show typical, well-defined morphological changes, including plasma membrane blebbing, cellular shrinkage, and chromatin condensation (Kerr *et al.,* 1972; Wyllie *et al.,* 1980). Biochemically, these changes are associated with an early redistribution of phosphatidylserine from the inner to the outer leaflet of the plasma membrane (Fadok and Henson, 1998; Denecker

et al., 2000), changes in mitochondrial membrane permeability (Kroemer and Reed, 2000), and caspase-dependent activation and nuclear translocation of an endonuclease resulting in internucleosomal DNA cleavage (Enari *et al.,* 1998). In an early phase of the apoptotic process, cells signal their apoptotic state to their environment, mainly by the exposure of phosphatidylserine at the cell surface resulting in recognition and engulfment by phagocytes (Savill and Fadok, 2000). Uptake of apoptotic cells causes an antiinflammatory response in the macrophage by reducing IL-1 and TNF production and enhancing IL-10 secretion of activated macrophages (Fadok *et al.,* 1998; Gao *et al.,* 1998).

Necrosis is characterized by cytoplasmic swelling and is therefore also related to oncosis, irreversible damage of the plasma membrane, and organelle breakdown (Grooten *et al.,* 1993; Fiers *et al.,* 1999). Exposure of phosphatidylserine on the plasma membrane is a late phenomenon coinciding with plasma membrane permeabilization and the absence of internucleosomal DNA fragmentation (Denecker *et al.,* 2001b). Necrosis results in a spill of the intracellular content in the environment and consecutive inflammation.

Until recently, necrosis was described as a result of traumatic injury or exposure to high concentrations of noxious agents rather than the result of an underlying genetic program. However, recent reports indicate that necrotic cell death can also occur during normal cell physiology and development (Chautan *et al.,* 1999; Kitanaka and Kuchino, 1999). *In vitro* studies on cell lines indicate the existence of a necrotic signaling pathway (Kawahara *et al.,* 1998; Vercammen *et al.,* 1998a,b; Holler *et al.,* 2000; Matsumura *et al.,* 2000). Little is known about the molecular mechanisms of initiation and execution of the necrotic cell death program.

The apoptotic process is better understood in molecular terms. A real break through occurred with the identification of cysteinyl aspartate-specific proteinases or caspases as the initiators and the central executioners of the apoptotic program. In most cell types, members of the TNF receptor superfamily initiate a caspase-dependent apoptotic signaling pathway, although in some cell lines an active caspase-independent necrotic process is induced in conditions in which caspases are blocked.

B. Death Receptor-Induced Apoptosis

Caspases are the key effector molecules on which presumably all apoptotic pathways converge. Evidence for the involvement of caspases in the process of apoptosis derived from the finding that a serpin-like proteinase inhibitor, the cowpox virus CrmA (cytokine response modifier A), could suppress Fas- and TNF-induced cell killing (Enari *et al.,* 1995; Los *et al.,* 1995; Tewari and Dixit, 1995). Later, it was demonstrated that CrmA specifically blocks caspase-8 and caspase-1 (Zhou *et al.,* 1997).

Caspases are a family of evolutionarily conserved cysteine proteases that proteolyze target substrates at specific aspartate residues (Nicholson and Thornberry,

1997; Earnshaw *et al.,* 1999). Based on database searches, two families of caspase-like proteins were recently identified and designated as paracaspases and metacaspases (Uren *et al.,* 2000). Paracaspases are found in mammalia and *Dictyostelium,* and metacaspases are found in plants, fungi, and protozoa. To date, 14 mammalian caspases have been cloned (Van de Craen *et al.,* 1997, 1998a,b; Earnshaw *et al.,* 1999). The reason for pleiad of mammalian caspases is not clear. The observation that no caspase knockout described to date abolishes all apoptosis supports the idea that in different tissues and in response to different stimuli, different apoptotic pathways are used consisting of different caspases (Earnshaw *et al.,* 1999). In fact, the most serious apoptotic phenotypes are seen in caspase-3 (Kuida *et al.,* 1996) and caspase-9 (Hakem *et al.,* 1998; Kuida *et al.,* 1998) gene disrupted mice displaying hyperplasia in the brain but no clear effects in other tissues (Table II). This indicates that caspases may act in a redundant way during development. Another reason for the variety of caspases is that they may function as sensors of proper organelle function (e.g., caspase-9 at the mitochondria and caspase-12 at the endoplasmatic reticulum) (Li *et al.,* 1997; Nakagawa *et al.,* 2000).

1. Initiation of Signaling

Two pathways of caspase activation have been described in the case of Fas-induced apoptosis (Scaffidi *et al.,* 1998, 1999). In so-called type I cells, the extrinsic death signal is initiated by the generation of a large amount of active caspase-8 at the DISC within seconds, followed by a rapid cleavage of caspase-3 and other downstream executioner caspases (Fig. 2). However, in so-called type II cells, hardly any DISC is formed, resulting in a weak and delayed activation of caspase-8. In order to activate the downstream caspases in the latter case, the released cytochrome c from the mitochondria is required for the activation of procaspase-9 and subsequent activation of caspase-3 (Li *et al.,* 1997). The intrinsic apoptotic pathway involves the formation of a proper apoptosome complex (Cain *et al.,* 2000). Thus, type I cells start with the formation of a proper receptosome complex resulting in the direct activation of downstream caspases and the formation of the apoptosome complex. For unknown reasons, type II cells have no clear receptosome complex formation and are strictly dependent on the postmitochondrial apoptosome complex in order to activate downstream caspases. As a consequence, Bcl-2 and Bcl-X_L are efficient inhibitors of type II apoptosis but do not affect type I apoptosis. Activation of PKC by PMA was only found to inhibit Fas-mediated apoptosis in type II cells, consistent with the view that only cells whose apoptosis depends on the apoptogenic activity of mitochondria are sensitive to PKC-mediated effects (Scaffidi *et al.,* 1999). Three procaspases contain homologous motifs, such as DED in the case of procaspase-8 and -10 or CARD in the case of caspase-2, in the prodomain by which they associate with similar motifs in TNF and Fas adaptor proteins FADD and RAIDD, respectively (Fernandes-Alnemri *et al.,* 1996; Vincenz and Dixit, 1997; Ahmad *et al.,* 1997; Duan and Dixit, 1997). These caspases participate in

the initiation of the apoptotic cascade and are therefore called initiator caspases, but only in the case of caspase-8 is this supported by *in vivo* data (Muzio *et al.,* 1996). Indeed, TNF- and Fas-induced cell death are completely ablated in caspase-8-deficient cells (Varfolomeev *et al.,* 1998) (Table II).

2. Activation of Downstream Caspases in Apoptotic Cell Death

In vitro, caspase-8 is able to activate most other caspases (Srinivasula *et al.,* 1996; Muzio *et al.,* 1997; Van de Craen *et al.,* 1999). Within cells, however, caspase-8 seems to act in a more restricted manner, resulting only in the proteolytic activation of caspase-3 and -7. Caspase-3, in turn, activates caspase-6 (Hirata *et al.,* 1998). As already mentioned, procaspase-9 is activated in the apoptosome complex through the release of mitochondrial cytochrome c. The molecular link between cytochrome c release and caspase-8 is delivered by the caspase-8-mediated activation of Bid, a proapoptotic member of the Bcl-2 family (Li *et al.,* 1998; Luo *et al.,* 1998). Recently, it was shown that lysosomal proteases may also be implicated in the proteolytic activation of Bid leading to cytochrome c release (Stoka *et al.,* 2000).

The final outcome of the apoptotic proteolytic cascade is the cleavage of cellular substrates by these effector caspases. A long list of substrates of caspases has been reported (Earnshaw *et al.,* 1999; Utz and Anderson, 2000), although little is known about the *in vivo* relevance and the functional consequence of these proteolytic events. Most cleavages result in a loss of function. However, remarkable gain-of-function caspase-dependent proteolysis of substrates has been reported. One of the most dramatic activities of caspases, however, is the cleavage of ICAD (inhibitor of caspase-activated DNAse)/DFF-45 (DNA fragmentation factor 45), the inhibitor of the endonuclease CAD (Liu *et al.,* 1997; Sakahira *et al.,* 1998). Upon induction of apoptosis, ICAD is cleaved by caspase-3, which allows the DNAse CAD to translocate to the nucleus and to induce internucleosomal DNA fragmentation, the hallmark of apoptosis (Enari *et al.,* 1998). Cells lacking caspase-3 or ICAD do not show DNA laddering induced by Fas activation (Woo *et al.,* 1998; Zhang *et al.,* 1998b).

Given the potentially devastating effect of inadvertent caspase activity, processing and activation of these cysteine proteases are tightly regulated at different levels (Earnshaw *et al.,* 1999). First, death receptor/FADD-induced recruitment and proximity-induced activation of procaspase-8 are prevented by FLIP (other names include I-FLICE, CASH, Flame-1, Usurpin, CASH, MRIT, and CLARP) (Goltsev *et al.,* 1997; Hu *et al.,* 1997; Irmler *et al.,* 1997; Srinivasula *et al.,* 1997; Rasper *et al.,* 1998). Initially, there was controversy regarding the modulator role of FLIP since, depending on the cell type and splice variant, overexpression often increases apoptotic signaling by Fas (Han *et al.,* 1997; Inohara *et al.,* 1997; Shu *et al.,* 1997). The large FLIP variant has a caspase-8-like structure including a duplicated DED motif, but lacks the catalytic residues for enzymatic activity. Second, active caspases and caspase cascades are inhibited by interactions with IAP proteins, of which several mammalian homologs have been identified (Rothe *et al.,*

1995b; Roy *et al.,* 1995; Duckett *et al.,* 1996; Liston *et al.,* 1996; Uren *et al.,* 1996; Ambrosini *et al.,* 1997; Kobayashi *et al.,* 1999; Kasof and Gomes, 2000). It has been demonstrated that cIAP-1 and cIAP-2 directly inhibit caspase-3 and -7 (Deveraux *et al.,* 1997, 1998; Roy *et al.,* 1997; Kasof and Gomes, 2000) and that XIAP, cIAP-1, cIAP-2, and Livin also directly inhibit caspase-9 (Deveraux *et al.,* 1998; Kasof and Gomes, 2000). Third, caspase activation is also controlled at the level of the mitochondria by Bcl-2 family members, which prevent the release of cytochrome c.

Although caspases are the main proteases in apoptosis, other proteolytic cascades have been implicated. Recently, cytosolic calpains have been reported to activate caspase-12 during endoplasmatic reticulum stress-induced apoptosis (Nakagawa and Yuan, 2000). Moreover, calpains have also been reported to be involved in an opposite way by proteolytically inactivating caspases (Chua *et al.,* 2000), leading in some cell lines to caspase-independent cell death (Wolf *et al.,* 1999). Also, lysosomal proteases have been reported to play a role in apoptosis, probably by cleavage of BID (Deiss *et al.,* 1996; Stoka *et al.,* 2000), but little is known about the molecular mechanism of the release of these lysosomal proteases to the cytosol.

3. Role of Mitochondria in Apoptosis

The intrinsic activation of procaspases involves the participation of mitochondria. There is no doubt that mitochondria play an important role in the initiation and execution of the apoptotic cell death program, mainly through the activity of pro- and antiapoptotic members of the Bcl-2 family (Adams and Cory, 1998; Gross *et al.,* 1999a) and the release of mitochondrial proteins. The main molecular link connecting the DISC and mitochondria is the caspase-8-mediated cleavage of BID, a BH3-only member of the Bcl-2 protein family. Following TNF or Fas treatment, BID is cleaved at its N- terminus, generating a 15-kDa fragment. Consequently, truncated BID (tBID) translocates to the mitochondria, where it induces the release of cytochrome c (Li *et al.,* 1998; Luo *et al.,* 1998; Gross *et al.,* 1999b) by a mechanism that remains under investigation. One model holds that the exposed BH3 domain of tBID binds to and induces an allosteric, conformational activation of BAK, a resident mitochondrial Bcl-2 family member that oligomerizes and provides a pore for cytochrome c release (Wei *et al.,* 2000). tBID is not a pore-forming protein. Also, BAX, another proapoptotic Bcl-2 family member, is implicated because cells lacking both BAX and BAK, but not cells lacking only one of these components, are completely resistant to tBID-induced cytochrome c release and apoptosis (Lindsten *et al.,* 2000; Wei *et al.,* 2001). The mechanism by which tBID rapidly and selectively targets the mitochondria is not known. Recent studies on artificial membranes bearing the lipid composition of mitochondria, as well as on intact mitochondria, show that posttranslational N-myristoylation of tBID, following cleavage by caspase-8, serves as an activating switch for targeting tBID to mitochondria (Zha *et al.,* 2000). Others have provided evidence that

cardiolipin, which is present in mitochondrial membranes, mediates the mitochondrial targeting of tBID by binding to a specific domain in tBID (Lutter *et al.,* 2000). In the presence of Bcl-2 or Bcl-X_L, the cleavage and translocation of tBID still occur, but the release of cytochrome c is prevented. In certain cell types, however, the cells still die, suggesting that mitochondrial events other than cytochrome c release may be critical to cell death (Gross *et al.,* 1999b). Measurements of several mitochondrial parameters in Fas-activated hepatocytes indicated that the loss of cytochrome c caused respiratory inhibition, reflecting a blockade between respiratory complexes III and IV, a process that could be reversed by adding exogenous cytochrome c at early time points. A prolonged lack of cytochrome c, however, leads to irreversible mitochondrial damage, indicating that during the process of apoptosis mitochondria endure a progressive dysfunction (Mootha *et al.,* 2001).

Together with the apoptosis protease-activating factor 1 (Apaf-1), ATP, and cytosolic procaspase-9, cytochrome c forms the so-called "apoptosome complex" (Cain *et al.,* 1999, 2000) (Fig. 2). A multistep process is proposed whereby catalytically active processed or unprocessed caspase-9 initially binds Apaf-1 and consequently recruits caspase-3 via interaction between the active site cysteine in caspase-9 and a critical aspartate in caspase-3 (Bratton *et al.,* 2001). Caspase-3 then activates procaspase-2, -6, -8, and -10, resulting in a feedback amplification loop (Slee *et al.,* 1999; Sun *et al.,* 1999). Apaf-1, caspase-9, and caspase-3 knockout mice show remarkably similar phenotypes, confirming that these proteins act in a linear activation pathway (Table II). They all display excessive neuronal cells in the brain and die 1 or 2 days after birth (Earnshaw *et al.,* 1999). Fas-induced apoptosis was markedly reduced in Apaf-1-deficient fibroblasts (Cecconi *et al.,* 1998), although T cells remain sensitive to Fas-induced killing (Yoshida *et al.,* 1998). BID-deficient mice are resistant to anti-Fas-induced hepatocyte apoptosis (Yin *et al.,* 1999): Mitochondrial dysfunction was delayed, no cytochrome c was released, and activation of downstream effector caspases was reduced. These examples demonstrate how the same death signal may use different proteolytic pathways in different cells.

Mitochondria contain other apoptogenic factors in addition to cytochrome c that are released upon death induction. In some cell lines these include procaspase-2 and -9 (Krajewski *et al.,* 1999; Susin *et al.,* 1999a) and apoptosis-inducing factor (AIF), a 57-kDa flavoprotein (Susin *et al.,* 1999b). AIF induces peripheral chromatin condensation and DNA fragmentation in fragments larger than 50 kb (Susin *et al.,* 2000). Recently, another mitochondrial protein released during apoptosis was identified: DIABLO (direct IAP-binding protein with low pl)/Smac (second mitochondria-derived activator of caspase), a protein that promotes caspase activation by eliminating IAP inhibition (Du *et al.,* 2000; Verhagen *et al.,* 2000). In addition, approximately 100 other proteins were identified by mass spectrometry to be released from atractyloside-treated mitochondria *in vitro,* but the relevance of these proteins in the process of apoptosis remains elusive (Patterson *et al.,* 2000; Spahr *et al.,* 2000).

C. Mechanism of Necrotic Cell Death by DD Receptors

TNF and Fas have the ability to induce either apoptosis or necrosis, depending on the cell type and on the availability of active caspases. In L929 fibrosarcoma cells transfected with human Fas, TNF-R1 initiates a necrotic signaling pathway, whereas Fas ligation by monoclonal anti-Fas antibody or FasL leads to apoptosis (Vercammen *et al.,* 1997). Pretreatment of these cells with the broad-spectrum caspase inhibitor zVAD-fmk or the cowpox virus inhibitor CrmA blocks Fas-induced apoptosis and initiates the necrotic cell death pathway (Vercammen *et al.,* 1998a). Moreover, these caspase inhibitors rendered the cells more sensitive to TNF-induced necrosis, suggesting that caspases may even be implicated in an anti-necrotic pathway (Vercammen *et al.,* 1998b). This TNF-induced necrosis in L929 cells is due to reactive oxygen species formation in the mitochondria (Schulze-Osthoff *et al.,* 1992), a process that is blocked by pretreatment with the oxygen radical scavenger butylated hydroxyanisole (Goossens *et al.,* 1995). The latter does not prevent anti-Fas-induced apoptotic cell death. TNF-mediated oxygen radical production has been suggested to result from enhanced electron flow through the electron transport chain complex I (Goossens *et al.,* 1999) and is reflected by the fact that complex I inhibitors delay TNF-mediated cell death (Schulze-Osthoff *et al.,* 1992, 1993). In L929 cells, relatively low levels of mitochondrial reactive oxygen species are detectable, but they are sufficient for TNF-induced necrosis (Goossens *et al.,* 1995; Vercammen *et al.,* 1998b; Fiers *et al.,* 1999).

Proteases other than caspases might be involved in death receptor-induced signaling to necrosis: *N*-tosyl-L-phenylalanine chloromethylketone, a serine protease inhibitor, inhibits necrotic cell death induced either by TNF or by a combination of anti-Fas and zVAD-fmk (Vercammen *et al.,* 1997, 1998a).

Several comparable *in vitro* models of caspase-independent cell death with necrotic morphology were published recently (Denecker *et al.,* 2001), and similar findings have been described in an *in vivo* system in which interdigital cell death in the development of the mouse embryo occurred through a necrotic, caspase-independent pathway by addition of a caspase inhibitor or by using Apaf-1 knockout mice (Chautan *et al.,* 1999).

In contrast to apoptotic cell death, little is known about the molecular mechanism of necrosis. A possible candidate, common to both the TNF and the Fas receptor complex, to initiate downstream signaling to necrosis is the adaptor protein FADD. Studies in Jurkat cells showed that enforced oligomerization of FADD in the presence of the caspase inhibitor zVAD-fmk or the use of cells deficient for caspase-8 result in necrotic cell death, whereas wild-type Jurkat cells die by apoptosis (Kawahara *et al.,* 1998). This is in agreement with the observation that overexpression of a FADD dominant-negative mutant, lacking the DED and thus blocking the recruitment of caspase-8, induces TNF-mediated necrosis in U937 and NIH3T3 cells (Khwaja and Tatton, 1999). These data suggest that FADD can mediate not

only caspase-dependent apoptotic signals but also caspase-independent necrosis. The bifurcation between both pathways might thus be situated at the level of this adaptor protein. FADD-DED would propagate apoptosis, whereas FADD-DD would initiate necrotic signaling (Boone *et al.,* 2000). Other researchers demonstrated that in Jurkat cells necrosis is initiated by the DED of FADD (Matsumura *et al.,* 2000). This discrepancy may reflect variation between cell lines.

Another study using FADD- and RIP-deficient Jurkat cells identified the kinase RIP as a crucial component of Fas and TNF-mediated caspase-independent necrosis (Holler *et al.,* 2000). FADD and RIP are recruited in the Fas DISC upon receptor interaction. The DED of FADD then binds procaspase-8, which leads to its activation and subsequent apoptosis, whereas the kinase RIP can phosphorylate an as yet uncharacterized target protein that triggers a signaling pathway that results in necrotic cell death. RIP kinase activity is essential to initiate necrotic signaling. In contrast to the studies described previously, FADD seems not to be required for the induction of necrosis. Since RIP is a key adaptor in the pathway mediating NF-kB activation and antiapoptotic gene induction, it is interesting to note that RIP can be cleaved by caspase-8, thus abolishing the induction of antiapoptosis factors and sensitizing cell death (Lin *et al.,* 1999; Martinon *et al.,* 2000).

Finally, bNIP3, a member of the Bcl-2 family that is expressed in mitochondria, has recently been described as a possible mediator of necrosis-like cell death through mitochondrial dysfunction and increased oxygen radical formation (Vande Velde *et al.,* 2000). The mechanism of bNIP3 action is independent of caspases and the Apaf-1/cytochrome c mitochondrial pathway and occurs before the appearance of nuclear damage. All ultrastructural features typical of necrotic cell death can be observed in bNIP3-expressing cells. Taken together, it is reasonable to postulate that some forms of necrosis-like cell death (e.g., in response to hypoxia) may be mediated by endogenous bNIP3 (Bruick, 2000).

V. Concluding Remarks

Despite increasing knowledge of the signaling proteins that are involved in the signal transduction pathways that are initiated by TNF receptors and Fas, the mechanisms that determine the final outcome for the cell are largely unknown. It should be stressed that much of our signaling models are still based on overexpression studies and the generation of specific cell lines that are deficient for a specific protein. Studies that examine endogenous protein–protein interactions in different cell types as well as the development of selective inhibitors will be crucial in more closely approximating the mechanisms of life and death. Moreover, temporal and spatial regulation of protein expression is likely to be a challenge in the near future.

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