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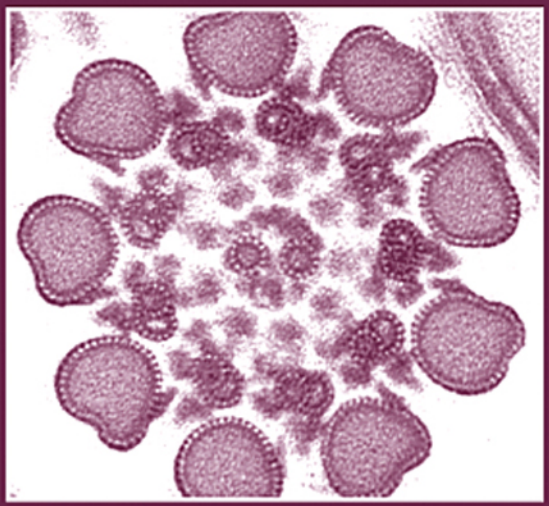
## A SURVEY OF CELL BIOLOGY

Edited by
Kwang W. Jeon



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A Survey of
# Cell Biology

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# A Survey of Cell Biology

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*This volume of* International Review of Cytology *is dedicated to the memory of Prof. Vladimir Pantic, who passed away on May 19, 2006. He was a valued advisor and board member from 1974 to 2006. His contributions will be missed.*

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# Biology of Langerhans Cells and Langerhans Cell Histiocytosis

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Langerhans cells (LC) are epidermal dendritic cells (DC). They play an important role in the initiation of immune responses through antigen uptake, processing, and presentation to T cells. Langerhans cell histiocytosis (LCH) is a rare disease in which accumulation of cells with LC characteristics (LCH cells) occur. LCH lesions are further characterized by the presence of other cell types, such as T cells, multinucleated giant cells (MGC), macrophages (MΦ), eosinophils, stromal cells, and natural killer cells (NK cells). Much has been learned about the pathophysiology of LCH by studying properties of these different cells and their interaction with each other through cytokines/chemokines. In this review we discuss the properties and interactions of the different cells involved in LCH pathophysiology with the hope of better understanding this enigmatic disorder.

**KEY WORDS:** Langerhans cell, Langerhans cell histiocytosis cell, Langerhans cell histiocytosis, Cytokines, Bystander cells. 

## I. Introduction

Much progress has been made in the field of dendritic cell (DC) biology in the past few years, including a significant expansion of our knowledge on epidermal DC, also known as Langerhans cells (LC). Studies on origin and lineage of DC revealed that these antigen-presenting cells can develop from myeloid as well as lymphoid progenitors (Shortman and Caux, 1997). This finding raised the question whether LC can also be generated from different precursors, which was found to be the case in mice (Anjuere *et al.*, 2000).



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Further insight into regulation of migration, maturation, and antigen presentation by LC is of particular interest for the understanding of the disease Langerhans cell histiocytosis (LCH), which is characterized by the accrual of cells with a LC phenotype (Arceci *et al.*, 2002; Laman *et al.*, 2003). Recent studies on phenotype and function of Langerhans cell histiocytosis cells (LCH cells) (Geissmann *et al.*, 2001), as well as cytokine and chemokine interactions between different types of cells within LCH lesions (Annels *et al.*, 2003; Egeler *et al.*, 1999), have shed more light on the pathogenesis of LCH, and may be useful for developing an immunotherapeutic strategy for this disease.

## II. Properties of Langerhans Cells (LC)

### A. Origins, Development, and Distinguishing Markers

LC are DC of the skin (Girolomoni *et al.*, 2002) located in the epidermis. LC represent approximately 2% of all epidermal cells in the skin (Bauer *et al.*, 2001). There are two different theories on the origin of LC, based on whether the cell of origin is a myeloid or lymphoid precursor. Evidence for a DC precursor from a myeloid lineage derives, in part, from human and mouse *in vitro* DC differentiation assays (Ardavin *et al.*, 2001). A novel mouse bone marrow (BM) progenitor cell, named macrophage (MΦ) DC progenitor (MDP) was described by Fogg *et al.* MDP express CD117 (c-KIT) and the chemokine receptor CX3CR1 but not markers of lineage-committed precursors ($Lin^-$). *In vivo* experiments showed that MDP give rise to monocytes, subsets of MΦ and $CD11c^+$ $CD8\alpha^+$ and $CD11c^+$ $CD8\alpha^-$ DC (Fogg *et al.*, 2006). Zhang *et al.* showed that murine BM-derived $Lin^-$ $c\text{-}KIT^+$ hematopoietic progenitor cells (HPC) cultured in the presence of GM-SCF, TNF-α, and SCF differentiated into $CD11b^-$ immature DC. Further stimulation with GM-CSF and TNF-α resulted in LC-like cells (Zhang *et al.*, 1998). *In vitro* stimulation of $Lin^-$ $c\text{-}KIT^+$ murine BM-derived HPC with GM-CSF and TGF-α has been shown to result in differentiation into monocyte/MΦ cell types. Further stimulation of these monocyte/MΦ-like cells with GM-CSF and TNF-α also resulted in LC-like cells (Zhang *et al.*, 1999).

In contrast to mice, human $CD34^+$ HPC from cord blood stimulated with GM-CSF and TNF-α develop along two independent DC pathways (Fig. 1). After 5–7 days of culture, two distinct precursor populations, based upon either CD1a or CD14 expression, can be identified. It should be noted that these precursors are not able to proliferate but are only able to differentiate, whereas precursors lacking CD1a and CD14 can proliferate. Under further stimulation with GM-CSF and TNF-α over 12 days, $CD1a^+$-derived cells differentiate into typical LC characterized by the expression of

E-cadherin, Lag antigen, and Birbeck granules (BG). $CD14^+$-derived cells lack LC characteristics. Instead they express markers of dermal (interstitial) and blood DC, such as CD2, CD9, CD68, and Factor XIIIa (Caux *et al.*, 1996). Jaksits *et al.* have demonstrated that human $CD34^+$ HPC, when stimulated with GM-CSF and TNF-α, give rise to $CD14^+CD11b^-$ and $CD14^+CD11b^+$ cells. Further stimulation of these subpopulations with GM-CSF, TNF-α, and TGF-β showed that only the $CD14^+CD11b^-$ cells differentiated into CD1a and E-cadherin expressing LC (Jaksits *et al.*, 1999). Human peripheral blood $CD14^+$ monocytes, as well as human peripheral blood $CD1a^+CD11c^+$ DC cultured in the presence of GM-CSF, TGF-β, and IL-4, have also been found to differentiate into LC (Geissmann *et al.*, 1998; Ito *et al.*, 1999).

DC derived from the lymphoid lineage are called thymic DC. Functional studies showed that these thymic DC have a different function. Compared to myeloid-derived DC, these cells seem to be involved in the regulation of the immune response of $CD4^+$ T cells and presentation of self-antigen to developing thymocytes, leading to deletion of self-reactive T cells (Austyn, 1998; Shortman and Caux, 1997; Suss and Shortman, 1996). Multipotent lymphoid precursors, termed $CD4^{low}$ precursors, have been shown to be able to generate lymphoid CD8 expressing DC, T cells, B cells, and NK cells (Ardavin *et al.*, 1993; Moore and Zlotnik, 1995). In regard to whether some LC are derived from the lymphoid lineage, Anjuere *et al.* demonstrated that mouse epidermal LC negative for CD8 and leukocyte function-associated antigen 1 (LFA-1) showed a maturation-induced expression of CD8/LFA-1 following migration into the lymph nodes (Anjuere *et al.*, 1999), suggesting that some LC are possibly derived from the $CD8^+$ lymphoid subset (Anjuere *et al.*, 2000). This observation led to the question of whether multipotent lymphoid precursors would be able to generate epidermal LC. To test this hypothesis, $CD4^{low}$ lymphoid precursors and $CD44^+$ $CD25^+$ pro-T cell precursors were isolated from Ly 5.2 mice and transferred intravenously into γ–irradiated Ly 5.1 recipient mice. Analysis of the Ly 5.1 mice showed that the LC were reconstituted by both donor-derived precursor populations, confirming the hypothesis (Anjuere *et al.*, 2000).

The ontogeny of LC in mice has also illustrated important DC lineage relationships. Dendritic epidermal leukocytes (DEL) are considered LC precursors. These cells are $ADPase^+$ $CD11b^+F4/80^+CD32^+$MCH class $II^-$ $CD205^-CD207^-CD90^-CD3^-$ cells and reside in the murine fetal epidermis (Chang-Rodriguez *et al.*, 2004; Dewar *et al.*, 2001; Elbe *et al.*, 1989; Kobayashi *et al.*, 1987; Reams and Tompkins, 1973; Romani *et al.*, 1986; Schweizer and Marks, 1977; Tripp *et al.*, 2004). Chang-Rodriguez *et al.* have shown that serum-free DEL cultures, supplemented with GM-CSF, SCF, FLT3L, and TGF-β1, developed into colonies with LC morphology. Furthermore, they demonstrated that 20–35% of all MHC class $II^+$ neonatal DEL were in G2/M

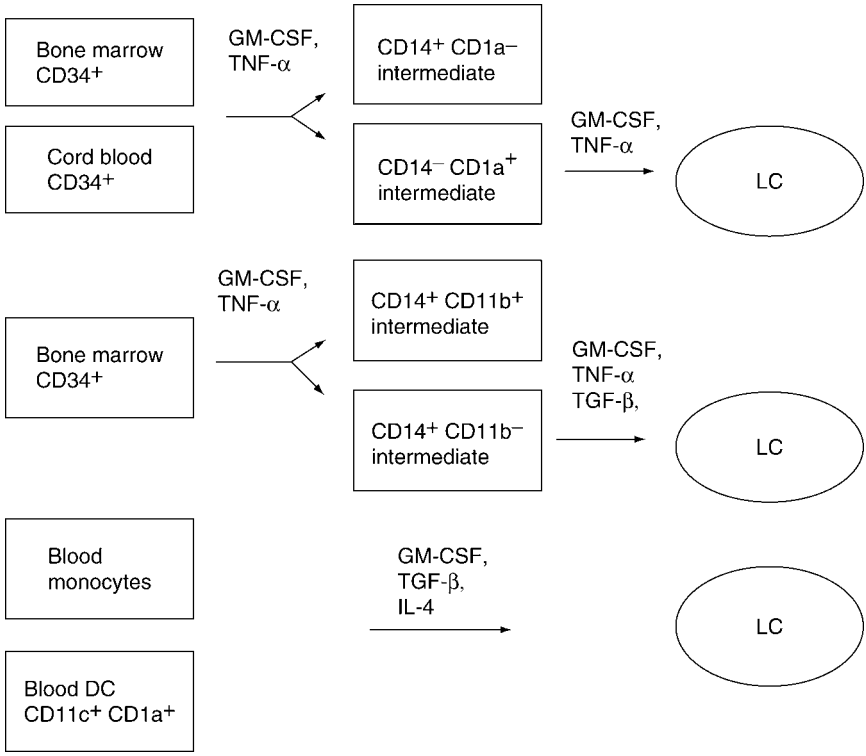


FIG. 1 *In vitro* differentiation pathways for LC in humans. The cytokines required for differentiation are indicated. Evidence for the cited pathways are described in Caux *et al.*, 1996; Geissmann *et al.,* 1998; Ito *et al.*, 1999; Jaksits *et al.*, 1999. (Adapted from Ardavin *et al.*, 2001, with permission from Elsevier).

phase of the cell cycle, indicating a high turnover of LC precursors in neonatal epidermis. Five percent of LC in the adult epidermis proved to be in the cell cycle, indicating that LC divide in the skin (Chang-Rodriguez *et al.*, 2005). These data are consistent with results from other investigators (Czernielewski and Demarchez 1987; Czernielewski *et al.*, 1985; De Fraissinette *et al.*, 1988; Merad *et al.*, 2002; Miyauchi and Hashimoto, 1989a,b). To test whether LC can develop from skin precursors *in vivo*, whole fetal skin was grafted onto adult severe combined immunodeficient (SCID) mice. This led to the appearance of dendritic MHC class $II^+$ donor cells in the epidermis (distinguished from recipient cells by selective staining of MHC class II haplotypes), expressing CD205 (DEC205) and CD207 (Langerin), both characteristic for LC (Chang-Rodriguez *et al.*, 2005).

TGF-β1 and macrophage inflammatory protein (MIP)-3α/CC chemokine ligand (CCL20) are known to have particularly prominent roles in LC

function. Evidence for an essential role of TGF-β1 in the development of LC was demonstrated by studies using TGF-$\beta^{-/-}$ mice. These specific mice lack LC in the epidermis. This abnormality was not due to the inflammatory wasting syndrome, commonly seen in these TGF-$\beta1^{-/-}$ mice. With inflammatory wasting syndrome, infiltrating lymphocytes destroy LC but TGF-$\beta1^{-/-}$ mice treated with rapamycin, an immunosuppressive agent, also lacked LC in the epidermis (Borkowski *et al.*, 1996; Koch *et al.*, 2000). Furthermore, lethally irradiated TGF-$\beta1^{+/+}$ recipients were able to produce LC upon being transplanted with bone marrow of TGF-$\beta1^{-/-}$ mice. This suggests that a paracrine effect of TGF-β1 is necessary for LC formation (Borkowski *et al.*, 1997a). The skin from TGF-$\beta1^{-/-}$ mice does not contain an excess of TNF-α and IL-1, cytokines known to have a role in the migration of LC from the epidermis (Borkowski *et al.*, 1997b). These findings suggest that LC precursors are normally present in TGF-$\beta1^{-/-}$ mice, but in the absence of TGF-β1, they are unable to enter the epidermis and/or differentiate into LC (Thomas *et al.*, 2001).

Radeke *et al.* (2005) have described overlapping pathways of sphingosine 1-phosphate (S1P) and TGF-β as potent chemoattractants of the phenotypically immature cells of the XS52 cell line (a murine epidermal-derived DC line, which retains important features of resident LC [Takashima *et al.*, 1995; Xu *et al.*, 1995]). Furthermore, they identified that the transcription factor, Smad3, plays a key role in the transmission of signaling effects of both S1P and TGF-β in this cell line (Radeke *et al.*, 2005). In humans, the development of LC has been shown by Strobl *et al.* to be dependent on TGF-β, which causes $CD34^+$ HPC cultures to develop into cells with highly immunostimulatory capacity, strong CD1a expression and Lag reactivity (Strobl *et al.*, 1996). These findings are specific for epidermal/mucosal LC and DC populations from the lymph node and the thymus (Kashihara *et al.*, 1986).

The chemokine MIP-3α/CCL20 is produced by epithelial cells, particularly at sites of inflammation. It plays a central role in the regulation of LC precursor recruitment and acts as a potent chemoattractive molecule via its cognate CC chemokine receptor (CCR6), expressed by LC precursors (Baba *et al.*, 1997; Dieu-Nosjean *et al.*, 1999, 2000; Tohyama *et al.*, 2001).

The BG is a pentilaminar cytoplasmic organelle that is unique to LC (Birbeck *et al.*, 1961). Therefore it may be used to identify LC in tissues other than the epidermis (Duijvestijn and Kamperdijk, 1982; Fokkens *et al.*, 1991; Hoshino *et al.*, 1970; Lenz *et al.*, 1993; Macatonia *et al.*, 1987; Millonig *et al.*, 2001; Schon-Hegrad *et al.*, 1991). Langerin (CD207), a type II $Ca2^+$–dependent C-type lectin displaying mannose-binding specificity, plays an important role in the formation of BG. Valladeau *et al.* (2000) identified and characterized this molecule and found it is constitutively associated

with BG and the anti-Langerin antibody is internalized into these organelles. Furthermore, they have shown that transfection of fibroblasts with Langerin cDNA resulted in BG formation, suggesting that expression of the Langerin gene is sufficient to drive BG formation (Valladeau *et al.*, 2000). Consistent with these findings, Verdijk *et al.* have demonstrated that a heterozygous mutation in the Langerin gene, resulting in an amino acid change from tryptophan to arginine at position 264 in the carbohydrate recognition domain of the Langerin protein, is responsible for the lack of BG in LC (Verdijk *et al.*, 2005). In addition to inducing BG formation, Langerin is involved in the trafficking of exogenous mannosylated ligands from the cell surface into intracellular BG compartments. From this finding, it can be speculated that BG may have a storage function; antigen internalized via Langerin is stored in the BG, until the LC enters T cell–rich areas in draining lymph nodes. BG disappear there and the antigen is re-exported to the cell surface (Valladeau *et al.*, 2003).

Besides Langerin, human LC also express other type II C-type lectins such as Dectin-1 (Ariizumi *et al.*, 2000), which recognizes β-glucan and mediates the production of TNF-α in response to live yeast pathogens (Brown *et al.*, 2003). Dectin-1 transfectant cells have been shown to trigger up-regulated expression of CD25, CD69, and CD40L (CD154) on T cells and to increase their IFN-γ production. Furthermore, Dectin-1 transfected cells have been shown to be involved in the induction of proliferation of $CD3^+$, $CD4^+$, and $CD8^+$ T cells (Grunebach *et al.*, 2002). Dectin-2 (exclusively found in LC) is a type II, C-type lectin which may be involved in T cell polarization into Th1 or Th2 cells. (Gavino *et al.*, 2005). Of the type I C-type lectins, DEC-205 (CD205) is expressed on human LC. Langerin and DEC-205 appear to be inversely regulated. The expression of Langerin is moderately down-regulated whereas expression levels of DEC-205 increase upon DC maturation. Within the epidermis, Langerin, Dectin-1, and DEC-205 may mediate the uptake of microbes and microbial particles (Ebner *et al.*, 2004).

Human LC express a number of markers that are consistent with their origin and function, such as the myeloid markers CD13 and CD33 and the leukocyte marker CD45. Different types of α-integrins such as CD11c, CD49c-f, β-integrins, adhesion molecules such as CD15s, CD40, CD44, CD50, CD54, cutaneous lymphocyte antigen (CLA), E-cadherin (CD324), CD95, CD95L, and CD120b are also found on LC (Peiser *et al.*, 2003; Valladeau and Saeland, 2005). Human LC, in line with being antigen-presenting cells, express MHC class I and class II molecules, CD74 (class II-specific chaperone, Ii, invariant chain), CD1a, CD1c, and, depending upon maturation stage, CD80, CD83, and CD86 (Elder *et al.*, 1993; Rattis *et al.*, 1996).

Finally, human LC express a variety of receptors such as the receptors for IL-1, IL-6, tumor necrosis factor-α (TNF-α), granulocyte/MΦ colony-stimulating factor (GM-CSF), and interferon γ (IFN-γ). Furthermore, FcγRI

(CD64), FcγRII receptor (CD32) (Tigalonowa *et al.*, 1989), and the FcεRI receptor (CD23) are expressed (Bieber *et al.*, 1989; Lappin *et al.*, 1996; Larregina *et al.*, 1996; Romani and Schuler, 1992), the latter being responsible for binding IgE.

## B. Immunologic Functions

### 1. Immature LC

Immature LC are the sentinel antigen-presenting cells of the epidermis and are pivotal to the initiation of immune responses (Shelley and Juhlin, 1976). They can use different pathways to recognize and capture antigens. Soluble antigen is internalized via two distinct mechanisms. Small molecules are taken up via clathrin-coated pits (micropinocytosis). Larger particles are internalized by cytoskeleton-dependent membrane ruffling (macropinocytosis) (Swanson and Watts, 1995). Phagocytosis is used for the uptake of a variety of antigens. Reis e Sousa *et al.* have shown that freshly isolated mouse LC are able to internalize latex beads, *Saccharomyces cerevisiae*, *Staphylococcus aureus*, *Cryptosporidium parvum*, and the yeast wall compound zymosan (Reis & Sousa *et al.*, 1993). Pathogen uptake through receptor-mediated endocytosis is done by the different C-type lectins, antigen-presenting molecules, and Fc receptors expressed by LC. Langerin captures glycolipids derived from *Mycobacterium leprae* (Hunger *et al.*, 2004), and human papilloma virus 6bL1 virus-like particles (HPV 6bL1 VLP) (Yan *et al.*, 2004). It also binds efficiently to both HIV-1 and HIV-2 gp120 proteins (Turville *et al.*, 2002) and a Lewis X-related sequence (Galustian *et al.*, 2004).

CD1a, a sialoglycoprotein associated with β2-microglobulin (β2-m), is abundantly expressed on LC (Fithian *et al.*, 1981). CD1a belongs to the CD1 family of MCH class I–like proteins and functions in the presentation of foreign lipids and glycolipids to T cells (Porcelli and Modlin, 1999). CD1a is internalized by common receptor-mediated endocytosis and accumulates in BG together with Langerin (Hanau *et al.*, 1987). Langerin is responsible for internalization of microbial glycolipids to BG. The glycolipids are then loaded onto CD1a for presentation to T cells (Hunger *et al.*, 2004).

After uptake, antigen is processed into fragments that can be recognized by effector cells of the adaptive immune system. The deposition of the antigen in the lymphoid organs is essential for the generation of primary immune responses (Zinkernagel, 1996), thus requiring the migration of LC. This phenomena was first observed in a contact sensitivity model in guinea pigs by Silberberg-Sinakin *et al.* They described the presence of LC in the afferent lymphatics of the skin in response to the application of a contact allergen (Silberberg-Sinakin *et al.*, 1976). The initiation of mobilization and

migration of LC is well documented in mice. TNF-α, an inducible product of keratinocytes, and IL-1β, which is found to be a constitutive and inducible product of LC themselves, are the key molecules (Cumberbatch *et al.*, 2000). It has been shown in mice that skin sensitization is associated with the up-regulated expression of LC-derived IL-1β (Enk and Katz, 1992). This cytokine has two functions. First, it acts in an autocrine fashion on LC as a stimulus for migration through the type I IL-1 receptor (IL-1RI). Second, IL-1β provides the stimulus for an increased production of TNF-α by keratinocytes (Enk *et al.*, 1993). TNF-α provides the signal for migration through type 2 receptors for TNF-α (TNF-R2) (Wang *et al.*, 1996).

The regulation of LC migration in humans is most likely similar to that observed in mice. Groves *et al.* (2005) have shown that intradermally administered TNF-α induced a dose- and time-dependent decrease in $CD1a^+$ epidermal LC numbers and an increase in dermal $CD1a^+$ cells, suggesting migration of LC away from the epidermis (Groves *et al.*, 1995). Cumberbatch *et al.* have shown that TNF-α caused a reduced frequency of $HLA\text{-}DR^+$ epidermal LC (Cumberbatch *et al.*, 1999). Experiments with intradermal administration of IL-1β in humans have established that IL-1β is able to stimulate migration of LC away from the epidermis and that this process is associated with local release of TNF-α. Further, additional evidence was found that topical administration of the iron-binding protein, LF, abrogates not only IL-1β–mediated LC migration but also reduces the local release of TNF-α protein. This suggests that LF may inhibit LC migration through its ability to control local production of TNF-α (Cumberbatch *et al.*, 2003).

LC are physically connected to keratinocytes by E-cadherin bonds. An additional role for TNF-α and IL-1β is down-modulation of E-cadherin expression, allowing LC to exit the epidermis (Schwarzenberger and Udey, 1996). At the level of cell adhesion, $\alpha_6$ integrins are directly involved in the regulation of LC migration from the epidermis. Price *et al.* have demonstrated the anti-$\alpha_6$ integrin antibody, GoH3, which blocks binding to laminin, completely prevented the spontaneous migration of LC from skin explants *in vitro* and the rapid migration of LC from mouse ear skin induced after intradermal administration of TNF-α *in vivo* (Price *et al.*, 1997).

Several chemokine receptors are involved in the migration of LC, such as CCR7 and CCR5. Evidence for a CCR7 role in LC migration was found in $CCR7^{-/-}$ mice, which show severely impaired migration of LC to draining lymph nodes (Forster *et al.*, 1999). A similar observation was made in *plt/plt* mice. These mice are homozygous for the *p*aucity of *l*ymph node *T* cell (plt) mutation, with the consequence that naïve T cells are unable to home to lymph nodes and lymphoid regions of the spleen (Nakano *et al.*, 1997). *plt/plt* mice lack expression of the CCR7 ligands CCL19 and CCL21-Ser. Further investigation revealed that CCR7 is required for LC migration

into dermal lymphatics under inflammatory conditions. Under steady-state conditions, a CCR7-based mechanism is also effective in directing a subpopulation of LC to the skin-draining lymph nodes. Because this subpopulation of cells is believed to play a role in peripheral tolerance, this observation may suggest a role of CCR7 in maintenance of peripheral tolerance (Ohl *et al.*, 2004).

Evidence for a specific role for CCR5 was found in the investigation of LC migration in the inflamed cornea model. In this study, thermal cautery of the cornea led to an increase in MHC class $II^+$ LC in the central areas of the corneal epithelium, which were associated with increased expression of the chemokines RANTES, MIP-1α, MIP-1β, eotaxin, and MIP-2. At the same time, CCR1 (e.g., RANTES, MIP-1α, and MCP-3), CCR2 (MCP-1, -2, and -3), and CCR5 (e.g., RANTES, MIP-1α, and MIP-1β) receptor genes showed increased expression. The functional relevance of CCR1, CCR2, and CCR5 was assessed by studying the recruitment of MHC class $II^+$ LC in $CCR1^{-/-}$, $CCR2^{-/-}$MIP-$1\alpha^{-/-}$, MIP-$1\alpha^{-/-}$, and $CCR5^{-/-}$ mice, compared with respective wild-type mice. These studies revealed that MHC class $II^+$ LC in the corneal epithelium were significantly, but not entirely, suppressed in $CCR5^{-/-}$ mice compared with wild-type, control animals. No significant differences were found in $CCR1^{-/-}$, $CCR2^{-/-}$ MIP-$1\alpha^{-/-}$, and MIP-$1\alpha^{-/-}$ mice when compared with controls. Furthermore, it was found that blockade of the CCR5 ligands, RANTES, and MIP-1β decreased the number of MHC class $II^+$ LC in the epithelium, demonstrating the functional relevance of CCR5 in the recruitment of MHC class $II^+$ LC (Yamagami *et al.*, 2005).

Matrix metalloproteinases (MMPs) cleave the various components of the extracellular matrix. Several studies have shown that MMP2, MMP3, and MMP9 play a role in migration of LC (Kobayashi *et al.*, 1999; Lebre *et al.*, 1999; Ratzinger *et al.*, 2002; Wang *et al.*, 1999). Protein kinase C (PKC) acts by transducing extracellular signals across the cell surface. PKC also contributes to the promotion of LC migration (Halliday and Lucas, 1993; Reynolds *et al.*, 1995). Epidermal PKC-β and PKC-$\beta_2$ have been found to be exclusively expressed in LC (Goodell *et al.*, 1996; Koyama *et al.*, 1991).

Finally, there are several proteins involved in the homing of migrating LC to draining lymph nodes. CD44 is an integral membrane protein that binds a variety of ligands. Numerous CD44 isoforms are being generated by extensive alternative splicing and post-translational modifications, which explains the array of potential CD44 ligands, as well as its varied biologic functions (Isacke and Yarwood, 2002). Several studies using $CD44^{-/-}$ mice and models of contact hypersensitivity (CHS) and LC migration have shown contradictory results, reporting normal (Schmits *et al.*, 1997) as well as impaired CHS responses (Kaya *et al.*, 1999) in $CD44^{-/-}$ mice. Mummert *et al.* (2004) demonstrated that LC migration from the epidermis in $CD44^{-/-}$ mice was

similar to that in wild-type mice. However, when fluorescein-5-isothiocyanate (FITC) was topically applied and the number of $CD11c^+/FITC^+$ cells in the draining lymph nodes counted, a significantly lower number of cells was found in the $CD44^{-/-}$ mice. This suggests that CD44 plays an essential role in at least LC homing to lymph nodes (Mummert *et al.*, 2004).

## 2. Mature LC

During migration, immature LC lose antigen uptake and processing properties as they differentiate into mature cells specialized for T cell stimulation (Banchereau and Steinman, 1998; Steinman, 1991). This process of maturation/activation is induced by several mediators. Gatti *et al.* (2000) have demonstrated that maturation of $CD34^+$-derived human LC was induced by TNF-α, lipopolysaccharide (LPS), and CD40L based on the profile of surface markers (increased surface expression of MHC class II and upregulation of CD80, CD86, and CD40). They found that each stimulatory agent was able to generate cells with markedly different morphologies, and, in part, different levels of functionality. TNF-α–treated cells did not develop further than the "intermediate" DC phenotype, resulting in a consistently diminished T-cell stimulatory activity in allogeneic assays. LPS and CD40L drove cells to complete maturity. Next to being competent APC, the LPS and CD40L-stimulated cells also produced IL-12, which is critical for the induction of Th1 T-cell responses (Gatti *et al.*, 2000). Consistent with this finding, Berthier-Vergnes *et al.* have shown that TNF-α enhances phenotypic maturation of highly purified LC from human skin. CD40, CD54, CD83, CD86, CCR7, and DC-LAMP are significantly up-regulated. In contrast with the finding of Gatti *et al.*, TNF-α strongly enhanced the allostimulatory capacity of LC. Furthermore, TNF-α induced LC to produce both IFN-γ-inducible-protein IP-10/CXCL10, a Th1-attracting chemokine, and IL-12p40 (p40 subunit of IL-12) (Berthier-Vergnes *et al.*, 2005).

Maturation of LC can also be induced following recognition of pathogen-associated molecular patterns (PAMP) by toll-like receptors (TLR). Mitsui *et al.* have shown that murine immature LC express mRNA encoding TLR2, TLR4, and TLR9 proteins but not TLR7. Furthermore, it was demonstrated that TLR2, TLR4, and TLR9 interaction with their corresponding ligands (SAC, LPS, and CpG) induced IL-6 and IL-12p40 production by the LC and inhibited TARC production; the only exception was that LPS showed no effect on IL-12p40 production (Mitsui *et al.*, 2004).

CD40 and its ligand, CD40L (CD154) are members of the TNF superfamily (Aggarwal, 2003). CD40L is mainly expressed by activated T cells (Armitage *et al.*, 1992). A study on CD40 expressing LC derived from $CD34^+$ HPC showed that CD40/CD40L cross-linking resulted in enhanced survival of LC, permitting prolonged LC/T cell interactions. Furthermore,

CD40/CD40L binding triggered LC maturation, evident from maintenance of high levels of MHC class II antigens and up-regulation of co-stimulatory molecules, such as CD80 and CD86 (Caux *et al.*, 1994). Peiser *et al.* have shown that CD40L-stimulated human epidermal LC induce T-cell proliferation and stimulation of naïve, allogeneic $CD4^+$ T cells to produce IFN-$\gamma$. Noteworthy is the finding of weak IL-12p70 (heterodimeric form of IL-12) production by these LC, even after stimulation with TLR-ligands, suggesting that natural LC produce an as-yet unidentified T-cell activating factor (Peiser *et al.*, 2004). A similar observation was done by Ratzinger *et al.* who demonstrated that LC had the most significant CTL stimulatory activity, even without production of IL-12p70. Significant IL-15 secretion by LC could possibly be an explanation for this observation (Ratzinger *et al.*, 2004).

## III. Langerhans Cell Histiocytosis Cell (LCH Cell)

### A. Features of LCH

LCH, formerly known as histiocytosis-X, eosinophilic granuloma, Abt-Letterer-Siwe disease, and Hand-Schüller-Christian disease (Coppes-Zantinga and Egeler, 2002), is a rare disorder of unknown etiology. The annual incidence of LCH has been reported to be 3–7 cases per million people (Carstensen and Ornvold, 1993; Kaatsch *et al.*, 1994; Nicholson *et al.*, 1998). It affects all age groups, but the features are much better defined in children than in adults (Arico, 2004). LCH may present with either involvement of a single site or as a multisystem disease. The most common sites include skin, bone, and lymph nodes. Multisystem disease affects at least two different organs but can involve any organ system.

The clinical spectrum of LCH is variable; patients can present with mild discomfort related to affected organs as well as generalized symptoms including fever and failure to thrive. A definitive diagnosis is made based on characteristic histology of involved tissue as well as the presence of BG-positive or CD1a-positive LC (Chu *et al.*, 1987) (Figs. 2 and 3).

The outcome of LCH varies. In some cases, the disease can resolve spontaneously, but it can also disseminate and cause damage to vital organs with a fatal outcome (Arceci *et al.*, 2002). Patients with limited involvement of LCH, particularly of the skeleton, have a good prognosis, sometimes requiring only local treatment interventions such as surgical curettage or steroid injections into bone lesions. However, patients with multisystem disease will nearly always benefit from systemic therapy, which usually includes a combination of corticosteroids and chemotherapeutic agents (Arceci *et al.*, 2002).

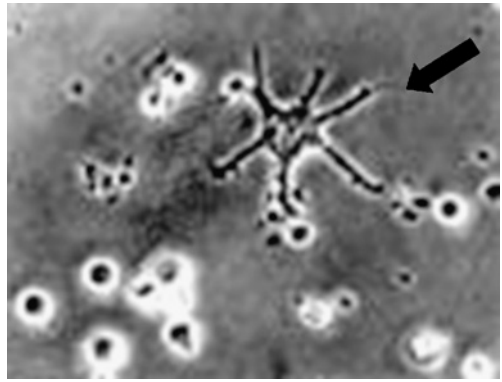

FIG. 2 DC with typical dendritical processes. (Photograph courtesy of Dr. J. J. Mulé.)

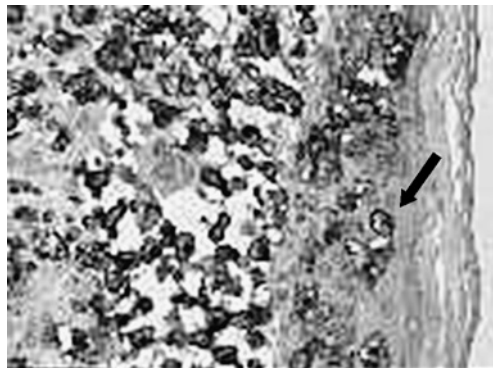

FIG. 3 Lesional LCH cells stained for CD1a and lack dendritic morphology. (Photograph courtesy of Dr. H. Kutzner.)

## B. LCH Cell versus LC

### 1. Morphology and Immunophenotype

Similar to LC, hematoxylin-eosin–stained LCH cells have moderate amounts of homogeneous pink cytoplasm. The nucleus is usually folded, indented, or lobulated; shows an irregular granular chromatin pattern; and several small nucleoli. In contrast to LC, LCH cells are rounded and lack typical dendritic extensions (Schmitz and Favara, 1998).

In a study on the phenotype of LCH cells in patient samples, a 100% concordance of CD1a and Langerin expression was found. CD68 co-expression was found on $CD1a^+$ $Langerin^+$ LCH cells in all patients, an association which was earlier described by other investigators (Hage *et al.*, 1993; Ornvold *et al.*, 1990).

$CD1a^+$ LCH cells isolated from bone and lymph node lesions also showed CD14 co-expression. (Geissmann *et al.*, 2001). Normal LC express little to no CD14 (Davis *et al.*, 1988), although Caux *et al.* have shown that $CD34^+$ HPC, cultured in the presence of GM-CSF and TNF-$\alpha$, develop into a distinct population of $CD1a^+$ $CD14^+$ double positive cells from day 7–10 of culture, which then further develop into either LC or dermal (interstitial) DC (Caux *et al.*, 1996). Murphy *et al.* have shown that decreased LC populations in the skin of patients after allogeneic BM transplantation were replenished by dermal and epidermal DC exhibiting co-expression of monocyte/MΦ and LC surface antigens (Murphy *et al.*, 1986). Assessment of MHC class II and co-stimulatory molecules expression showed that samples taken from patients with bone lesions/disseminated diseases expressed intracellular MCH class II, but almost never expressed CD83, CD86, and DC-LAMP (markers of mature DC). These findings reflect the immature state of differentiation of LCH cells in bone or disseminated lesions that resembles the phenotype of immature LC. In contrast, LCH cells from isolated skin lesions showed a more mature phenotype with expression of CD83, CD86, and DC-lamp but not CD14 (Geissmann *et al.*, 2001). Emile *et al.* have also demonstrated expression of CD80, CD86, CD24, and LFA-1 in LCH cells in skin lesions, suggesting an activated state. A few normal LC in these lesions, identified by their dendritic morphology, showed the same phenotype (Emile *et al.*, 1994), suggesting that besides the inflammatory milieu other factors are responsible for the development of LCH cells. De Graaf *et al.* concluded that the phenotype of the LCH cell has characteristics of epidermal LC as well as activated LC. They found expression of CD1a, CD62L/L-selectin, and the presence of BG, all characteristic for epidermal LC as well as strong expression of CD58, CD54/ICAM-I, CD49d/VLA-4, $\beta$1, and $\beta$2 integrin chains of integrins, which are characteristic of activated LC (de Graaf *et al.*, 1995). High expression of CD80 and CD86 in pulmonary LCH cells has been confirmed by Tazi *et al.*(1999). CD40 appears to be more prominently expressed on LCH cells than on LC (Egeler *et al.*, 2000; Geissmann *et al.*, 2001). The finding of a high number of CD40L-expressing T cells in the same LCH lesions suggests a potential role for the CD40–CD40L interaction in the pathogenesis of LCH (Table I).

## 2. Cell Cycle-Related Proteins

LCH cells have been found to be clonal in both localized and multisystem forms of the disease, with the exception of smoking-related adult pulmonary LCH (Willman *et al.*, 1994; Yousem *et al.*, 2001; Yu *et al.*, 1994). Familial cases of LCH have also been documented (Arico *et al.*, 1999). Based on these findings, it has been hypothesized that a genetic alteration affecting cell proliferation, cell cycle regulation and/or apoptosis might be responsible for the development of LCH. Schouten *et al.* have investigated the expression

TABLE I
Characteristic Markers of Epidermal LC, Activated DC, and LCH Cells

| | Epidermal LC | Activated lymph node DC | LCH cells |
|---|---|---|---|
| **DC markers** | | | |
| **Immature** | | | |
| CD1a | + | − | + |
| Langerin | + | − | + |
| Birbeck granules | + | − | + |
| CD14 | + | − | + |
| CD68 | ± | − | + |
| CLA | + | − | + |
| CCR6 | + | − | + |
| **Mature** | | | |
| CD83 | − | + | − or ± |
| Fascin | − | + | − or + |
| DC-LAMP | − | + | − or ± |
| CCR7 | − | + | − or + |
| **Cell activation** | | | |
| CD25 | − | + | + |
| CD116 | ± | + | + |
| **Antigen presentation** | | | |
| MHC class II | ± (intracellular) | ++ (surface) | + (intracellular) |
| CD40 | + | ++ | ++ |
| CD80 and CD86 | − | + | ± or + |
| APC function | ± | ++ | − or + after stimulation with CD40 |
| **Adhesion molecules** | | | |
| E-cadherin | ++ | − or ± | − or ± |
| CD49d | − | + | + |
| CD54 | ± | ++ | + or ++ |
| CD2–CD58 | − or ± | ++ | + or ++ |

APC, antigen-presenting-cell; CCR, chemokine receptor. Adapted from Laman *et al.*, 2003, with permission from Elsevier.

of the proliferation marker Ki-67 in addition to several gene products that regulate proliferation and apoptosis, including TGF-β receptor I and II, p53, MDM2, Bcl2, p21, p16, and Rb. They showed that Ki-67 is highly expressed in LCH lesions, indicating that the cell cycle is unlikely to be blocked in LCH cells. (Schouten *et al.*, 2002). A similar finding was reported by other

investigators (Bank *et al.*, 2003; Hage *et al.*, 1993). Furthermore, Schouten *et al.* demonstrated that in more than 90% of the cases, the expression of TGF-β receptor I and II, p53, MDM2, Bcl2, p21, p16, and Rb was detected in LCH cells. Bcl-2 was also found to be expressed in many lesional cells. These findings suggest that the cellular mechanisms that detect DNA damage and control proliferation and apoptosis are activated in LCH cells (Schouten *et al.*, 2002). Savell *et al.* have also demonstrated Bcl-2 expression in LCH cells, whereas normal LC do not express similar levels of Bcl-2. Southern blot hybridization analysis revealed no evidence for Bcl-2–gene rearrangements, suggesting that LCH cells may respond to signals that regulate normal Bcl-2 expression (Savell *et al.*, 1998). Björk *et al.* showed that *in vitro* cultured DC up-regulate Bcl-2 expression after CD40-ligation by T cells (Bjorck *et al.*, 1997). The presence of p53 in LCH cells, in contrast to the absence in LC, has also been reported by other investigators. Gene mutations in p53 have not been detected (Bank *et al.*, 2002; Petersen *et al.*, 2003; Weintraub *et al.*, 1998).

Fas (CD95, APO-1) and its ligand, Fas-L (CD95L), are also known to be mediators of apoptosis (Nagata and Suda, 1995). A relatively high percentage of LCH cells express both Fas and Fas-L. The frequency of Fas/Fas-L co-expression was found to be higher in single-system disease (69%) compared to multi-system lesions (46%). The presence of Fas and Fas-L in a LCH cell can lead to autocrine-mediated, apoptotic signals (Petersen *et al.*, 2003). Because only a part of the LCH lesions resolve spontaneously, it could be that co-expression of Fas and Fas-L contributes to spontaneous regression of early solitary LCH lesions. Older LCH lesions with a more fulminant disease stage may have found mechanisms to circumvent this apoptotic pathway. FADD/Fas-associated death domain–containing protein, FLICE/FADD-like interleukin-1β–converting enzyme (both pro-apoptotic) and FLIP/FLICE-inhibitory protein (anti-apoptotic) are proteins involved in the Fas signaling cascade. All three proteins were found to be highly expressed in LCH cells, supporting their potential importance in LCH (Bank *et al.*, 2005). *In situ* hybridization showed that the proliferation-stimulating oncogenes, *c-myc* and H-*ras,* had increased mRNA expression in LCH cells. Their elevated expression was only observed in the terminal proliferative phase, and not in the early phases of the disease (disease stages were based on clinical history, histology, and cytology) (Abdelatif *et al.*, 1990).

These observations show that inhibitory as well as stimulatory pathways of cell proliferation and apoptosis are expressed in LCH cells. Expression of p53, p21, p16, and Rb indicate that these proliferation inhibitory pathways are activated. In contrast, the high-level expression of Bcl-2, the presence of MDM2 (a p53 inhibitor), and the increased mRNA expression of *c-myc* and H-*ras,* would support survival and proliferation. Despite the presence of proliferation markers, the number of mitosis observed in LCH cells is usually low (Bank *et al.*, 2003; Laman *et al.*, 2003; Schmitz and Favara, 1998).

No gene mutations have thus far been reported in the cell cycle-related proteins studied to date. Together with the inability to propagate LCH cells *in vitro* for long periods of time or in immunodeficient mice, these findings suggest that the cellular micro-environment also plays an important role in the maintenance and progression of lesions (Schouten *et al.*, 2002).

## C. Chromosomal Alterations and Genomic Instability in LCH Cells

Because a genetic component has been suspected to play a role in the pathogenesis of LCH, chromosomal studies were undertaken. A cytogenetic investigation of 5 LCH lesions, each from a different patient, showed one chromosomal clonal abnormality in one case and non-clonal abnormalities in four cases. The clone was found to be a t(7;12)(q11.2;p13) translocation (Betts *et al.*, 1998). Scappaticci *et al.* performed chromosomal analyses on phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes (PBLs) of 11 patients with multisystem and five patients with single system LCH. A hundred (100) metaphases from each case were analyzed for the presence of chromatid and/or chromosomal breaks, 20 metaphases were also analyzed for the presence of chromosomal breaks. They reported that 11 patients (69%) had chromatid and chromosomal breaks in 7–45% of cells, with a significant difference between multisystem and single system disease (13.4% in multisystem versus 6.2% in single system, $p = 0.003$). Polyploid cells were observed in some cases and structural rearrangement was found in one case during the active phase of the disease (Scappaticci *et al.*, 2000).

Using both comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) analyses, the extent of somatic genetic changes in LCH lesions of the bone was assessed. Losses of DNA sequences were found on chromosomes 1p, 5, 6, 7, 9, 16, and 22q. For chromosome 1, the alterations were restricted to the 1p36 band in 6 out of 7 cases. The remaining case showed a larger deletion along the p21-p36 segment. A gain of DNA copy number was seen on chromosomes 2q, 4q, and 12. The highest frequencies of LOH were found on chromosome 1p region (3 of 7 cases) and on chromosome 7 (4 cases). Allelic losses were detected on chromosomes 9 (1 case) and 22q (2 cases). The region 1p35-p36.3, which contains several putative tumor suppressor genes (Weith *et al.*, 1996), was deleted in all cases. These findings suggest that chromosome 1 might harbor a tumor suppressor gene(s) which is involved in the development and progression of LCH (Murakami *et al.*, 2002). Another interesting observation was the alteration on chromosome 22q; 22q12 contains the neurofibromatosis type 2 gene (Ruttledge *et al.*, 1994), whose product, neurofibromin-1 (NF-1), a RAS GTPase, is involved in inactivating RAS. Mutations in NF-1 have been linked to not only neurofibromatosis type I but also to myeloproliferative disorders such as

juvenile myelomonocytic leukemia (Leung *et al.*, 2003). McClain *et al.* have shown in microdissected samples from paraffin-embedded LC and LCH tissues from three patients significantly higher levels of IL-13, NFκB, TNRF p55, RANK-L, CD40-L, and TGF-βR in LCH involving liver and spleen compared to LCH tissue taken from bone lesions (McClain *et al.*, 2005).

Researchers (Dacic *et al.,* 2003) have investigated the genotype of pulmonary LCH by CGH and LOH. The most frequently altered chromosomal locus was 22q (41% of cases). Locus 9p, which contains the tumor suppressor gene, p16, showed an allelic imbalance in 36% of the cases and locus 17p in 30% of the cases. The finding that pulmonary LCH is not a monoclonal disease (see later) suggests that chromosomal aberrations may not contribute in the pathogenesis of most cases of pulmonary LCH (Dacic *et al.*, 2003).

### D. Clonality of LCH Cells

A central question about LCH is whether it is a neoplastic or a reactive disorder. Neoplasms usually originate from a single clone of cells (Knudson, 1985) and can therefore be distinguished from reactive disorders, which are most commonly polyclonal. Clonality can be assessed by molecular analysis of X-chromosome inactivation patterns in female subjects (Vogelstein *et al.*, 1987). Using such an assay on the human androgen-receptor (HUMARA) gene locus, Willman *et al.* detected clonal cells in lesions of 9 of 10 patients. In 1 patient, clonality could not be determined definitely due to constitutional skewing of X-chromosome inactivation. Yu *et al.* demonstrated that CD1a-positive LCH cells from 3 patients with multisystem disease had a non-random X-chromosome-inactivation pattern, whereas CD1a-negative cells in the same tissue showed a random pattern. These combined results suggest that LCH is a result of a clonal expansion of LCH cells (Willman *et al.*, 1994; Yu *et al.*, 1994).

In contrast, in a study in which the HUMARA assay was applied to pulmonary LCH, 71% of the LCH nodules were found to be nonclonal. Furthermore, clonal LCH cells usually appeared in association with the presence of nonclonal LCH cell populations. These results indicate that pulmonary LCH is not uniformly a clonal proliferative process but mostly reactive. There may be some cases in which clonal evolution occurs, however (Yousem *et al.*, 2001).

## IV. Cytokine and Chemokine Networks

### A. Cytokine Expression

Cytokines are hormones that mediate communication between cells of the immune and hematopoietic systems (Kishimoto *et al.*, 1994). They function mostly in extracellular signaling through autocrine and paracrine pathways.

Cytokines can stimulate production of other cytokines, providing a cascade mechanism for amplifying biological responses under physiological circumstances. Such a cascade can also contribute to the development of pathologic conditions (Kannourakis and Abbas, 1994; Leonard, 2003).

In LCH lesions, the levels of cytokines have been demonstrated. De Graaf and colleagues have shown high levels of cytokines, including IL-1α, IL-1β, IL-4, GM-CSF, TGF-α, TGF-β, TNF-α, and IFN-γ, in LCH lesions. A small percentage of normal LC in healthy epidermis and mucosa expressed the same cytokines, while LC in lymph nodes with dermatopathic reaction expressed none of these cytokines. MGC, particularly found in bone lesions, have also been shown to express variable staining for all studied cytokines except bFGF (de Graaf *et al.*, 1996). Egeler *et al.* have demonstrated the presence of the hematopoietic growth factors, IL-3, IL-7, and GM-CSF, the lymphocyte regulatory cytokines, IL-2, IL-4, and IL-10 and the inflammatory regulators, IL-1α, and TNF-α as well as effector cell-activating cytokines, IL-5 and IFN-γ in LCH lesions. IL-1 was synthesized only by LCH cells. T cells produced IL-2, IL-4, IL-5, and TNF-α. IL-10 and GM-CSF were expressed by all cell types in equal amounts. MΦ appeared to be the main source of IL-7. Eosinophils were associated with the expression of IL-5, IFN-γ, GM-CSF, IL-10, IL-3, and IL-7 (Egeler *et al.*, 1999). A study investigating the expression of TNF-α, IL-11, and LIF by LCH cells from 9 patients demonstrated that TNF-α was expressed in lesions from all patients, while IL-11 was found in LCH cells of 7 of 9 and LIF expressed in 6 of 9 patients (Andersson By *et al.*, 2004). Emile *et al.* have shown that the GM-CSF receptor is present on LCH cells (Emile *et al.*, 1995).

## B. Chemokine Expression

Chemokines are small molecules that have a central role in inflammatory responses. They trigger activation and migration of lymphocytes and phagocytic cells, including LC. During different stages of development, LC synthesize a distinct pattern of functional chemokines and chemokine receptors. To determine whether chemokines might play a role in the pathogenesis of LCH, Annels *et al.* have investigated the presence of several chemokines and cognate receptors expressed by LCH cells. They reported that LCH cells in all lesions expressed CCR6, but not CCR7, markers for immature and mature DC respectively. Furthermore, they demonstrated that LCH cells produced the ligand for CCR6, CCL20/MIP-3α. CCL5/RANTES, and CXCL11/I-TAC, are both involved in recruitment of immature LC as well as other cell types to sites of inflammation. These results suggest that LCH cells are in an immature state of differentiation and synthesize chemokines

that may recruit and retain lesional cells (Annels *et al.*, 2003). Other investigators have reported that LCH cells co-express CCR6 and CCR7. This pattern of co-expression would be consistent with LCH cells migrating to tissues that express cognate ligands, such as CCL20/MIP-3α in bone and skin, and CCL19/ELC and CCL21/SLC in secondary lymphoid organs (Fleming *et al.*, 2003). It is unclear why these two studies have reported contradictory results, but methodologic differences may in part account for the differences.

## C. Mechanisms of Tissue Damage in LCH

Increasing evidence exists to implicate the involvement of cytokines in the tissue damage seen in LCH. Several studies have shown that a variety of cytokines are produced in LCH lesions. The presence of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL5, IL-7, IL-10, IL-11, LIF, GM-CSF, TGF-α, TGF-β, TNF-α, and IFN-γ have been reported (Andersson By *et al.*, 2004; de Graaf *et al.*, 1996; Egeler *et al.*, 1999). Besides LCH cells, the LCH lesions contain MGC, MΦ, neutrophils, eosinophils, lymphocytes, plasma cells, and occasional mast cells, which all can contribute to the production of cytokines (Kannourakis and Abbas, 1994).

Several cytokines have been found to play a role in bone damage resulting in characteristic osteolytic lesions. Purified LCH cells from bone lesions have been found to produce IL-1 and prostaglandin E2 (PGE2). IL-1 and PGE2 are known for their ability to stimulate bone resorption (Bockman and Repo, 1981; Gowen *et al.*, 1983). IFN-γ has been found to be produced by LCH cells, T cells, as well as macrophages in LCH lesions from the bone (Egeler *et al.*, 1999). Arenzana-Seisdedos *et al.* have demonstrated in an experiment in which LCH cells were stimulated with LPS, that pretreatment with IFN-γ significantly increased the IL-1 production by LCH cells. IFN-γ is, therefore, likely to contribute to the formation of bone lesions (Arenzana-Seisdedos *et al.*, 1986). Da Costa *et al.* have shown that the osteoclast-inducing cytokines RANKL and M-CSF are highly expressed by LCH cells and T cells in LCH lesions (da Costa *et al.*, 2005). TNF-α, IL-11, and LIF are also potent activators of osteoclast activity, thus suggesting that they too play a role in the bone involvement of LCH (Andersson By *et al.*, 2004).

Several studies have demonstrated that TGF-β, combined with GM-CSF, can induce LC development from various precursor cells, such as HPC (Caux *et al.*, 1999; Riedl *et al.*, 1997; Strobl *et al.*, 1996), peripheral blood monocytes (Geissmann *et al.*, 1998), peripheral blood $CD1a^+/CD11c^+$ cells (Ito *et al.*, 1999), and dermal resident $CD14^+$ cells (Larregina *et al.*, 2001). On the other hand, TGF-β can also induce LC from HPC in a culture in combination with M-CSF or IL-3 in the absence of GM-CSF (Mollah *et al.*, 2003).

IL-3 stimulates the generation and differentiation of the precursor cells of all hematopoietic lineages as well as displays a number of synergizing activities with other cytokines (Holldack *et al.*, 1992; Maurer *et al.*, 1993). In addition, GM-CSF plays an important role in recruitment of LC into different tissues (Kaplan *et al.*, 1992). Because high levels of TGF-β, IL-3, and GM-CSF have been reported in LCH lesions, it has been hypothesized that these cytokines contribute to the proliferation and differentiation of LCH cell progenitors and the accumulation of LCH cells (Egeler *et al.*, 1999). TGF-β has also been found to be a key factor in fibrogenesis (Border and Noble, 1994) and might, therefore, play a role in the development of the fibrosis observed in some LCH lesions of bone, liver, and lungs (Schmitz and Favara, 1998).

TNF-α, GM-CSF, and IL-3 together with several other cytokines function as chemoattractants for eosinophils, neutrophils, MΦ, and $CD34^+$ LC precursors (Caux *et al.*, 1992). TNF-α has other properties that could contribute to formation of LCH lesions, such as the stimulation of LC formation from $CD34^+$ progenitors (Reid *et al.*, 1992) and inhibition of the progression of mature LC to spontaneous apoptosis (Ludewig *et al.*, 1995). Survival of LC is likely supported by IL-1α, GM-CSF, and IL-3 (Ludewig *et al.*, 1995; Reid *et al.*, 1992). Taken together, these effects could promote survival and accumulation of LCH cells in LCH lesions.

Elevated soluble IL-2r (sIL-2r) serum levels correlate with the extent of the disease in patients with LCH (Rosso *et al.*, 2002; Schultz *et al.*, 1998). Expression of IL-2 in LCH lesions has also been reported (Egeler *et al.*, 1999). Because IL-2, as well as IL-2r, are products of activated T cells, this may be the cellular source for elevated levels of both proteins in LCH. LCH cells express inflammatory chemokines as well, including CCL20/MIP-3α, CCL5/RANTES, and CXCL11/I-TAC. These chemokines may be involved in recruiting more LCH cells and other types of inflammatory cells to LCH lesions. For instance, CCL20/MIP-3α has been found to be an important chemoattractant for memory T cells (Liao *et al.*, 1999), which are abundantly present in LCH lesions (Annels *et al.*, 2003; Laman *et al.*, 2003; Liao *et al.*, 1999). CCR6, the receptor for CCL20/MIP-3α, has been found on infiltrating T cells at sites of LCH activity, suggesting that LCH cells may be able to maintain disease activity (Annels *et al.*, 2003).

## V. Bystander Cells

### A. Interaction Between LCH Cells and T Cells

Lymphocytes account for approximately 20% of the lesional cells in LCH tissues (Favara and Steele, 1997). About 80% of these T cells are $CD4^+$ and express $CD45RO^+$, which indicates that they are memory cells (Annels *et al.*, 2003;

Laman *et al.*, 2003). Different possible mechanisms of interaction between LCH cells and T cells have been described. CD40 ligation on normal LC leads to up-regulation of various co-stimulatory molecules and adhesion molecules such as MHC class II, CD80, CD86, CD54, CD58, and IL-2R. As a result of this up-regulation, LC are capable of triggering proliferative responses and IFN-γ production by T cells (Cella *et al.*, 1996). In turn, IFN-γ enhances LC synthesis of IL-1α. (Santiago-Schwarz *et al.*, 1994). CD40L on activated T cells leads to their activation and enhanced production of cytokines, such as IL-2, IL-4, IL-5, and IFN-β (Grewal and Flavell, 1998). Furthermore, Ludewig *et al.* have demonstrated that soluble CD40L released by T cells has a long-lasting anti-apoptotic effect on LC (Ludewig *et al.*, 1996).

LCH cells expressing CD40 and $CD3^+$ T cells expressing CD40L have been found in close juxtaposition in different LCH tissues. This finding suggests cellular interactions, including CD40-CD40L binding (Egeler *et al.*, 2000; Tazi *et al.*, 1999), that could lead to local proliferation of LCH cells as well as the characteristic expression of cytokines in LCH lesions (Egeler *et al.*, 1999, 2000). Another mechanism of interaction between LCH cells and T cells involves chemokines. LCH cells in LCH lesions of bone, skin, and lymph node have been found to express CCL20/MIP3α. This chemokine is the ligand for CCR6, which can attract cells expressing the cognate receptor. T cells present in the same lesional tissues appeared to express CCR6. In the same study, LCH cells were also shown to express the ligand CXCL11/I-TAC. Its receptor, CCR3, was found on lesional T cells. Both of these observations suggest that LCH cells may recruit T cells to sites of disease activity (Annels *et al.*, 2003) (Fig. 4).

## B. Multinucleated Giant Cells

MGC have been observed in a variety of infectious and noninfectious granulomatous disorders (Okamoto *et al.*, 2003). Based on morphology, they are generally classified into Langhans' giant cells, foreign body giant cells (FBGCs), osteoclasts, and HIV-1-induced $CD4^+$-T cell–derived syncytia (HIV-S) (Anderson, 2000). MGC originate from fusion of monocytes or macrophages, but little is known about the mechanisms of the fusion process itself. Various stimuli have been shown to induce *in vitro* formation of MGC from human blood monocytes, such as the use of conditioned medium, the addition of different cytokines, the addition of lectins, alone or in combination with IFN-γ, as well as the addition of antibodies or phorbol myristate acetate (PMA). *In vivo* experiments have shown that freshly infiltrating monocytes play a key role for the formation of MGC. Generation of MGC requires only a few days, suggesting that fusion occurs before infiltrating monocytes could have developed into mature MΦ (Most *et al.*, 1997).

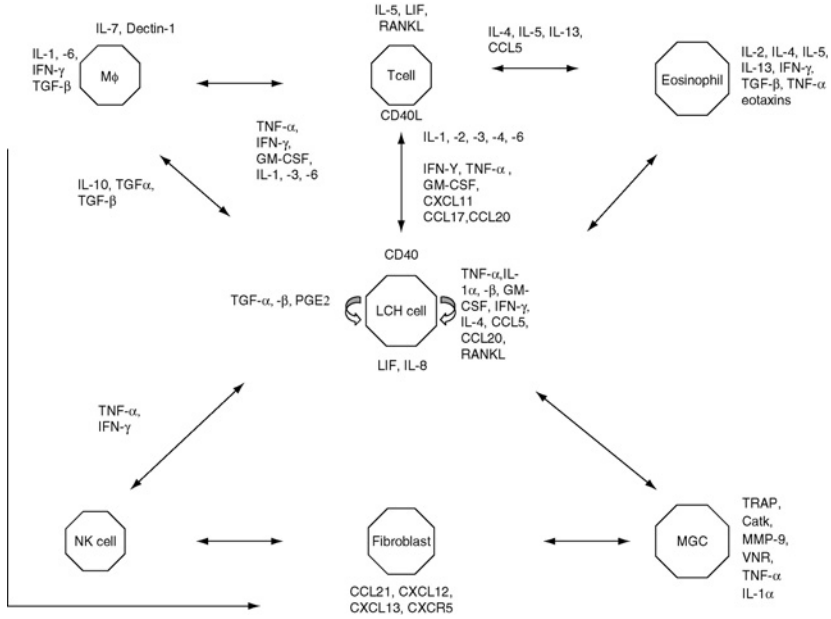


FIG. 4 The interactions between LCH cell and bystander cells. The main cytokines and chemokines, which play a role in LCH lesions, are shown. CatK, Cathepsin K; MMP-9, matrix metalloproteinase-9; TRAP, tartrate-resistant acid phosphatase; VNR, vitronectin receptor. (Adapted from Laman *et al.*, 2003, with permission from Elsevier.)

The functions of MGC have not been definitively proven and may be quite variable. MGC in granulomas could act as a disposal mechanism for dead MΦ and enhance the phagocytosis of pathogens involved in granuloma formation (Most *et al.*, 1997). Osteoclasts are the resorptive cell of bone, playing a central role in the formation of the skeleton and regulation of its mass (Teitelbaum, 2000).

The presence of MGC in LCH lesions has long been recognized (Ide *et al.*, 1984). The precise role of these cells in LCH is unknown. Da Costa *et al.* have shown that MGC in ostotic, as well as in non-ostotic LCH lesions, are osteoclast-like MGC using multicolor immunohistochemical analysis for the typical osteoclast markers CD68, tartrate-resistant acid phosphatase (TRAP), vitronectin receptor (VNR), and the osteoclast-secreted enzymes cathepsin K (CatK) and matrix metalloproteinase-9 (MMP-9) (da Costa *et al.*, 2005).

This observation of osteoclast-like MGC in ostotic lesions as well as in non-ostotic LCH lesions led to the question of their origin. Triple immunofluorescent staining of MGC revealed co-expression of $CD68^+$, $CatK^+$, and

$CD1a^+$ in 1 out of 3 skin lesions and 2 out of 4 lymph node lesions. Ruco *et al.* also reported 1 case of $CD1a^+$ MGC in an LCH biopsy specimen of the mandible and cervical lymph node. However, none of the ostotic lesions contained $CD1a^+$ MGC. These data suggest that MGC in bone LCH display a normal osteoclast phenotype, but also suggest either a possible existence of a lineage relationship between LCH cells and MGC in non-ostotic lesions (da Costa *et al.*, 2005; Ruco *et al.*, 1993). Further characterization of MGC has demonstrated strong expression of ICAM-1 (CD54), an adhesion molecule expressed on monocytes during aggregation to form MGC (Fais *et al.*, 1994). Because MGC in the LCH lesions thus far reported did not express the proliferation marker, Ki67, it could be hypothesized that these cells are formed by fusion of terminally differentiated, resident monocytes-MΦ.

Various cytokines play an important role in the formation of MGC, including IFN-γ, IL-3, IL-4, and GM-CSF. These cytokines are abundantly expressed in LCH lesions (Egeler *et al.*, 1999). The receptor activator of NF-κB ligand, RANKL/osteoprotegerin ligand (OPGL), has been reported to play a critical role in MGC formation as well (Lacey *et al.*, 1998; Yasuda *et al.*, 1999), because it can induce osteoclastogenesis with the aid of M-CSF (Namba *et al.*, 2001).

Da Costa *et al.* have shown that the majority of LCH cells and T cells in LCH lesions express RANKL. They have also demonstrated that the RANK receptor was present on a high percentage of LCH cells and on $CD68^+$ cells in all lesions RANKL was present. Moreover, they observed M-CSF to be expressed by $CD1a^+$ cells in bone lesions, in 1 out of 3 skin lesions, as well as in 3 out of 4 lymph node lesions. These findings suggest that high amounts of osteoclast-inducing cytokines present in LCH lesions are responsible for MGC formation (da Costa *et al.*, 2005). Rivollier *et al.* have observed that stimulation of immature DC with M-CSF and RANKL resulted in the acquisition of osteoclast functions. This process was more evident when IL-1 and TNF-α were added to the culture medium. These results not only support the importance of cytokines in MGC formation, but also demonstrate the existence of a significantly cellular plasticity of some differentiated myeloid phagocytes (Rivollier *et al.*, 2004).

Besides expression of various matrix-degrading enzymes, MGC are also capable of producing inflammatory cytokines. Hernandez-Pando *et al.* have shown by immunohistochemistry as well as by *in situ* RT-PCR that MGC in an *in vivo* mouse model produced IL-1α and TNF-α from 21 days after local injection of nitrocellulose. After 45 days, the expression of these cytokines decreased, but the transcription of TGF-β remained high. These results suggest that MGC could contribute to the cytokine production in LCH, possibly resulting in tissue damage and fibrosis (Hernandez-Pando *et al.*, 2000).

## C. Macrophages

LCH lesions are characterized by the infiltration of MΦ. Circulating myeloid DC precursors like monocytes and lineage-negative ($lin^-$) HLA-DR+ $CD11c^+CD1a^+$ cells can be induced to differentiate into MΦ in response to several cytokines, such as M-CSF. Rolland *et al.* have reported increased populations of myeloid DC precursors in the blood of LCH patients (Rolland *et al.*, 2005). This observation could be explained by increased serum levels of FLT3-ligand, which is known to mobilize DC subsets (Pulendran *et al.*, 2000). More than 2-fold elevations of serum levels of M-CSF have also been found in patients with LCH (Rolland *et al.*, 2005). If increased serum levels are caused by elevated production of these cytokines in LCH lesions, this could be an explanation for the influx of MΦ into lesional sites.

MΦ produce several cytokines. In LCH lesions of bone and lymph node, IL-10 expressing MΦ were found in close contact with LCH cells and T cells. IL-10 is known for its inhibitory effect on inflammation as well as maturation of immature LC. MΦ may, by secretion of IL-10, contribute to the maintenance of LCH cells in an immature stage of differentiation (Egeler *et al.*, 1999; Geissmann *et al.*, 2001). IL-7 production by MΦ has also been reported (Egeler *et al.*, 1999). IL-7 has been found to be an important factor in osteoclastogenesis and bone resorption. Weitzmann *et al.* have demonstrated that IL-7 *in vitro* promotes osteoclast-formation via actions on T cells. This cytokine appears to have a direct effect on T cells, by inducing up-regulation of RANKL as well as M-CSF secretion by T cells (Weitzmann *et al.*, 2000). These findings are supported by an *in vivo* study on T cell–deficient nude mice. Athymic mice did not undergo bone loss after IL-7 injection. However, T cell–repleted nude mice showed IL-7 induced bone loss, concurrent with induction of RANKL and TNF-α secretion by T cells (Toraldo *et al.*, 2003). Because both these cytokines are known to induce osteoclastogenesis, this cascade could lead to enhanced bone resorption in LCH lesions.

MΦ express a wide range of non-opsonin pattern recognition receptors enabling them to interact with their environment. Of these receptors, four types might be important in the role of MΦ in the pathophysiology of LCH. Seven transmembrane receptors (TM7) transduce signals for a wide variety of stimuli. F4/80 (homologous to human EMR1) belongs to a subfamily of TM7 receptors, and besides MΦ, it is also expressed by LC and eosinophils. A possible role for F4/80 in retention and/or adhesion of MΦ in specific tissues has been suggested based on the finding that LC completely down-regulate this receptor upon migration to T cell–enriched areas of the spleen and lymph nodes (McKnight and Gordon, 1998). Further investigation on EMR1 will reveal whether this receptor contributes to accumulation of MΦ in LCH lesions.

The scavenger R (SR) family of proteins consists of different members including CD163 (SR-A), CD36 (SR-B), and CD68 (SR-D). In general, these

receptors are involved in interactions with microorganisms. CD36 has also been found to be involved in clearance of apoptotic cells. CD163 binds to hemoglobin-haptoglobin complexes. Screening for CD163 in 1,105 human malignancies has revealed that this receptor provides a greater degree of specificity than CD68 for cells from the monocyte/histiocyte lineage. Interestingly, CD163 was also found to be expressed in 3 of 5 screened LCH lesions (Nguyen *et al.*, 2005).

Dectin-1 is type II C-type lectin-like receptor, which interacts with β-glucan polysaccharides (Herre *et al.*, 2004). Dectin-1 and TLRs have been shown to function synergistically. For instance, Dectin-1 enhances TLR signaling through NF-κB, leading to the production of inflammatory cytokines such as IL-12 and TNF-α. In turn, TLR signaling enhances responses that are triggered by Dectin-1, such as the production of ROS (Gantner *et al.*, 2003). Dectin-1 also recognizes an endogenous ligand on T cells (Herre *et al.*, 2004) and is, therefore, able to stimulate proliferation of $CD3^+$, $CD4^+$, and $CD8^+$ T cells (Grunebach *et al.*, 2002). The mannose receptor (MR) has been found to participate in clearance of endogenous glycoproteins, pathogen recognition, and antigen presentation by MΦ. MR might also be involved in the process of lymphocyte exit from lymph nodes through interaction with L-selectin (Taylor *et al.*, 2005).

## D. Eosinophils

Eosinophils are frequently observed in LCH lesions, which led to the term, eosinophilic granuloma. Their function as predominant cells in allergic airways has been well described and may provide clues as to their potential role in LCH.

Eosinophils are mainly produced in the bone marrow from IL-3, IL-5, and GM-CSF–stimulated $CD34^+$, progenitor cells (Johansson *et al.*, 2004). Eosinophils constitutively express CCR3 and, to a lesser extent, CCR2 (Daugherty *et al.*, 1996; Heath *et al.*, 1997). Activation and migration of resting eosinophils to sites of inflammation are mainly regulated by the CCR3-specific ligand CCL11 (eotaxin), which has been shown to be produced by several cell types including endothelial cells, epithelial cells, fibroblasts, DC, and smooth muscle cells (Elsner *et al.*, 1996, 2001). Other CCR3-ligands with the same effect on eosinophils are CCL24 (eotaxin-2), CCL7 (monocyte chemotactic protein MCP-3), CCL13 (MCP-4), and CCL5 (RANTES) (Elsner *et al.*, 2004). Cytokines such as IL-4, IL-5, and IL-13, which are produced by Th2 cells, also play an important role in the activation and recruitment of eosinophils (Bousquet *et al.*, 1990; Kapsenberg *et al.*, 1992; Wierenga *et al.*, 1993). Once eosinophils have reached a site of inflammation, they release toxic chemicals such as eosinophilic protein (ECP) and ROS, the latter being responsible for tissue damage and propagation of inflammatory responses. Rumbley *et al.* have

demonstrated that eosinophils represent the major cytokine-producing cells in schistosome granuloma, secreting IL-2, IL-4, IL-5, and IFN-γ (Rumbley *et al.*, 1999). TGF-β, EGF, TNF-α, and IL-13 have also been reported to be produced by eosinophils. Moreover, eosinophils secrete other soluble mediators such as prostaglandins, leukotrienes, and platelet activating factor (PAF) (Schmid-Grendelmeier *et al.*, 2002; Wardlaw, 1999). T cells in LCH lesions are associated with high expression levels of IL-4 and IL-5 (Egeler *et al.*, 1999). Furthermore, LCH cells have been shown to express CCL5. T cells and LCH cells could therefore be involved in the recruitment and activation of eosinophils. Taking into account the wide variety of mediators produced by eosinophils, these may suggest that eosinophils could be involved in the maintenance of the cytokine production and, thereby, play a part in tissue damage and fibrosis in LCH lesions.

## E. Stromal Cells

Fibroblasts are the principal cells of stromal tissue. They are increasingly recognized as key regulators in inflammatory responses. In several immune-mediated inflammatory conditions, such as rheumatoid arthritis, autoimmune liver disease, and diabetes, local expansion and activation of fibroblasts in conjunction with abnormal persistence and recruitment of infiltrating inflammatory cells have been observed (Armengol *et al.*, 2003; Buckley, 2003; Green and Flavell, 2000). A possible mechanism by which fibroblasts contribute to these events may be based on "stromal address codes" (Parsonage *et al.*, 2005). The immunomodulatory functions of fibroblasts depend on the pathological condition of the tissue of their origin and their anatomical location (Brouty-Boyé *et al.*, 2000). In search of the relationship between fibroblast function and gene expression profiles, Chang *et al.* obtained gene expression patterns of 50 fibroblast cultures from different anatomical sites by using cDNA microarrays. Approximately 2 million gene expression measurements revealed that the different fibroblasts cultured exhibited unique RNA expression patterns corresponding to their tissue of origin. Further investigation for the role of HOX genes in topographic differentiation of fibroblasts showed that these genes can be used to predict their anatomical origin. Thus, the gene expression profile of fibroblasts could be used as an "address code" (Chang *et al.*, 2002). Parsonage *et al.* demonstrated that fibroblasts isolated from diverse tissues exhibited similar patterns of gene expression. However, stimulation with various cytokines showed different transcriptional profiles, resulting in secretion of different products involved in inflammation. This suggests that the identity of resident tissue fibroblasts may depend on inflammatory cytokines that are characteristic for different tissues.

Fibroblasts are involved in the accumulation, survival, and differentiation of tissue-infiltrating leukocytes by expressing a specific spectrum of chemokines (Parsonage *et al.*, 2005). Therefore, it could be speculated that diseased stromal tissue could secrete inappropriate amounts of inflammatory mediators. This could lead to aberrant lymphoid proliferation, resulting in persistent pathological conditions such as observed in chronic inflammation (Hjelmström, 2001). Evidence supporting this theory has been found in various diseases, including the synovium of rheumatoid arthritis, the salivary glands in Sjögren's syndrome, and *Helicobacter pylori*-induced chronic gastritis. Expression of homing chemokines such as CCL21, CXCL12, CXCL13, and its receptor CXCR5, together with features of lymphoid proliferation, have been observed in patients exhibiting these pathologies (Amft *et al.*, 2001; Hjelmström, 2001; Mazzucchelli *et al.*, 1999).

Fibroblasts are also known to mediate T-cell survival (Scott *et al.*, 1990). Piling *et al.* have demonstrated that fibroblasts prevent activated T cells from undergoing apoptosis by secreting type I IFN. A subset of activated T cells return to the resting G0/G1 cell cycle phase, thus preserving immunological memory by antigen-specific T cells (Pilling *et al.*, 1999). Fibroblasts also prevent apoptosis of neutrophils (Wang *et al.*, 2003). These events may lead to a persistent leukocyte infiltrate with the consequence of increased production of inflammatory products, resulting in chronic inflammation and fibrosis (Buckley *et al.*, 2001).

In LCH, infiltrating fibroblasts are specifically seen in late-stage fibrosis. Uebelhoer *et al.* have investigated the effect of alveolar MΦ-derived platelet-derived growth factor (PDGF) on fibroblast activation. In their study, they stimulated a human lung fibroblast cell line with alveolar MΦ supernatant from LCH patients or healthy controls. Fibroblasts incubated with supernatant showed a higher mitotic rate and higher collagen production (Uebelhoer *et al.*, 1995). Other investigators have reported additional MΦ-derived products, such as IL-1, IL-6, IFN-$\gamma$, and TGF-$\beta$, capable of stimulating fibroblasts *in vitro* (Cantin *et al.*, 1988; Kelley, 1990; Ross *et al.*, 1986). The inflammatory conditions of LCH lesions, along with the presence of activated fibroblasts that produce inflammatory mediators and recruit leukocytes, are likely to play an important role in tissue damage observed in LCH.

## F. Natural Killer Cells

NK cells play an important role in immuno-surveillance. Upon activation, they release cytokines and chemokines involved in inflammatory reactions. They also are capable of killing tumor cells and virus-infected cells (Biron, 1997; Moretta *et al.*, 2002; Trinchieri, 1989). Another interesting property of NK cells, especially in view of LCH, is their interaction with DC. Both NK

and immature DC are commonly observed in inflammatory sites. Their association promotes a series of events that involve changes in NK cells as well as immature DC (Della Chiesa *et al.*, 2005; Moretta, 2002).

NK cells and DC can influence each other's activation and maturation. In a study by Ferlazzo *et al.*, NK cells were cultured in the presence of either immature or mature DC. After 5 days of incubation, active DNA synthesis and NK cell expansion were observed under both conditions. These findings suggest that DC, either in immature or mature differentiative states, can stimulate proliferation and expansion of NK cells. Furthermore, in response to this stimulation, NK cells secrete IFN-$\gamma$ and acquire cytolytic activity (Ferlazzo *et al.*, 2002). Vitale *et al.* have demonstrated that only a minor subset of NK cells is involved in DC-induced activation. This subset is characterized by a $CD56^+$ $CD16^-$ HLA-class I–specific inhibitory receptor CD94/$NKG2A^+$ (natural killer group protein 2-A) $KIR^-$ (killer Ig-like receptor) phenotype (Vitale *et al.*, 2004). The natural cytotoxicity receptor (NCR), NKp30, is a crucial triggering molecule for interactions between NK cells and DC, because addition of an anti-NKp30 monoclonal antibody abrogates NK cell–mediated killing of immature DC as well as IFN-$\gamma$ production by NK cells. Interestingly, NK cells appear to be able to kill immature DC but not mature DC. Della Chiesa *et al.* have shown that the NK cell subset with a CD94/$NKG2A^+KIR^-$ surface phenotype displayed spontaneous cytolytic activity against autologous immature DC. A comparative analysis of the expression of HLA class I subtypes HLA-A, -B, -C, and -E on immature DC and mature DC showed that all HLA class I molecules were highly up-regulated in mature DC as compared with immature DC. Addition of anti-HLA class I Mabs could restore lysis of mature DC. These data suggest that mature DC are protected from NK-induced cytotoxicity by up-regulation of HLA class I molecules. Moreover, this also implies that NK cells regulate the number or type of maturing DC by eliminating DC that under-express HLA class I molecules and, thereby, control the amplitude of DC responses (Della Chiesa *et al.*, 2003; Ferlazzo *et al.*, 2001; Moretta, 2005).

These properties of NK cells may have interesting therapeutic implications for LCH. For example, NK cells could potentially be targeted against LCH cells to induce cell lysis or differentiation, thereby, providing a cell-based adoptive therapeutic approach.

## VI. Conclusions

Comparison of LC and LCH cells reveals important differences in morphology, phenotype, and function. LCH cells are phenotypically immature LC, but are not able to act as APC without stimulation by CD40 (Geissmann

*et al.*, 2001). The mechanisms regarding how LCH cells develop are still not completely understood. However, several mechanisms might play important roles, including: (1) multiple chromosomal alterations that have been found in LCH cells; (2) clonal proliferation of LCH cells, although definitive and consistent molecular events leading to clonal proliferation, are unclear; (3) high levels of cytokines are secreted by LCH cells as well as by bystander cells in LCH lesions. These cytokines could contribute to the proliferation and differentiation of LCH cell progenitors and could play a role in preventing LCH cells from maturing.

Future studies on the biology of normal LC and LCH cells should enable us to better understand the pathology and mechanisms responsible for the development of LCH and its variable clinical course observed in patients. Key to such studies will be the ability to propagate in culture LCH cells that retain their *in vivo* characteristics. Such studies are also likely to lead to more rational and effective treatment strategies for patients with LCH.

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# Unusual Axonemes of Hexapod Spermatozoa

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Hexapod spermatozoa exhibit a great variation in their axoneme structure. The 9+2 pattern organization is present in a few basal taxa and in some derived groups. In most hexapods, a crown of nine accessory microtubules surrounds the 9+2 array, giving rise to the so-called 9+9+2 pattern. This general organization, however, displays a number of modifications in several taxa. In this review, the main variations concerning the number and localization of the accessory tubules, microtubular doublets, central microtubules, dynein arms, and axonemal length are summarized. We discuss the phylogenetic significance of all this structural information as well as the current hypotheses relating the sperm size and sperm polymorphism with reproductive success of some hexapod species. Also described are the biochemical data and the motility patterns which are currently known on some peculiar aberrant axonemes, in light of the contribution these models may give to the comprehension of the general functioning of the conventional 9+2 axoneme. Finally, we summarize methodological developments for the study of axoneme ultrastructure and the new opportunities for the molecular analysis of hexapod axonemes.

**KEY WORDS:** Axonemes, Sperm cells, Microtubules, Sperm ultrastructure, Hexapods, Dynein, Accessory tubules, Central pair complex. 

## I. Introduction

Insects constitute the largest and most diversified group in the animal kingdom. Several factors could have underlain such a widespread and successful radiation. First of all, the age of the group, which represents one of the most



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ancient terrestrial animal taxa; the recent discovery of the supposed winged insect *Rhyniognatha hirsti* from Early Devonian indicates, in fact, that insects evolved during the Silurian, at the onset of the terrestrial ecosystem. This early appearance on the Earth allowed them to take advantage of all the new opportunities offered by the presence of vascular plants and angiosperms (Grimaldi and Engel, 2003) and to achieve this by exploiting several innovations in their ground plan. First, the acquisition of a protective exoskeleton that, defending them against dehydration, gave insects, along with the differentiation of an efficient osmoregulatory system, the chance to expand and colonize dry environments. Second, the differentiation of wings for the flight that, unlike birds, was realized without the loss of functionality of other appendages, thus improving their ability to colonize new habitats and to escape more efficiently from predators; furthermore, the ability to bend the wings along the body allowed insects to perform walking without difficulties. Finally, the appearance of holometaboly was another favorable innovation that allowed insects both to accomplish a faster transformation of immatures and to exploit differential diets at the immature and adult stages; this, along with the impressive reproductive capability of insects, made them the true rulers on the Earth.

All the aforementioned achievements occurred in insects concomitantly with a particularly high speciation rate, due to their short generation lifespan, that allowed the accumulation of mutations and a faster genetic divergence. Barriers to fertilization evolved rapidly between diverging populations. Genes mediating sexual reproduction have been shown to diverge faster than genes expressed in nonreproductive tissues; in fact, proteins specifically expressed in the male and female reproductive tracts of *D. melanogaster* exhibit a greater divergence than nongonadal proteins (Civetta and Singh, 1995; Thomas and Singh, 1992). Spermatozoa exhibit a rapid and often bizarre diversification, indicating that they are a constant target for intense selection.

It appears therefore reasonable to relate the great variation in sperm structure and axonemal pattern observed in insects with their very high number of species. There is no other group of animals displaying such a great variability in sperm architecture as insects, ranging from a conventional flagellated sperm to an aflagellated immotile cell, from minute—only a few microns long—to giant spermatozoa reaching a length of about 6 cm. Interestingly, however, sperm variability within insects is independent of the size of the group in which it occurs, for example, the dipteran family Cecidomyiidae shows a greater number of sperm models than larger orders, such as Lepidoptera or Hemiptera.

We present here an overview of the current ultrastructural information available on insect sperm axonemes and discuss this from two main points of view. First, the contribution that the analysis of axonemal organization may provide to the comprehension of the phylogenetic relationships existing

among different insect taxa. Second, the possible benefit that the use of some insect modified axonemes as biological models can contribute to the analysis of peculiar aspects of the cell biology of flagellar axoneme. A few examples for each of these main points are then proposed in the last part of this review.

## II. Structure of Axonemes

### A. Conventional Axonemes: From 9+2 toward 9+9+2 Pattern

A 9+2 axoneme can be observed in the sperm cells of the basal hexapod Collembola (Fig. 1A), as well as in some higher insects where it represents, however, a derived condition as the consequence of a secondary reduction. This occurs in all members of Mecoptera and Siphonaptera (Fig. 1B), in a few isolated species among basal Diptera, such as the fungus gnat *Keroplatus reamurii* (Dallai *et al.*, 1995a), and in the lepidopteran Micropterygidae (Sonnenschein and Häuser, 1990). In all these species, the axoneme shares all the features exhibited by the evolutionarily conserved 9+2 organization expressed in the flagella of other widely divergent phyla, that is it consists of nine peripheral microtubular doublets arranged around two central singlet microtubules (Dallai and Afzelius, 1990; Witman *et al.*, 1972). Each doublet is formed by a complete microtubule—the A-tubule, containing 13 tubulin protofilaments—and by an incomplete microtubule with a C-shaped cross section—the B tubule, containing 10 protofilaments plus a smaller filament located at the junction with the A tubule (Dallai and Afzelius, 1990), so that the number of units in the B subtubule wall is sometimes given as 11 (Fujiwara and Tilney, 1975; Nojima *et al.*, 1995). The B-tubule curvature corresponds to that observed in native tubules with 16 protofilaments (Afzelius *et al.*, 1990). The two central tubules consist of 13 protofilaments. The central microtubules and the peripheral doublets are interconnected by a series of projections, including dynein arms, radial spokes, and the central pair projections (Fig. 1B).

Except for the few examples just mentioned, the flagellar axoneme of insects is generally provided with a further set of nine microtubules that encircle the axoneme and are usually referred to as accessory tubules, giving rise to the so-called 9+9+2 axonemal organization. The occurrence of accessory tubules has to be regarded as a typical feature of insect flagellar axoneme; the structural and molecular evidences available on this peculiar microtubular array, as well as the motility pattern of the 9+9+2 axoneme are described in this section.

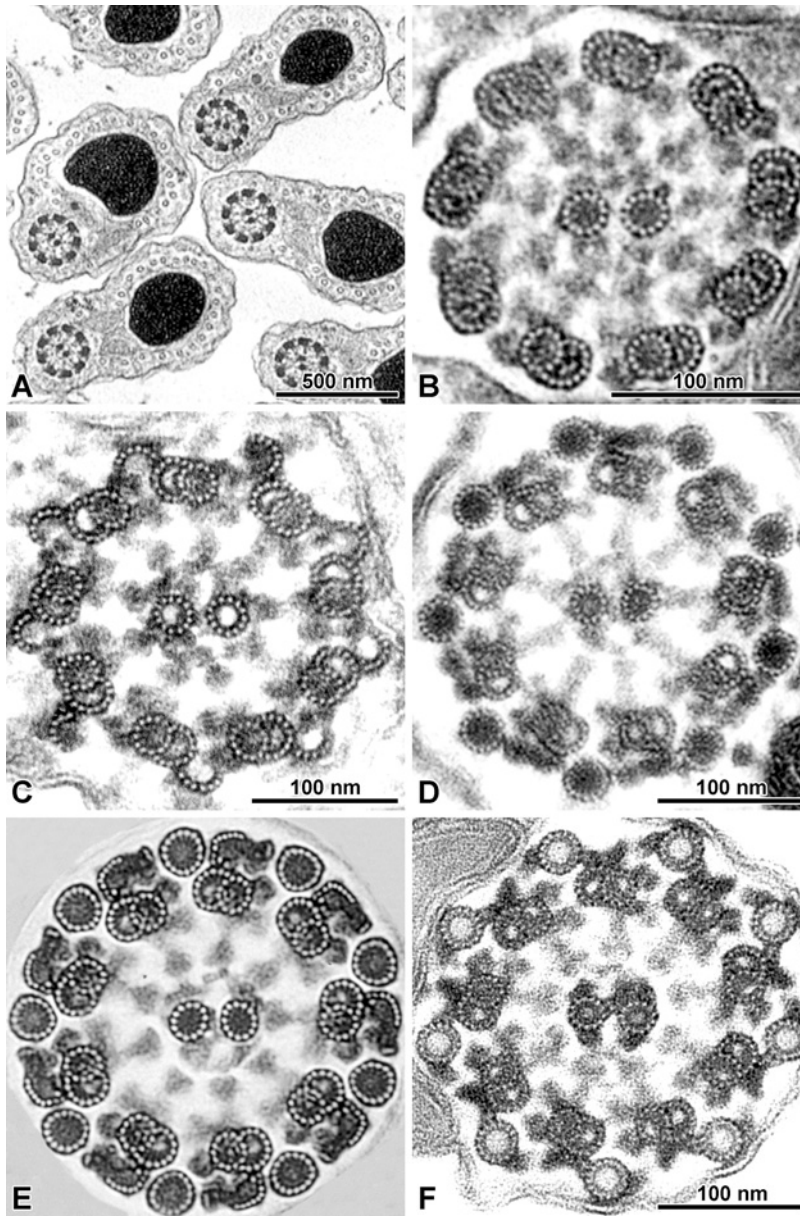


FIG. 1 (A) Cross-section through aged spermatids of the basal wingless hexapods (Collembola) showing a 9+2 axoneme pattern. (B) The simple 9+2 axoneme of the holometabolic flea *Ctenocephalides felis* (Siphonaptera) spermatozoon. (C) The accessory microtubules grow during spermiogenesis from the B tubule of each axoneme doublet, as occurs in the wingless insect *Campodea* sp. (Diplura). (D–F) When present, the accessory microtubules are generally

## 1. Peculiar Synapomorphic Character: Accessory Tubules

The accessory tubules are synapomorphic structures present both in the wingless diplurans and in insects (Dallai and Afzelius, 1999). In the past, the occurrence of nine peripheral microtubules around the 9+2 flagellar axoneme of the velvet worm *Euperipatoides leuckarti* had suggested the existence of a close relationship between onychophorans and insects; however, it was later demonstrated that this feature is due to a convergence process (Dallai and Afzelius, 1993a), because the additional tubules are assembled through different mechanisms in the two taxa. In fact, in onichophorans they originate from cytoplasmic microtubules, whereas in hexapods (with the exclusion of Protura and Collembola which lack these structures) the accessory tubules are formed during spermiogenesis (Cameron, 1965; Dallai and Afzelius, 1993b) as short outgrowths from the B tubule of each doublet, at a position corresponding or close to protofilament no. 4 (Witman *et al.*, 1972) (Fig. 1C). These projections, which are oriented opposite to dynein arms and consist initially of only a few protofilaments, progressively grow giving origin to hook-shaped structures that finally, at the end of this process, close on themselves and detach from the doublets, giving rise to complete independent microtubules; later, an electron-dense material is deposited in the intertubular space between two adjacent accessory tubules (Fig. 1C). Growth and detachment of accessory tubules do not proceed synchronously on all the doublets. Both the final number of protofilaments in the accessory tubules and the amount of the intertubular material may vary among different taxa, and these features are commonly used as valuable characters in the study of phylogenetic relationships among taxa (Fig. 1D–F).

Besides their usefulness as phylogenetic characters, accessory tubules have also attracted much interest as native models for the study of the structural properties of tubulin polymers exhibiting different numbers of protofilaments. Accessory tubules can measure about 25 nm and consist of 13 protofilaments, as occurs in Diptera, or they can be wider, up to 34 nm, and consist of 19 protofilaments, as happens in the caddisfly *Odontocerum albicorne*, or finally they can reach the unique dimension of 44 nm, as occurs in the so-called "macrotubules" of the paraspermatozoa of the neuropteran *Perlamantispa* (=*Mantispa*) *perla* (Dallai *et al.*, 2005) (Fig. 3G). Independent of the number of protofilaments, optical diffraction patterns on negatively stained tubules

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orderly arranged outside the 9+2 axoneme and are separated from each other by dense, more (E) or less (D/F) expanded intertubular material. (D) The zygentoman *Lepismodes inquilinus.* (E) the mantodea *Mantis religiosa* (computer-aided elaboration). (From Dallai and Afzelius, 1999). (F) The fungus gnat nematoceran dipteran *Neoplatyura nigricauda*. In these three species accessory microtubules have 16 protofilaments in their tubular wall, as occurs in the majority of pterygotan taxa (From Dallai and Afzelius, 1999).

have revealed usual spectral lines at 8 and 4 nm, corresponding to the tubulin dimer and monomer repeat, respectively (Lanzavecchia *et al.*, 1994).

The first results on protofilament handedness obtained by tannic acid fixation of accessory tubules from a stick insect were reported by Afzelius (1988) and successively extended to other insect species (Dallai *et al.*, 1993a; Lanzavecchia *et al.*, 1994); later, Hirose *et al.* (1997) employed cryo-electron microscopy for the analysis of accessory tubules after decoration with the tubulin-motor proteins ncd and kinesin from the cricket *Acheta domestica*.

Accessory tubules consisting of 13 protofilaments, such as in the dipluran *Japyx solifugus*, possess the same straight organization exhibited by cytoplasmic and *in vitro*-polymerized microtubules (Chrétien and Wade, 1991; Wade and Hewat, 1994), with protofilaments running parallel to the tubule axis and a three-start helix arrangement of tubulin monomers, consistent with a regular B-lattice model (Dallai and Afzelius, 1999). On the contrary, the accessory tubules of the nematoceran dipteran *Exechia seriata* and of the caddis fly *Limnephilus bipunctatus*, exhibiting respectively 16 and 19 protofilaments, have skewing protofilaments (Lanzavecchia *et al.*, 1994), as it had been predicted by Wade and Chrétien (1993) for microtubules with more than 13 protofilaments. Spectral peak phase analysis has indicated the presence of a four-start helix disposition of dimers, staggered like in the B-lattice model (Chrétien and Wade, 1991; Wade and Hewat, 1994). The twisting of protofilaments has been suggested to be required for the accommodation of the extra tubulin molecules that have to be arranged at each helix turn (Wade *et al.*, 1990). Interestingly, a variation in protofilament number from the centriolar region toward the tail tip has been observed in accessory tubules with more than 16 protofilaments (Afzelius *et al.*, 1990; Dallai *et al.*, 1993a).

The molecular mechanisms underlying such a high degree of variability in the number of protofilaments that constitute the accessory tubules has not yet been definitely clarified; however, a genetic and structural study on *Drosophila* transgenic mutants, co-expressing the moth *Heliothis virescens* ortholog along with the fly testis-specific $\beta_2$ tubulin, has suggested that the number of protofilaments is specified by some β-tubulin sequence motives; in fact, the 16-protofilament structure typical of the moth was imposed on *Drosophila* accessory tubules, which are normally formed by 13 protofilaments (Raff *et al.*, 1997). Similarly, the presence of a luminal filament within the accessory tubules, which constitutes a common feature of *Drosophila* spermatozoa, seems also to depend on the expression of the appropriate β-tubulin sequence (Fackenthal *et al.*, 1995).

Only limited biochemical data have been reported on accessory tubules; however, the available evidence indicates that they are the most stable microtubular components within the axoneme. In fact, accessory tubules resist treatments, such as thermal exposition and sarkosyl extraction, that

induce a complete depolymerization of both central singlets and doublet microtubules (Pirner and Linck, 1995; Stephens *et al*., 1989). This behavior fits well with some observations made on particular degenerative processes that in some insect species affect axonemal microtubules but not accessory tubules (Dallai and Afzelius, 1999; Giuffrida and Rosati, 1993). On the basis of this differential stability, it was possible to achieve the biochemical fractionation of *Apis* spermatozoa and the separation of accessory tubules from axonemal microtubules, thus allowing a biochemical and immuno-electron microscopic study of the distribution of the various tubulin posttranslationally modified isoforms in the two classes of microtubules (Mencarelli *et al.*, 2000). The results of this study have demonstrated the close relativeness of accessory tubules to axonemal rather than to cytoplasmic microtubules, given that they contain the whole set of posttranslational modifications that characterize the axoneme, although the overall molecular heterogeneity of tubulin appears to be lower in accessory tubules than in axonemal microtubules. In particular, the tyrosination and glycylation extent of accessory tubules correlates with the tyrosinated and glycylated tubulin isoform content of axonemal B subfibers; this observation confirms at the molecular level the morphogenetic origin of the accessory tubules as outgrowths of the B subtubules that had been evidenced at the ultrastructural level. Interestingly, the existence of significant qualitative and quantitative differences in the tubulin isoform content of accessory tubules and axonemal microtubules was also observed.

These results suggest that the differential segregation of posttranslationally modified tubulin isoforms between accessory tubules and axonemal microtubules contributes to the structural and functional specialization of the different microtubular classes expressed in the insect sperm axoneme. Because the functional role of tubulin posttranslational modifications has not been clearly defined, it is difficult to attribute a precise functional meaning to the previously reported biochemical differences between accessory and axonemal microtubules. Given that most modifications affect the structure of the 15 amino-acid C-terminal sequence of the tubulin molecule, which is involved in its capability to interact with other proteins (Boucher *et al.*, 1994; Bré *et al.*, 1996; Gagnon *et al.*, 1996; Larcher *et al.*, 1996), it is conceivable that the lower level of tubulin heterogeneity exhibited by accessory tubules could be related to the less complex protein interactions they are involved in with respect to axonemal microtubules. The currently available data do not provide any clue to clearly understand the functional role that accessory tubules play in the physiology of insect sperm flagella. Given their great stability, the most plausible function seems to be that of a passive mechanical role, either as structures that act to reinforce the long flagellum or as cytoskeletal elements that amplify the mechanical power generated by the axoneme.

## 2. Motility Pattern of the 9+9+2 Axoneme

Notwithstanding the peculiarity of the 9+9+2 axonemal organization and the abundance of structural information on the modifications it has undergone in different insect groups, only a few studies have been performed on the motility pattern of insect sperm flagella and data are quite fragmentary and limited to a very few species. However, on the basis of available evidence, it seems that a quite general feature of the 9+9+2 sperm motility is the occurrence of a complex tridimensional, helicoidal beating pattern originated from two superimposed types of waves, both arising from behind the sperm head and spreading toward the posterior tail tip. A major helical wave, characterized by a large amplitude and a low frequency, thus coexists with a minor wave, which exhibits a low amplitude and a high beat frequency. This kind of motility has been more accurately described in the phasmid *Bacillus rossius* (Baccetti *et al.,* 1973a), in the coleopterans *Tenebrio molitor* (Baccetti *et al.*, 1973b) and *Aleochara* sp. (Werner *et al.*, 1999, 2002) as well as in the tephritid dipterans *Ceratitis capitata* and *Bactrocera* (=*Dacus*) *oleae* (Baccetti *et al.*, 1989), but it is likely to also be shared by other species (Phillips, 1974). A reverse backward motility was also described in *Ceratitis*, with the tail tip progressing anteriorly and pulling the cell body (Baccetti *et al.*, 1989). The double wave motility has been proposed to be raised by the contemporary activity of an active component—the minor wave, generated by the axoneme activity—and of other passive, elastic components of the flagellum (Werner *et al.*, 2002) which would impose some mechanical restriction to the axonemal movement thus originating the major, large waves. In this sense, it is interesting to note that most insect sperm flagella contain additional components—accessory tubules and the asymmetrically disposed accessory bodies and mitochondrial derivatives—that, given their structure, can be supposed to affect the mechanics of motility. As a matter of fact, an almost planar beating has been observed in the 9+2 spermatozoa of the siphonapteran *Ctenocephalus canis* (Baccetti, 1972).

A double-wave movement is also exhibited by the spermatozoa of several species of *Drosophila;* some functional differences have been evidenced between short and long spermatozoa in these sperm dimorphic species in the passage from male to female storage organs (Bressac *et al.*, 1991). *In vitro, Drosophila* spermatozoa are, however, apparently unable to perform a forward-progressing motility, even though they are motile within both the male and female storage organs. In this respect, it is important to consider the possibility that the sperm motility might differ when these cells are observed *in vitro* or *in vivo*, not only for the different environmental conditions, but also for the mechanical restrictions imposed by the narrow reproductive ducts. It is also important to note that the contribution of flagellar motility to the transport of sperm cells to the spermatheca appears to be reduced in

several insect groups; in dipterans, for example, active movement of sperm seems to play a minor role in spermathecal filling and is probably assisted by female processes (Arthur *et al.*, 1998; Linley and Simmons, 1981; Simmons *et al.*, 1999).

## B. Structural Variations in Hexapod Axonemes

Although the 9+9+2 organization is the most widely expressed among hexapods, many insect orders show a more or less diffuse tendency toward a progressive axonemal degeneration and express axonemes in which one or more of the structures involved in sperm motility are modified or lost, up to the complete loss of the whole axoneme. The cellular and molecular basis of insect axonemal variability are still under debate. It seems reasonable to argue that the accumulation of mutations affecting the axoneme structure could have been at least partly facilitated by the concomitant development of specialized reproductive features that help in sperm transfer, namely a peculiar structure of the reproductive ducts, elongate copulatory organs, spermatophores, sperm storage organs, etc.; these are frequently observed in species expressing unconventional axonemes and could have allowed the occurrence of the increasingly more drastic alterations in the 9+9+2 axonemal organization and in its motility. Also, in some insect groups an atypical spermatogenetic process occurs that could possibly contribute to the production of abnormal sperm models. A clear example is provided by the families Sciaridae and Cecidomyiidae, in which a major part of germ cell chromosomes is lost during the first meiotic division (White, 1977); such a loss of genetic information might affect both the axoneme morphogenesis and function. Finally, it is interesting to note that in the evolution of some insect groups, the introduction of a first modification in the basic axoneme structure is often followed by or is concomitant with the appearance of further alterations; for example, the loss of the central apparatus is often related, initially, to the absence of one or both dynein arms and, in more advanced species within the same group, to even more profound modifications of the axoneme pattern, such as those in the number and disposition of doublets.

### 1. Number of Flagella and Axonemes

The flagellar axoneme of insect sperm cells is usually assembled from the single centriole inherited by each of the spermatid cells at the end of spermiogenesis; in fact, contrary to what happens in most animals, in insects no centriole duplication occurs at the second meiotic division (Callaini *et al.*, 1999; González *et al.*, 1998). In some insect groups, however, the spermatogenesis proceeds in an aberrant way and two centrioles are present in the

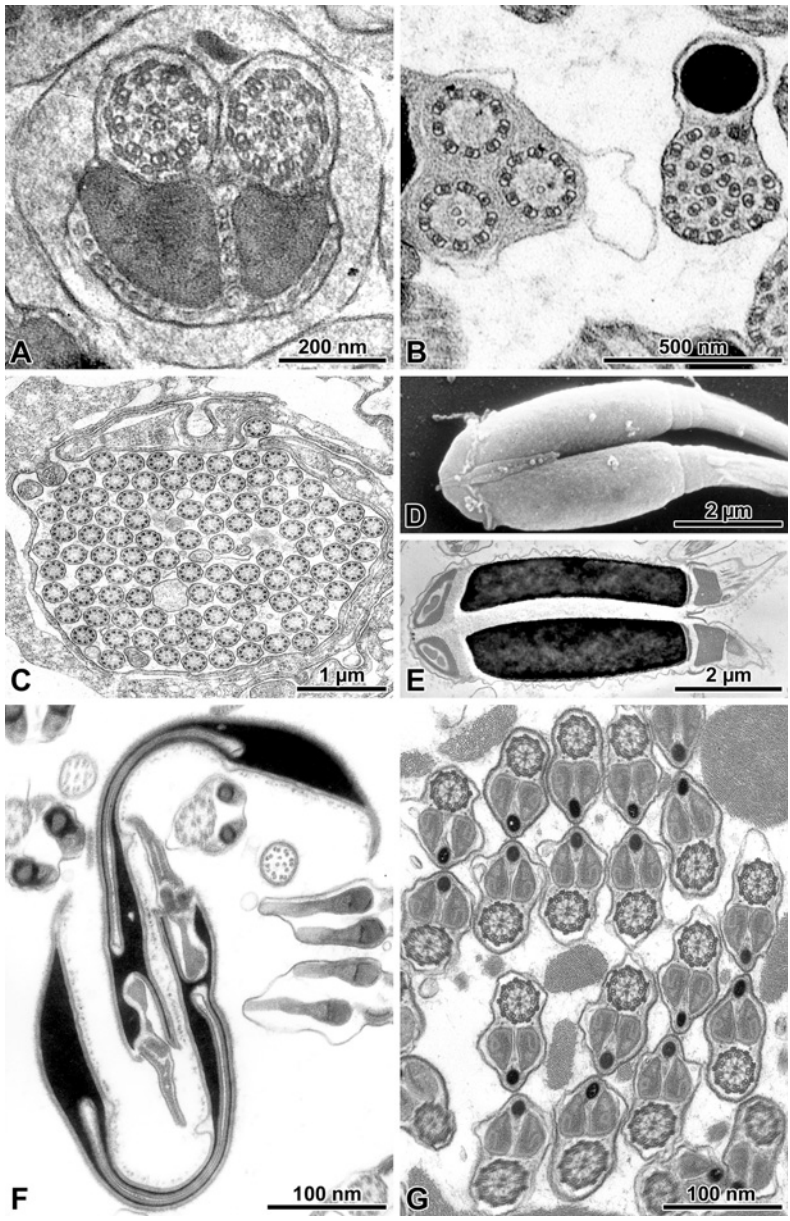


FIG. 2 (A) Cross-section through an aged spermatid of the *Menopon gallinae* (Mallophaga). Two axonemes in a single sperm flagellum are visible. (B) Cross-section through two different stages of the thrips *Haplothrips simplex* (Thysanoptera) sperm maturation. On the left, three axonemes 9+2 (or sometimes 9+0) are visible within the same aged spermatid; on the right, the final structure deriving from the amalgamation of the three axonemes is recognizable.

spermatids; as a consequence, the sperm flagellum consists of two axonemes. This has been shown to occur in the sucking and biting lice Anoplura and Mallophaga (Baccetti *et al.*, 1969) as well as in thrips (Fig. 2A). An evolutionary trend toward the acquisition of a biaxonemal sperm flagellum occurs also in the related book lice (Psocoptera). In the thrips *Haplothrips simplex* (Thysanoptera), a third centriole is assembled in the early spermatids before spermiogenesis and the sperm flagellum contains three axonemes (Paccagnini *et al.*, 2006); in this group, the final axoneme structure is very peculiar, because it derives from the fusion and rearrangement of three 9+0 axonemes (Fig. 2B). In the gall-midge lasiopterid *Semudobia betulae,* two 9+0 axonemes are also assembled (Dallai and Mazzini, 1989).

Other insects assemble more than one sperm axoneme, but in these species each axoneme is part of a distinct flagellum. This is the case of the multiflagellated spermatozoon of the termite *Mastotermes darwiniensis* (Fig. 2C) and of the binucleated, biflagellated spermatozoon of *Tricholepidion gertschi*, the only living species of Zygentoma Lepidotrichidae (Fig. 2D, E). Actually, the latter is a sort of "siamese twin" spermatozoon, because it results from the association of two sperm cells through a membrane fusion process occurring in the deferent ducts between the two sperm head regions at the end of a conventional spermiogenesis (Dallai *et al.*, 2001); in the female genital tract, the two cells are supposed to separate before fertilization. In a few other examples the association between two spermatozoa is achieved by the production of cementing material, as occurs in the water beetles *Dytiscus marginalis* and *Hydaticus seminiger* (Fig. 2F), or by differentiating a type of junctional contact, as it has been described in some members of the wingless Zygentoma (Dallai and Afzelius, 1984, 1985; Dallai *et al.*, 2004a) (Fig. 2G). All these cases of "double" spermatozoa have to be considered as peculiar adaptations for sperm transfer. In fact, insects have often exploited methods to increase the efficiency of sperm transfer through aggregations of spermatozoa in different structures (spermatodesms, spermatolophids, spermatophores) (Dallai *et al.*, 2002; Proctor, 1998; Schaller, 1979).

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(C) Cross-section through sperm region beneath the nucleus of the termite *Mastotermes darwiniensis* (Isoptera); many flagellar axonemes with a 9+0 pattern are visible. (From Baccetti and Dallai, 1978). (D, E) SEM and TEM views of the biflagellated spermatozoon of the wingless *Tricholepidion gertschii* (Zygentoma). Note that the two apical acrosomes and the two nuclei are surrounded by the same plasma membrane, as the result of a sperm fusion process that occurs between the anterior regions of two sperm cells after spermiogenesis, during the sperm storage into the seminal vesicles. (D, E: from Dallai *et al.*, 2001). (F) Cross-section through the joint spermatozoa of *Hydaticus seminiger*, joined by a cementing material localized between the two heads. (From Dallai and Afzelius, 1985). (G) Cross-section through the spermatozoa of the zygentoman *Allacrotelsa kraepelini*, joined by a junctional contact between their plasma membranes.

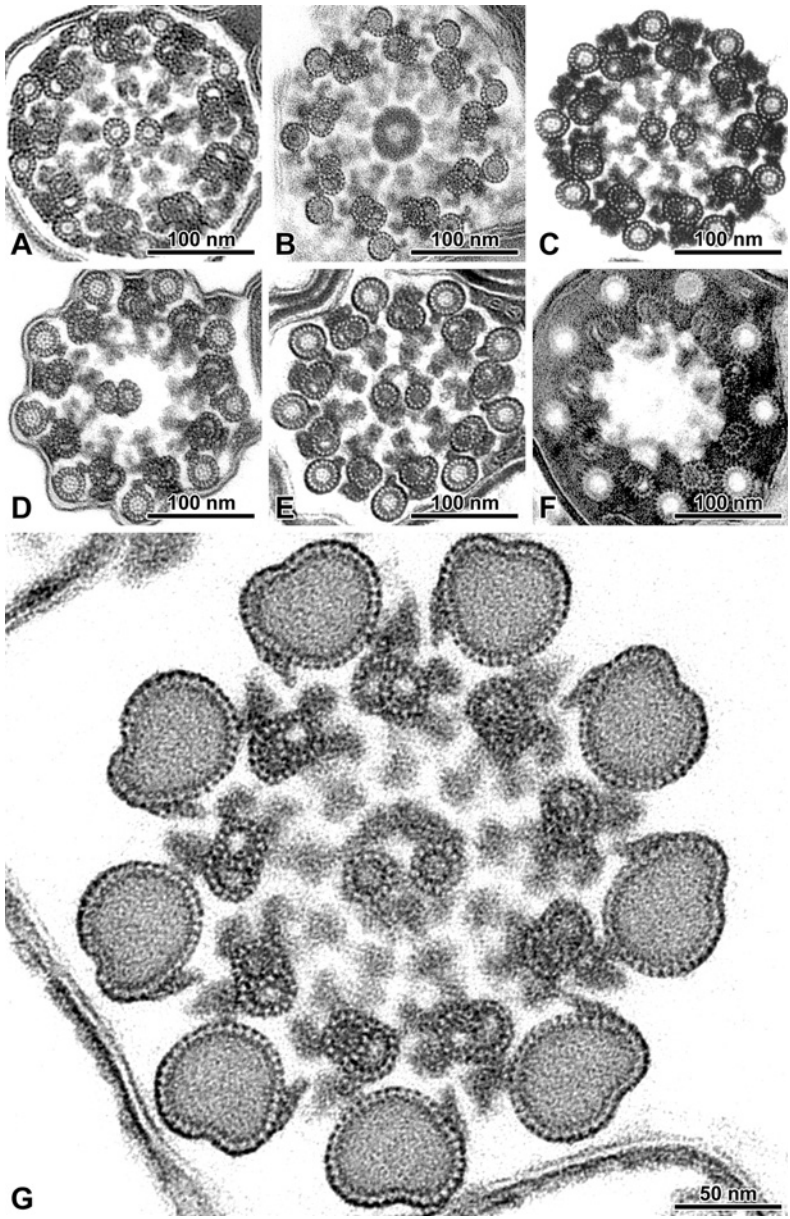


FIG. 3 Cross-sections through the sperm flagellar axonemes of insects belonging to several pterygotan orders to show the accessory tubules provided with different numbers of protofilaments. (A) The brachyceran dipteran *Scatophaga* sp. (Scatophagidae) with 13 protofilaments. (B) The nematoceran dipteran *Bibio marci* (Bibionidae) with 15 protofilaments. (C) The stick insect *Baculum* sp. (Phasmatodea) with 17 protofilaments. (B, C: from Dallai and Afzelius, 1999).

## 2. Protofilament Number of Accessory Tubules

The most diffuse number of protofilaments in insect accessory tubules is 16, and this structure can be considered as a feature of the sperm ground plan of the group (Fig. 1D–F). However, as we have already reported in the previous section, tubules constituted by a different number of protofilaments have also been found in some species.

Accessory tubules formed by 13 protofilaments, (i.e., sharing the structure of cytoplasmic microtubules expressed in other cell types) are present in the basal diplurans *Japyx solifugus* and *Campodea* sp., as well as in the paleopteran insect Ephemeroptera. Among neopterans, Psocoptera, Phthiraptera (Mallophaga and Anoplura), and the higher dipterans Brachycera, all possess 13-protofilament accessory tubules (Fig. 3A). Besides being formed by the same number of protofilaments, peculiar distinctive features mark the accessory tubules in each one of these insect groups; thus, in mayflies a set of seven orderly arranged filaments are internally associated with the tubular wall (Fig. 7D); book lice (Psocoptera) as well as biting and sucking lice (Mallophaga and Anoplura) possess elliptical tubules, while the accessory tubules of brachyceran dipterans contain a dense central filament (Fig. 3A).

Accessory tubules with 14 protofilaments have not yet been described in any taxon. Tubules provided with 15 protofilaments are commonly found among several nematoceran dipteran families, such as Chironomidae, Bibionidae, Culicidae, and Simulidae (Fig. 3B), while tubules consisting of 17 protofilaments have been described formerly in the stick insect *Baculum* by Afzelius *et al.* (1990), and successively in other Phasmatodea (Fig. 3C), in the caddisflies Rhyacophilidae (Dallai *et al.*, 1995b), considered to be a basal trichopteran family, and in the spermatozoa of some Strepsiptera (Dallai *et al.*, 2003). Since these groups do not share a recent phylogenetic origin, the occurrence of a common accessory tubule structure has to be considered as the result of a parallel independent acquisition.

A high variability and an increasing number of protofilaments have been detected in the accessory tubules of trichopterans; indeed, this group is one of the insect orders expressing the highest number of modified axonemal models, as it can be inferred also from the following paragraphs concerning other

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(D) The caddisfly insect *Leptocerus tineiformis* (Trichoptera, Leptoceridae) with 18 protofilaments. Note the eccentric position of the two central tubules and also the lack of the sheath surrounding these microtubules. (E) The caddisfly insect *Potamophylax cingulatus* (Trichoptera, Limnephilidae) with 19 protofilaments. (F) The caddisfly insect *Sericostoma italicum* (Trichoptera, Sericostomatidae) with 20 protofilaments at the centriolar level. (D–F: from Dallai and Afzelius, 1994). (G) The paraspermatozoa of the neuropterid *Perlamantispa perla* (Planipennia) have accessory microtubules with 40 protofilaments. These "macrotubules" have a larger diameter (up to 55 nm) and are filled with a dense material; at first glance, they resemble the accessory fibers of higher vertebrates. (From Dallai *et al.*, 2005).

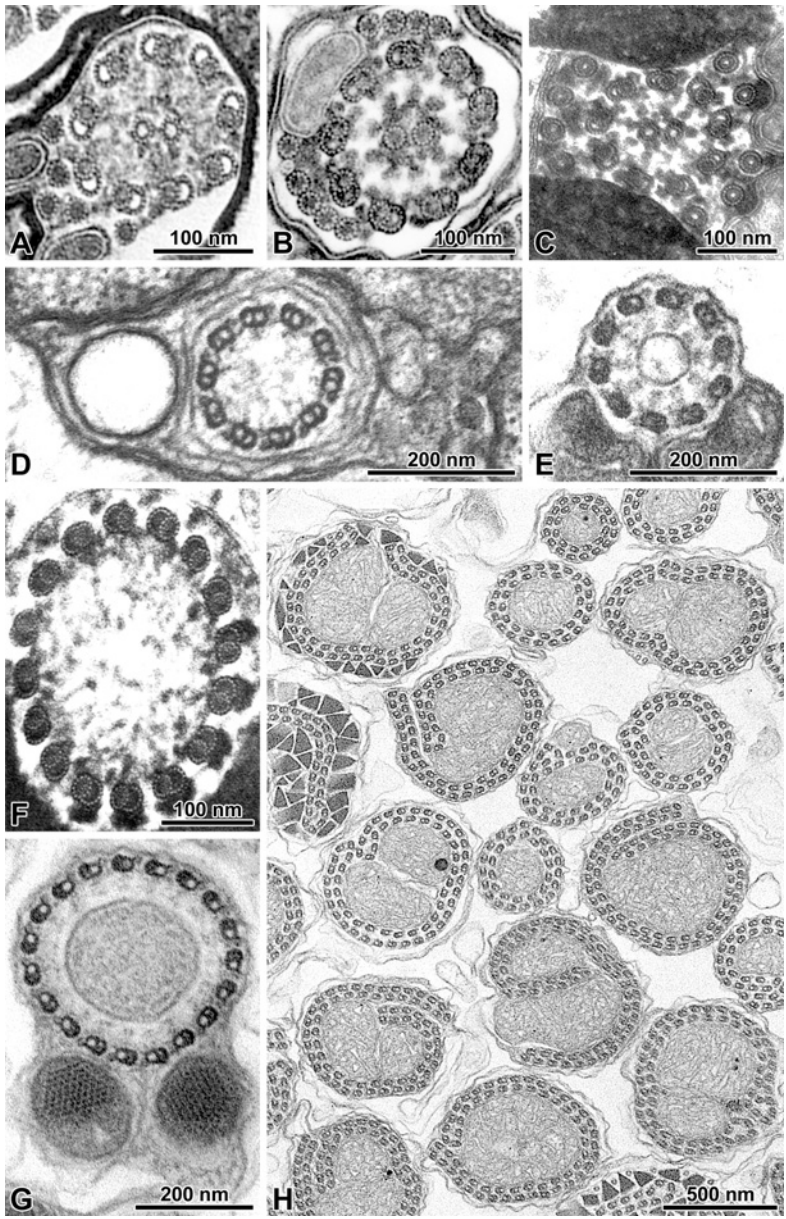


FIG. 4 (A) Cross-section of a *Japyx solifugus* (Diplura) spermatozoon. Five of the nine accessory tubules moved from their original position to a new localization close to the mitochondria; (B) Cross-section of a *Campodea* sp. (Diplura) spermatozoon. The nine accessory tubules are aligned on both sides of the mitochondrion. (C) Cross-section through a *Machilis distincta* (Microcoryphia) spermatozoon. The nine accessory tubules are organized

axonemal features. Within trichopterans, accessory tubules with 18 protofilaments have been described in the family Leptoceridae—which is characterized also by the peculiar eccentric position of the two central microtubules of the axoneme and by the absence of the central sheath (Fig. 3D)—and Glossosomatidae, while 19 protofilaments have been found in the members of three families—Limnephilidae, Goeridae and Odontoceridae (Fig. 3E)—and 20 protofilaments in the family Sericostomatidae (Fig. 3F) (Dallai and Afzelius, 1994, 1995).

As previously mentioned, a unique type of giant accessory tubules, called macrotubules, is expressed in the paraspermatozoa of the neuropterid *Perlamantispa* (=*Mantispa*) *perla* (Fig. 3G). These macrotubules, which at a first glance recall the accessory fibers of some vertebrates, comprise 40 protofilaments in their tubular wall; though their origin from the B-tubule has been well established (Dallai *et al.*, 2005), their mechanism of assembly and their structure would be worthy of further investigation.

### 3. Number and Localization of Accessory Tubules

In the 9+9+2 flagellar axoneme, accessory tubules are often shorter than microtubular doublets, so that in the proximal part of the axoneme, or toward the tail posterior tip, only a few accessory tubules are visible. For instance, the fungus gnat mycetophilids *Exechia seriata* and *E. fusca* have axonemes with only seven, rather than nine, accessory microtubules (Dallai *et al.*, 1995a), doublets 7 and 8 being apparently devoid of such tubules.

As a general rule, the accessory tubules are located in a precise position outside the microtubular doublets and are flanked by the intertubular material; thin connections have been observed between them and the B subtubules of doublets (Rosati, 1976). Nevertheless, in the flagellar axonemes of the apterygotan Diplura (both Japygina and Campodeina) (Fig. 4A, B) as well as of the machilid *Machilis distincta* (Microcoryphia=Archaeognatha), the accessory tubules lose their connections with the microtubular doublets during spermiogenesis and migrate to a different position (Dallai, 1972;

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in two opposite groups with respect to the 9+2 axoneme. (From Jamieson *et al.*, 1999). (D, E) Two examples of 10+0 axoneme patterns in the gall-midge fly spermatozoa of the subfamily Cecidomyiinae (Diptera, Nematocera); (D) *Stomatosema* sp. (E) *Xylopriona toxicodendri*. (F) Cross-section of the 16+0 axoneme of the basal wingless hexapod *Acerentulus confinis* (Protura). (G) The cecidomyiid fly *Anaretella* sp. (Diptera, Nematocera) has a sperm flagellar axoneme provided with about 20 microtubular doublets in a circle. In the center of this structure a single mitochondrion is visible. (H) Cross-section through the cecidomyiid fly *Lestremia cinerea* (Diptera, Nematocera) sperm axonemes, showing the two antiparallel microtubular rows surrounding mitochondria.

Wingstrand, 1973). Similarly, in *Machilinus kleinbergi* the accessory tubules move toward two opposite sides of the 9+2 axoneme (Fig. 4C). In the dipluran *Japyx solifugus*, five of the nine accessory tubules, longer than the others, shift on one side of flagellum close to the two mitochondria (Fig. 4A); similarly, in *Campodea* sp., the nine accessory tubules are arranged in a constant pattern, five on one side and four on the opposite side of the longest mitochondrion (Dallai and Afzelius, 1999).

In some hemipteran bugs, variations in the disposition of microtubules at the level of the end piece have been described (Folliot, 1970; Folliot and Maillet, 1970). A particular pattern has been observed in psillids and in *Euscelis lineolatus*. In the spermatozoa of these insects the posterior axonemal part is flattened and characterized by the presence of a single row of regularly alternating doublets and accessory tubules. In the membracid *Campylenchia latipes*, the flagellum tends to split into two parallel parts (Folliot, 1970) or, as in *Ceresa* sp. and in *Dalbulus maidis*, into four parts (Cruz-Landim and Kitajima, 1972; Phillips, 1969).

Accessory tubules often occur in number equivalent to that of microtubular doublets, so that any variation in their number is concomitant to a corresponding variation in the number of microtubular doublets. This occurs in the 13+13+0 axoneme of the trichopteran *Wormaldia copiosa* (Dallai *et al.*, 1995c) and in the aberrant axonemes of the dipteran family Sciaridae; in *Sciara coprophila*, about 70 doublets along with their accessory tubules are present (Fig. 6A) (Makielski, 1966; Phillips, 1965, 1966), while in *Rhynchosciara angelae* the axoneme consists of up to 350 doublets, each doublet being flanked by the related accessory tubule (Phillips, 1974; Shay, 1972, 1975).

## 4. Number and Disposition of Microtubular Doublets

The occurrence of extranumerary microtubular doublets has been described in several hexapod groups and, in some instances, the increase in doublet number has been so high to have generated a completely aberrant axonemal model. The first example can be found in proturans. Among the several modifications of the axonemal architecture exhibited by this group, the family Acerentomidae comprises species expressing a 12+0 (*Acerentulus tragardhi*), 16+0 (*A. confinis*) (Fig. 4F), and 14+0 (*Acerentomon majus*) axoneme, whereas the sperm flagellum of the proturan *Neocondeellum dolichotarsum* contains 13 doublets (Dallai *et al.*, 1992; Yin *et al.*, 1985); in these species the doublets are connected to each other by evident nexin links and inner dynein arms and to the plasma membrane by electron-dense bridges; the axoneme is motile (Dallai *et al.*, 1992). Thirteen doublets also constitute the flagellar axoneme of the trichopteran mayfly *Wormaldia copiosa*, arranged around a large central vesicle along with the related accessory tubules (Dallai *et al.*, 1995c).

An exceeding variation in the doublet number has been described in the dipteran nematoceran Cecidomyiidae; this is one of the greatest families and includes more than 4000 species belonging to three subfamilies: Lestremiinae, Porricondylinae, and Cecidomyiinae (Skuhravá, 1986). With respect to Lestremiinae, Cecidomyiinae are apomorphic in most somatic and spermatological characters (including the loss of the inner dynein arms, a unique feature in the animal kingdom) but are, however, more plesiomorphic than other insects, in having no crystallization of mitochondria. For the presence of a great number of microtubule doublets in the sperm axoneme, Cecidomyiidae recall the closely related family Sciaridae (Fig. 6A).

During the last 30 years, several spermatological works have reported the occurrence of numerous axonemal models in this family. While the flagellar axoneme of Porricondylinae usually consists of the regular nine peripheral doublets, an increased number of doublets is expressed in Lestremiinae. In this group, three species belonging to the tribe Micromyini (*Micromyia lucorum*, *Polyardis bispinosa,* and *Xylopriona toxicodendri*) share an axoneme consisting of 10 doublets surrounding a central cylinder (Dallai *et al.*, 1996a) (Fig. 4D, E); in the same subfamily, *Anaretella cincta* and another unclassified species of the same genus possess axonemes provided with 20–22 doublets arranged around a central cylindrical mitochondrion (Fig. 4G). A general consequence of the increased number of microtubular doublets is a larger size of the axoneme and an elliptical, rather than circular, outline. Finally, in different species of the genus *Lestremia* the number of microtubular doublets may vary from 65–70 to 150 (Fig. 4H); as a consequence of their great number, doublets are no longer circularly arranged, but rather they are arranged in antiparallel rows that surround a central group of mitochondria. It has been suggested that this peculiar axonemal pattern might have been derived from an almost circular axoneme similar to that of *Anaretella*; a progressive increase in the doublet number would then have been followed by a progressive flattening and bending of the microtubular lamina, up to the formation of two antiparallel rows of microtubule doublets (Fig. 5A–C) (Dallai *et al.*, 1996b).

The subfamily Cecidomyiinae continues the trend toward an extreme modification and variability of the axonemal pattern seen in Lestremiinae; the evolution of axonemal features in the four supertribes of this subfamily has been revised by Dallai *et al.* (1996c). *Didactylomyia longicauda* and *Stomatosema* sp. belong to the supertribe Stomatosematidae, the former species being provided with a 9+5 axoneme and the latter one with a 10+0 axoneme. Both dynein arms are present, thus setting this group in a separate position with respect to the other supertribes. The supertribes Asphondyliidi and Cecidomyiidi are characterized by a progressive increase in the number of doublets, by the absence of accessory tubules and central pair, and by the presence of the outer dynein arms only.

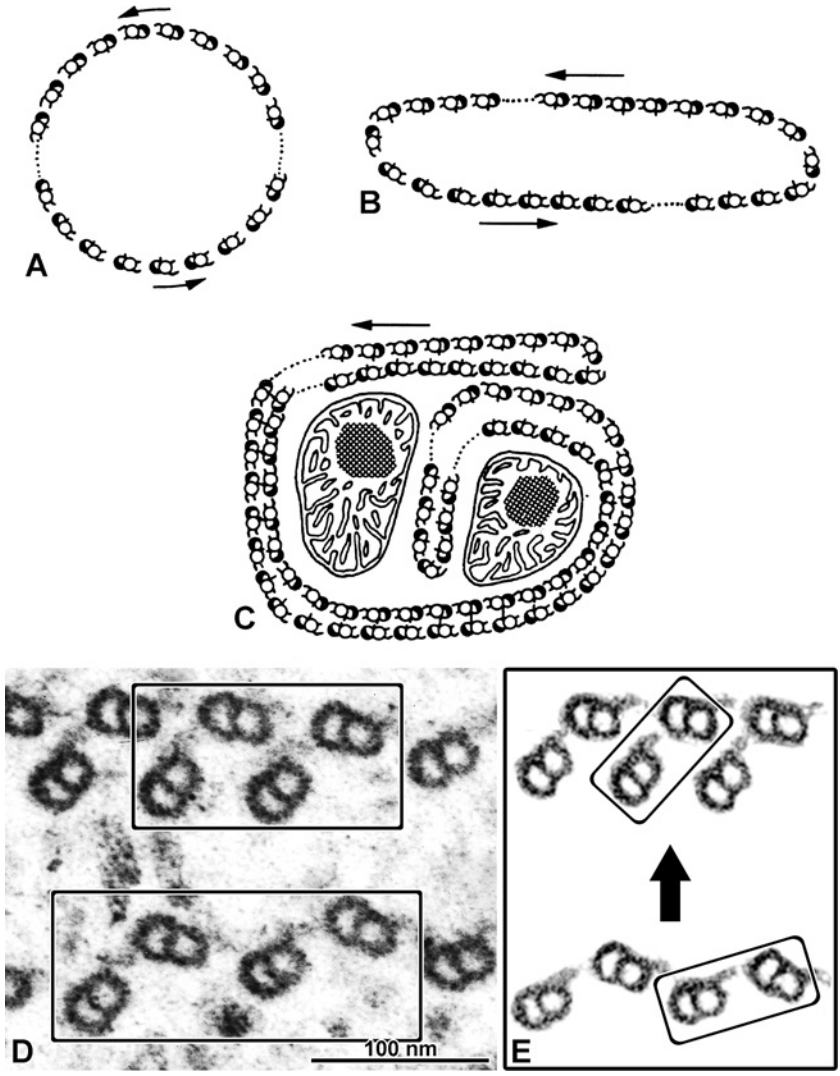


FIG. 5 (A–C) Schematic drawing of the possible origin of the aberrant giant axoneme of the gall-midge fly *Lestremia cinerea*. Starting from a circular axoneme consisting of 20 microtubule doublets, as that described in *Anaretella* (A), it may be hypothesized that a process of flattening (B) and folding of the axoneme might have given rise to the axoneme of *L. cinerea* (C). (From Dallai and Mazzini, 1983). (D, E) Detail of a cross-section of a spermatid of *Asphondylia ruebsaameni* showing the rearrangement that microtubular doublets undergo during spermiogenesis, from an original single, zig zag spiral to a double spiral arrangement. This process involves a rotation and a sliding of doublets.

The axoneme of Asphondyliidi consists of two parallel spirals of microtubular doublets surrounding a longitudinal axis of mitochondria (Fig. 6E, F). The number of doublets may vary from 30–70 in *Asphondylia jaapi* to about 2,500 in *A. ruebsaameni* (Fig. 6F) (Dallai, 1988). Other species, such as *Kiefferia pericarpiicola*, *Placochela nigripes*, *Daphnephila machilicola,* and *Asphondylia ilicicola* (Dallai, 1988; Dallai *et al.,* 1996c) have different numbers of doublets, but the general architecture of the axoneme is always preserved. This peculiar axonemal pattern consisting of two parallel spirals forming this peculiar axoneme is settled late during spermiogenesis by a mechanism of rotation and sliding of doublets, as shown in Fig. 5D, E.

In most species of the supertribe Cecidomyiidi, the sperm axoneme is formed by a single rather than by a double parallel row of doublets (Fig. 6B–D) (Mencarelli *et al.*, 2001), and may comprise from 40 doublets (*Mycodiplosis* sp.) (Dallai and Mazzini, 1980; Dallai *et al.*, 1997) to about 1,000 doublets (*Diplolaboncus tumorificus*) (Baccetti and Dallai, 1976). This is the so-called ''Sciara-like model,'' because of its resemblance to the sciarid sperm axoneme. A separate group of species, which are also commonly included by taxonomists in this supertribe, exhibits a different sperm shape, the so-called ''doublet-pocket model.'' Here, the sperm flagellum is in general short and flat, and the plasma membrane forms several small evaginations, each containing a microtubular doublet devoid of dynein arms. This model is shared by *Contarinia* (*C. floriperda*, *C. pulchripes*, *C. loti*), *Lestodiplosis* (*L.* sp.a), *Allocontarinia* (*A. sorghicola*) and *Myricomyia* (*M. mediterranea*) (Fig. 7A, B). The number of microtubular doublets may vary from 9 up to 14 (Dallai *et al.*, 1993b). The same model is present also in the lasiopterid *Rhyzomia* (Dallai *et al.*, 1996c).

The supertribe Lasiopteridi comprises species having spermatozoa provided with a 9+0 axoneme, which is immotile because of the absence of both dynein arms (Fig. 7C). Moreover, the sperm cytoplasm contains a great number of singlet microtubules, which often prolong into cell membrane expansions, giving the cell its characteristic shape.

The whole evidence summarized here clearly indicates that the assembly of an exceeding number of microtubular doublets has always occurred concomitantly with major defects in the assembly of the central pair complex, which is no longer present in any one of the aforementioned axonemal models. There is no available clue concerning the molecular defect(s) that underlie these phenotypes. Recently, the occurrence of 10-doublet axonemes devoid of the central singlets has been reported in *Drosophila* mutants carrying mutations in the axonemal $\beta_2$-tubulin molecule (Nielsen *et al.*, 2001); these data could suggest tubulin as one of the possible protein components that have been modified in these unorthodox axonemal models.

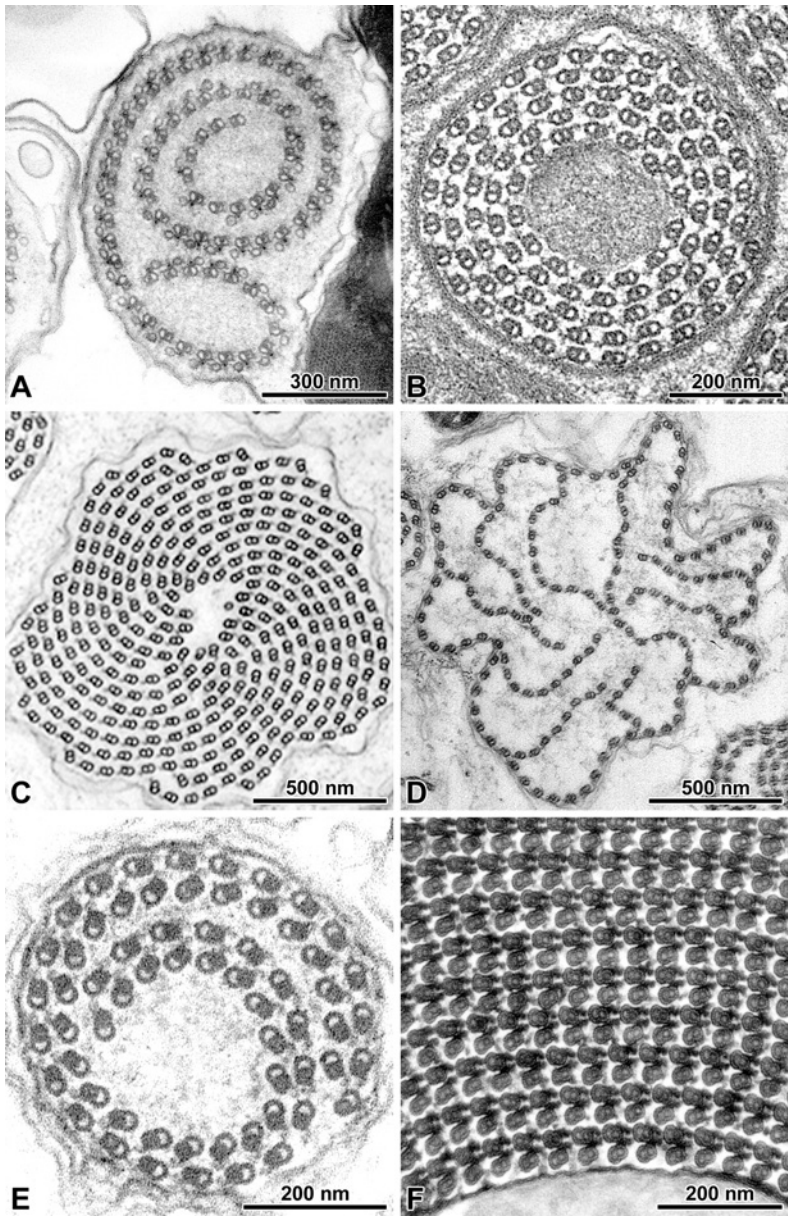


FIG. 6 (A) Cross-section of the sperm axoneme of *Sciara coprophila* (Diptera, Nematocera) consisting of microtubule doublets and accessory microtubules disposed in a spiral-like arrangement. (From Dallai *et al.*, 1973). (B–D) Cross-section of the sperm axonemes of three gall-midge flies of the subfamily Cecidomyiinae, supertribe Cecidomyiidi (Diptera, Nematocera). Many microtubular doublets are organized in a simple spiral in *Bremia* sp. (B), or in linear

## 5. Modification of the Central Complex

The most frequent alterations of the 9+9+2 pattern that occur in hexapod sperm axonemes concern the central apparatus, which may be modified, absent, or substituted by axial elements of different structure and nature. The frequency of such kind of axonemal modification among insects is quite remarkable, given the important role that the central apparatus plays in the regulation of axonemal motility and in the conversion of dynein-driven microtubule sliding into complex flagellar waveforms. As a matter of fact, mutations affecting the central pair structure and composition in the protist *Chlamydomonas* always result in flagellar paralysis (see Smith and Lefebvre, 1997). It is interesting to note here that these immotile mutants retain the ability to undergo microtubule sliding and can rescue motility, without restoring the missing structures, only if a further, suppressor mutation affecting other axoneme structures occurs (Huang *et al.*, 1982; Porter *et al.*, 1992, 1994; Rupp *et al.*, 1996); these suppressor mutations were found to alter outer or inner dynein arm components. Abundant and detailed information about the motility of insect sperm flagella carrying a modification of the central apparatus is not available; however, some data indicate that at least in some cases these spermatozoa are motile. In this respect, it is important to underline that in the evolution of some insect groups the modification of the central pair appears often to be followed by—or to be concomitant with—the loss of other axonemal components, giving rise to increasingly aberrant patterns.

An interesting series of progressive modifications of the axonemal architecture is provided by the dipteran family Cecidomyiidae; a detailed description of the axonemal patterns expressed in the three subfamilies of this group (Lestremiinae, Porricondylinae, and Cecidomyiinae) has been reported in the preceding paragraph. Here, we only analyze the different modifications of the central apparatus observed in these insects.

Within this group, the subfamilies Porricondylinae and Lestremiinae are commonly supposed to comprehend the most primitive species; among the former, *Diallactes* exhibits sperm cells with either 9+2 or 9+1 axonemes, whereas other species possess sperm flagella with 9+3 or 9+0 axonemes (Dallai and Mazzini, 1983; Dallai *et al.*, 1996d). In all the species belonging

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series disposed in a cartwheel array as in *Massalongia bachmaieri* (C), or in short, irregular centripetal rows in *Monarthropalpus flavus* (D). (E, F) Cross-section of the sperm axonemes of two gall-midge flies of the subfamily Cecidomyiinae (Diptera, Nematocera), supertribe Asphondilyiidi. Different numbers of microtubular doublets are present, always devoid of inner arms: about 60, as in *Placochela nigripes* (E) and about 2500, in *Asphondylia ruebsaameni* (F); given the dimension of its axoneme, only part of the section is shown here for the latter species.

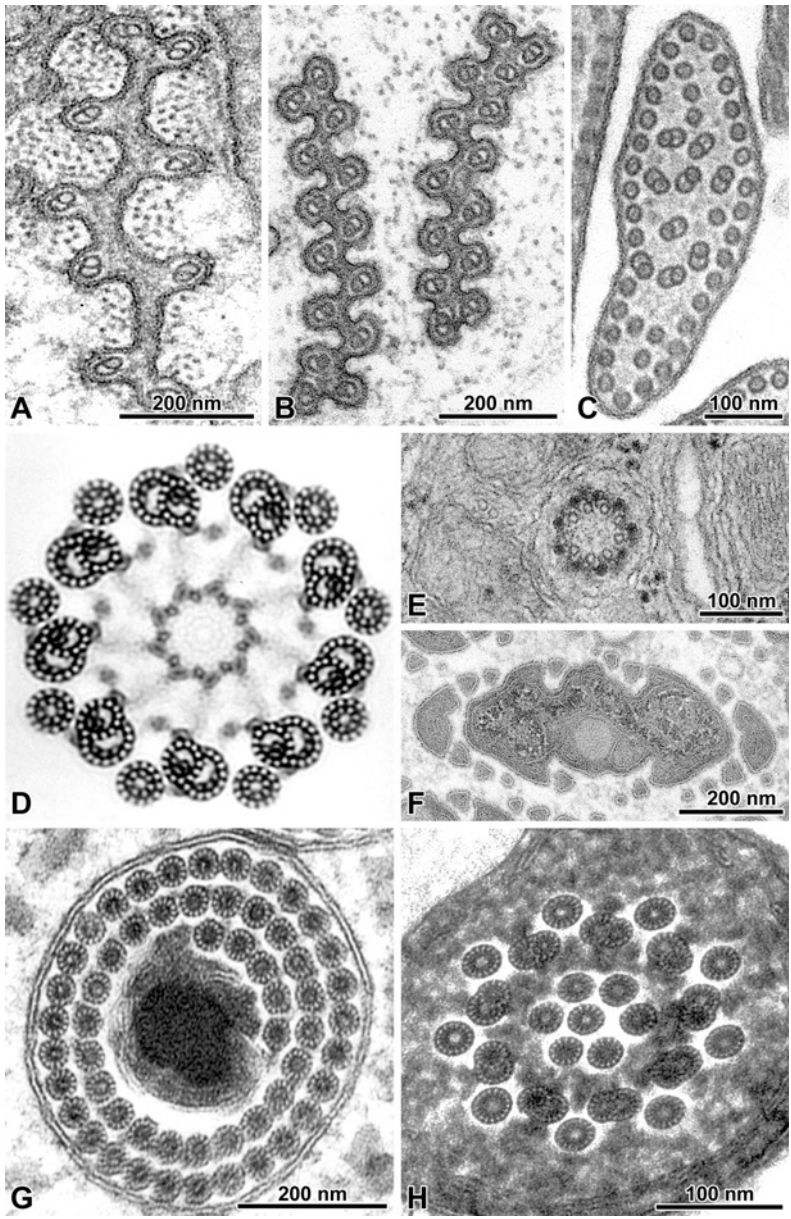


FIG. 7 (A, B) Cross-section of the flattened sperm axonemes of two gall-midge fly Cecidomyiidae (Diptera, Nematocera), which are provided with 9 and 14 microtubule doublets, in *Contarinia* sp. (A) and in *Myricomyia mediterranea* (B), respectively. Doublets are devoid of dynein arms and are hosted in plasma membrane pockets. (C) Cross-section of the immotile sperm axoneme of the gall-midge fly *Gephyraulus* sp. (subfamily Cecidomyiinae, supertribe

to Lestremiinae, the axoneme lacks the central microtubular pair and is characterized by either a 9+0 organization or, as previously mentioned, by an increasing number of microtubular doublets arranged either around a sort of central "cylinder" formed by a noncrystallized mitochondrion (Fig. 4G) or in double rows and containing up to 70–150 doublets (Fig. 4H) (Dallai *et al.*, 1996a,b). In both subfamilies, the axoneme maintain some—though reduced—degree of motility. In the more advanced subfamily Cecidomyiinae, the absence of both the central complex and the inner dynein arms leads first to the appearance of odd axonemal patterns and then to the complete loss of the axoneme. In some groups, giant axonemes consisting of a huge number of doublets, are present (Baccetti and Dallai, 1976; Dallai *et al.*, 1996c) (Fig. 6B, F). These spermatozoa exhibit a very peculiar kind of motility (see Chapter 4). Thus, in the evolution of this insect family, the disappearance of the central pair/radial spoke complex has been followed by the loss of one or both dynein arms and by profound alterations in the axonemal architecture.

Other nematoceran groups exhibiting modifications of the central apparatus comprise the families Simulidae and Culicidae (Culicomorpha) and the bibionomorph families Bibionidae and Sciaridae. The families Simulidae and Culicidae exhibit respectively three central microtubules (9+9+3) and the replacement of the central pair by a single central rod element (Baccetti *et al.*, 1974; Breland *et al.*, 1968; Clemens and Potter, 1967; Ndiaye *et al.*, 1996). In the bibionomorph family Bibionidae the central microtubules are either absent, along with the peripheral accessory tubules (9+0), or substituted by a prominent, about 80-nm wide, cylindric sheath sending projections toward the A-subtubules, as in *Bibio* (Fig. 3B) (Dallai and Afzelius, 1999; Trimble and Thompson, 1974). Sciaridae, possess a highly aberrant axoneme formed by many doublets and resembling the cecidomyiid axoneme, but with doublets provided with both dynein arms (Fig. 6A)

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Lasiopteridi) (Diptera, Nematocera). Nine scattered microtubular doublets, devoid of dynein arms, and several microtubules are visible (A and C: from Baccetti and Dallai, 1976). (D) Cross-section of the sperm axoneme of the mayfly *Cloeon dipteron* (Ephemeroptera) (computer-aided elaboration). Microtubular doublets are provided with inner dynein arms only, the central microtubules are missing and the accessory tubules have 13 protofilaments in their tubular wall; moreover, these tubules contain a structured internal material. (From Dallai and Afzelius, 1999). (E, F) Cross-sections of the sperm flagella of an early spermatid (E) and of a mature immotile spermatozoon (F) of the white-fly *Dialeurodes citri* (Homoptera). During spermiogenesis, the axoneme degenerates and in the mature spermatozoon it is no longer visible. (G) Cross-section of the sperm flagellum of *Comstockaspis perniciosa* (Coccoidea, Homoptera), showing the singlet microtubule spiral surrounding the nucleus. (H) Cross-section of the immotile sperm axoneme of the caddisfly *Philopotamus montanus* (Trichoptera, Annulipalpia). Microtubular doublets are devoid of dynein arms and surround 7 central tubules. (From Dallai *et al.*, 1995c).

(Dallai and Afzelius, 1990; Dallai *et al.*, 1973). A detailed analysis of the motility pattern has been carried out only in one species expressing a 9+9+0 (or 9+9+'1') axoneme, the mosquito *Aedes notoscriptus*; in this species, the sperm tail propagates two waves of different frequency and amplitude, that is, a fast, small-amplitude wave that is superimposed to a slow, large-amplitude wave, similarly to what has been described in the 9+9+2 axoneme of other insect species (Swan, 1981).

Alterations in the central apparatus are scattered among other insect orders. In particular, the central complex is completely absent in the proturan species *Acerentulus* and *Acerentomon* (12+0, 14+0, or 16+0) (Fig. 4F) (Baccetti *et al.*, 1973c; Dallai *et al.*, 1992), in which the central region of the axoneme may contain glycogen granules; in the coleopteran curculionoidean family Rhynchitidae (9+9+0) (Burrini *et al.*, 1988) and in the Psocomorpha (Psocoptera), in which it is replaced by a central rod (9+9 +'1') (King and Ahmed, 1989). In three families of trichopteran Annulipalpia: Philopotamidae, Polycentropodidae, and Hydropsychidae, the central pair is either absent or constituted by seven, instead of two, microtubules (Fig. 7H) or replaced by an axial vesicle. In this group both dynein arms are absent and a progressive loss of both the sperm flagellum and motility has been observed (Dallai *et al.*, 1995c).

The central apparatus is also absent in two insect groups characterized by a completely aberrant organization of the axoneme. The tysanopterans, with their peculiar flagellum containing three amalgamated axonemes, are still able to propagate fast waves (Dallai *et al.*, 1991); the coccoids (Hemiptera, Homoptera), which possess a motile apparatus consisting of a large number of singlets arranged in concentric rings or in spirals (Fig. 7G) (Robison, 1977; Swiderski, 1980; Swiderski and El Said, 1992; references in this review), and have a very peculiar kind of motility (Harris and Robison, 1973; Robison, 1966). In this last example, however, the motile apparatus cannot be defined as an axoneme, because the microtubule bundle does not grow from a centriolar structure (Robison, 1977 and our unpublished results).

Finally, a 9+9+0 axonemal pattern is shared also by most of the members of the paleopteran Ephemeroptera. In these species the central pair is substituted by a central cylinder about 600 Å in diameter, which carries nine projections radiating out to meet the subtubule A of the doublets (Fig. 7D). The outer dynein arms are absent (Baccetti *et al.*, 1969b; Phillips, 1969, 1970a,b; Dallai and Afzelius, 1990). The structure motility and the dynein heavy chain complement have been analyzed in two ephemeropteran species by Dallai *et al.* (Our unpublished results, see Section IIIC).

Although the data available on the pattern of motility exhibited by these modified axonemes provided structural variations of the central apparatus are extremely limited, it is interesting to note that several insect species have been able to overcome the restrictions that would have been likely to be

imposed on flagellar motility by modifications of the central apparatus and thus to maintain at least some capability of movement.

## 6. Loss of Dynein Arms, the Axoneme, and Impairment of Sperm Motility

As it has been already outlined, several hexapod groups are characterized by the loss of one or both dynein arms, a feature that can be found both in the regular 9+9+2 arrangement and in highly modified axonemes. The existing information concerning the absence of dynein arms, as well as the impairment of sperm motility attributable to the loss of the whole axoneme is reviewed here.

In the wingless Protura, many species possess aberrant, but still motile sperm axonemes, consisting of microtubular doublets provided with the inner dynein arms only; the derived families Eosentomidae and Sinentomidae are characterized by discoidal or globular immotile spermatozoa that have lost the whole axoneme. The 9+9+0 axoneme of most paleopteran Ephemeroptera is also endowed with inner arms only (Fig. 7D) (Dallai and Afzelius, 1990), but the family Leptophlebiidae possess aflagellated immotile sperm cells (Gaino and Mazzini, 1990, 1991).

An evolutionary trend toward the progressive loss of dynein arms, first, and successively of the whole axoneme has occurred also in Isoptera. The multiflagellated spermatozoon of *Mastotermes darwiniensis* contains 9+0 axonemes with doublets presumably provided with only the outer dynein arms (Fig. 2C), whereas the elongated spermatozoa of the families Hodotermitinae and Kalotermitidae are aflagellated and immotile, as well as the globular spermatozoa of Rhinotermitidae and Termitidae (Baccetti and Dallai, 1978; Baccetti *et al.*, 1981). Among the homopterans Auchenorrhyncha, we have already mentioned the presence in different species of a branching axoneme (Folliot and Maillet, 1970). Among the homopterans Sternorrhynca, the basal superfamilies Aphidoidea and Psylloidea express a 9+9+2 axoneme, whereas Aleyrodoidea assemble, during early spermiogenesis, a 9+9+0 axoneme devoid of dynein arms, which later degenerates into a solid rod (Fig. 7E, F); along with the axoneme, the mitochondria disappear as well (Baccetti *et al.*, 1977; Báo *et al.*, 1997; Dallai, 1979). In the most evolved superfamily Coccoidea, spermatozoa have lost a true flagellar axoneme and the sperm motility is supported by a series of orderly arranged singlet microtubules, surrounding an elongated nucleus (Fig. 7G) (Robison, 1970, 1972, 1977); these microtubules are connected to each other by projections endowed with an ATPase activity (Baccetti *et al.*, 1982), but no information is available on their protein content. The movement of this peculiar structure of the axoneme has been reported to be caused by the dislocation of microtubules along each row (Harris and Robison, 1973).

As already reported, among nematoceran Diptera the different taxa have achieved axonemal models exhibiting several variations compared to the conventional 9+9+2 pattern. Within the family Psychodidae, the basal subfamily Phlebotominae (Dallai *et al.*, 1984; Fausto *et al.*, 1995; Mazzini *et al.*, 1992) assembles a motile 9+9+0 sperm axoneme, while in the most evolved subfamily Psychodinae (Baccetti *et al.*, 1973d) the loss of the axoneme represents a common feature. In some of the already described gall-midge flies Cecydomyiinae, two supertribes, Asphondyliidi and Cecydomyiidi, have motile unusual axonemal models with outer dynein arms only, though Lasiopteridi possess nine disordered doublets without arms. Among related groups characterized by a flattened flagellum and by doublets devoid of arms, a *Lestodiplosis* species (*L.* sp.b) is aflagellated (Dallai *et al.*, 1993b).

In Trichoptera, the basal Integripalpia possess a 9+9+2 axoneme that lacks the outer dynein arms (Fig. 3D–F) (Dallai and Afzelius, 1994), while the more evolved Annulipalpia are characterized by immotile spermatozoa exhibiting several aberrant axonemal models; a modification of the central apparatus has been described for Philopotamidae and Polycentropodidae (see earlier), along with the absence of outer dynein arms, while Hydropsychidae possess a highly bizarre spermatozoon, with finger-like appendages that extrude from the cell body and contain microtubular doublets devoid of both outer and inner dynein arms; these doublets originate either from the centriole or from a dense material scattered in the cytoplasm (Dallai and Afzelius,1995; Friedländer and Morse, 1982). Spermatozoa from six species of Hydroptilidae are immotile and aflagellated (Dallai and Afzelius, 1995).

The many evidences described herein seem to indicate that when a first modification is introduced into the axoneme basic structure, this is often followed by an evolutionary trend toward a more and more profound alteration of the axoneme, up to its complete regression and the transformation of the spermatozoon in an aflagellate immotile cell.

## 7. Variations in Axonemal Length and Sperm Polymorphism

The interaction between males and females during fertilization is a matter of great interest in the field of reproductive biology and behavioral ecology. In general, in polyandric species in which females mate with more than one male, sperm competition selects males with the highest fertilizing capability (Civetta, 1999). In *Drosophila* females, engaged in multiple matings, sperm from the first mating are retained in the female only for a short period, while the second male is able to sire a more numerous progeny by inducing the female to store and preferentially use its own sperm or even to strongly displace already resident sperm. According to Birkhead (2004), females have evolved to keep sperm at arm's length, allowing them control over

which sperm will father their offspring. This is not the kind of situation that males or their sperm accept passively lying down. Their goal is to fertilize eggs and for every barrier evolved in females, it pays males to evolve a counter-strategy to bypass it.

In most animal phyla, males produce tendentially large numbers of tiny motile spermatozoa, low-investment gametes that they use with minimal frugality (Alexander and Borgia, 1979; Parker *et al.*, 1972), while females are more concentrated on the production of a few large eggs. In insects, however, recent studies have revealed the occurrence of a great interspecific variability in sperm number and morphology. Sperm variability has been described in hemipterans (Fig. 8A), coleopterans, hymenopterans, neuropterans (Fig. 8B), dipterans (Fig. 8C), and lepidopterans (Fig. 8D, E). Moreover, the current view on the evolution of male sexual strategy is contradicted by the existence of giant sperm (Fig. 8C). In fact, some *Drosophila* species produce a very low number of giant-sperm cells and use them with female-like judiciousness, carefully partitioning their limited quantity of sperm among successive females (Pitnick *et al.*, 1995a). As a matter of fact, the production of either giant sperm or heteromorphic sperm is costly. Pitnick *et al.* (1995a) have showed that *Drosophila* males producing giant sperm become reproductively mature later than males of related species producing normally sized spermatozoa (Pitnick and Markow, 1994a).

In many species characterized by sperm polymorphism, one type of sperm cells is not a fertilizing gamete, but still represents up to 95% of the sperm transferred to female (Bressac and Hauschteck-Jungen, 1996; Silberglied *et al.*, 1984; Sivinski, 1980; Snook, 1995, 1997). This occurs in lepidopterans, where males produce both apyrene, anucleated sperm and eupyrene sperm, which is the only functional sperm for fertilization (Fig. 8D, E) (Friedländer, 1983; Hamon and Chauvin, 1992; Sonneschein and Hauser, 1990). Apyrenic spermatozoa do not enter the *canaliculus fecundans* or the *vestibulum*, where the eggs are fertilized. As to the function of apyrenic spermatozoa of lepidopterans, it has been hypothesized that this sperm type might help eupyrenic spermatozoa to reach the female genital tract. (Friedländer and Gitay, 1972; Friedländer *et al.*, 2005). Quite recently, dimorphic sperm have been found in the neuropteroid *Perlamantispa* (=*Mantispa*) *perla* (Fig. 3G; Fig. 8B); the giant type of sperm is not the fertilizing gamete and is presumed to be involved in preventing remating by filling the spermatheca (Dallai *et al.*, 2005).

In *Drosophila*, the most studied insect, the sperm length ranges from 56 μm in *D. pseudoobscura* (Joly *et al.*, 1989) to about 60 mm in *D. bifurca* (Fig. 8C) (Pitnick *et al.*, 1995a); in the *D. obscura* group, the 19 species examined so far produce "short" and "long" sperm (Beatty and Sinhu, 1970; Snook, 1997), which have been proposed to be involved in immediate

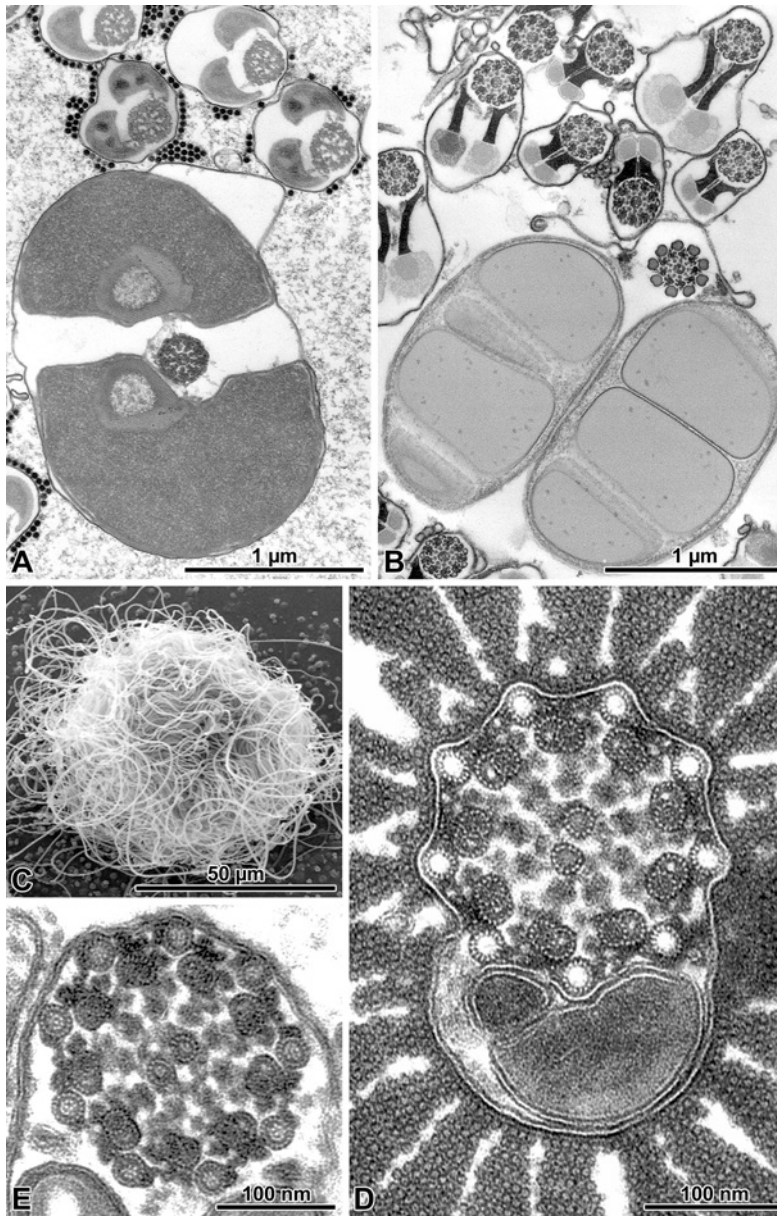


FIG. 8 (A, B) In the bug *Raphigaster nebulosa* (Heteroptera) (A), as well as in the neuropterid *Perlamantispa perla* (Planipennia) (B), two types of spermatozoa are present: the conventional euspermatozoa and the atypical giant paraspermatozoa. (From Dallai *e al.*, 2005; Jamieson *et al.*, 1999). (C) SEM micrograph of a *Drosophila bifurca* sperm ball taken from the seminal vesicle. A single spermatozoon is about 6-cm long; (D, E) Cross-sections of the flagellar

and delayed fertilization, respectively. In fact, short sperm do not show the modifications in the motility kinetics that are exhibited by long sperm; this sort of "overactivation" is supposed to confer on long sperm a better ability to survive (Bressac *et al.*, 1991).

The sperm heteromorphism and the cost associated with production of nonfertilizing gametes raises several questions regarding the adaptive value of this trait (Snook, 1997). Why do males produce different sperm types, if only one type is able to fertilize? What is the value of the production of multiple sperm types and that of the nonfunctional sperm? Some researchers have suggested that nonfertilizing sperm might either serve as a nutrient supply provided by the male to enhance female reproductive capabilities (Snook, 1997), or function in sperm competition (Cook and Gage, 1995; Snook *et al.*, 1994).

The hypothesis supporting a nutritive contribution of nonfertilizing sperm type would suggest that they are either used by functional sperm or they are incorporated into female somatic tissue and/or oocytes (Hanson *et al.*, 1952; Silberglied *et al.*, 1984). However, in *D. pseudoobscura*, Snook and Markow (1996) have found evidences that nonfertilizing sperm do not function as a nutrient reserve; furthermore, in *D. melanogaster* and *D. pachea*, the sperm tail persists throughout embryogenesis and is successively found in the larva midgut, from which it is finally expelled with feces (Pitnick and Karr, 1998). The same seems to be true for the apyrenic sperm of lepidopterans (Cook and Gage, 1995). As to the possibility that apyrene sperm might be involved in sperm competition, this would occur either by increasing female remating latency, by blocking sperm storage organs, or by displacing a former male's sperm (Silberglied *et al.*, 1984). In lepidopterans, stored sperm are associated with delaying female receptivity (Drummond, 1984); males transfer larger numbers of apyrenic sperm to younger compared with older females (Cook and Gage, 1995). Apyrene sperm, therefore, may maximize the first male's fitness by delaying female remating behavior because young females are more likely to engage in future copulation and have a higher reproductive potential than older females (Snook, 1997).

In species in which long sperm are produced, the evolution of these sperm types has been related to an increased motility: longer sperm would be faster than shorter sperm, thus being more efficient in reaching eggs (Briskie and Montgomerie, 1992, 1993; Gomedio and Roldan, 1991). This feature may have been indeed valuable in mammals and birds, but the giant sperm

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axoneme of the eupyrene (D) and apyrene (E) spermatozoon of the moth *Apopestes spectrum* (Lepidoptera, Dytrisia). Note the huge glycocalyx in the former spermatozoon and the lack of this feature in the apyrene spermatozoon. Both spermatozoa have a typical 9+9+2 axoneme, with accessory microtubules showing 16 protofilaments in their tubular wall. (From Dallai and Afzelius, 1999).

produced by some *Drosophila* species are unlikely to be associated with motility advantages. It has also been suggested that the selective advantage of producing long sperm could be related to some post-fertilizing function of these cells. Bressac *et al.* (1994) claimed that giant *Drosophila* sperm might ensure the input of paternal mitochondrial DNA. This possibility, however, seems unlikely because in the giant sperm only a small fragment of the entire sperm tail enters the egg (in *D. bifurca*, having 58.3-mm-long sperm, less than 3 mm of it enter the egg) (Pitnick *et al.*, 1995b).

There is another consideration that does not support the functional advantage of giant sperm, that is the reduction in the number of sperm cells transferred at each mating that occurs concomitant with the lengthening of the sperm. In fact, in *D. melanogaster*, which produces 1.9 mm long sperm, the number of progeny per mating is about 300–700; on the contrary, in *D. hydei*, the sperm of which is 23.3 mm long, its progeny declines to about 70 (Pitnick *et al.*, 1995b).

Also it has been suggested that the interspecific variation in sperm length might be related to postzygotic reproductive isolation. According to Karr (1991), fertilizing sperm assume a species-specific conformation inside embryos that may play a role in positioning the male pronucleus before karyogamy. Therefore, hybridization between species having different sperm length may not be possible because sperm are not properly positioned within the egg (Snook, 1997). This author analyzed the interspecific variation in sperm length in the species of the *D. obscura* group, characterized by sperm heteromorphism, with long and short sperm. She found that only 22% of the interspecific variation observed in long sperm could be correlated to the phylogenetic relationships occurring among species, whereas the size of short sperm was not significantly correlated with phylogeny. Thus, the two types of sperm are likely to have undergone a different selective pressure: long sperm would have evolved in response to fertilization demands, whereas short sperm might be related to other mechanisms affecting the evolution of male reproductive success (e.g. the increase of the female remating latency).

Mechanisms of sperm competition may also underlie the advantage provided by longer sperm, which can be supposed to be more efficient in preventing access to female storage organs by sperm of other males or in resisting displacement from female storage organs induced by the sperm of other males. An example is provided by those species in which the sperm are transferred to the female as a copulatory plug. This could be verified in the featherwing beetles (Dybas and Dybas, 1981), where the first mating male fills the female spermatheca, thereby preventing access by sperm from other potential competitors. This mechanism, however, seems not to be valid for all *Drosophila* species producing long sperm, as, for instance, *D. pachea*; in this species, overlapping ejaculates from successive matings would be stored by the females (Pitnick and Markow, 1994b).

## 8. Coevolution Sperm Length and Female Genital Tracts

In the discussion on the functional significance of sperm polymorphism and, more specifically, on that of longer sperm, a coevolution of sperm and female genital districts has been recently suggested. In a study performed on some *Drosophila* species of the *nannoptera* group, it has been found that the length of seminal receptacle varies exhibiting a positive relationship with sperm length (Miller and Pitnick, 2002; Pitnick and Markow, 1994b). A strict correlation between the length of *Drosophila* ventral receptacle and the sperm length was previously noticed by Hihara and Kurakowa (1987). If a male produces sperm that are longer than the female ventral receptacle, where the sperm are stored after mating, then these sperm might be unable to enter this district. On the contrary, if a male produces sperm shorter than the length of the ventral receptacle, these sperm can be stored, but after they would not be able to leave the storage organ (Pitnick and Markow, 1994b). A similar correlation between sperm and female genital tract has been demonstrated also in lepidopterans. At fertilization, eupyrenic sperm must migrate from storage down the female spermathecal duct to meet with the ovum. The frequent increase in the length of the female duct is associated with an increased length of eupyrenic sperm, suggesting a positive female influence on the sperm size; longer, more powerful sperm might have been selected to migrate and/or compete more successfully with other sperm, in their migration down along longer ducts. On the contrary, apyrene sperm length is not related to the dimension of female reproductive tract (Morrow and Gage, 2000). A similar coevolution between spermathecal and sperm lengths was also found in beetles of the *Bambara invisibilis* group. In these species, the correlation involves the diameter of the sperm and that of the spermathecal duct, rather than the length of sperm and of the spermathecal lumen. It has been proposed that this correlation could depend on the limited number of spermatozoa and eggs produced by these beetles, which would confer high adaptive value and selective advantage in fertilization; they could function to husband limited resources of gametes. Morphological coadaptation of sperm and spermathecae should function to preclude sperm displacement and to ensure sperm precedence to the first male mating with the female. Under this aspect, the system would function as a reproductive barrier of prezygotic type, preventing hybridization (Dybas and Dybas, 1981).

A clear coevolution between sperm morphology and female tracts is occurring in the psychodid flies of the subfamily Psychodinae. The males of this group have aflagellated immotile spermatozoa, characterized by elongate flattened nucleus and acrosome and by a bifurcated posterior end (Baccetti *et al.*, 1973d). At mating, the sperm, instead of being stored within the spermatheca, which is absent, are injected into the female distal genital tracts, where many can be found in the lateral oviduct and in the narrowed

duct lumen of ovariole peduncle (Burrini and Dallai, 1975). The spermatheca is instead present in members of the subfamily Phloebotominae, which are characterized by motile spermatozoa provided with flagellar axoneme.

## III. Functional and Biochemical Characterization of Aberrant Axonemes

### A. Posttranslational Modifications of Tubulin in Unorthodox Axonemes

The assembly and function of the evolutionarily conserved 9+2 axoneme is based on a multiplicity of protein interactions that must be both spatially and temporarily coordinated. As many as 250 different polypeptides have been detected in the 9+2 axoneme of *Chlamydomonas* flagella (Pazour *et al.*, 2005). In this organism, the molecular analysis of many mutant strains carrying different alterations in the axoneme structure has provided important information regarding the function of axoneme.

The organization of insect flagellar axonemes has been extensively analyzed at the ultrastructural level; however, the available molecular information is more limited and much work is still required for the comprehension of the molecular mechanisms that underlie the great variability of insect axonemal patterns. Available data come from analysis of *Drosophila* mutants, which have shown that some alterations of tubulin primary structure differentially affect the assembly and function of specific microtubular components within the axoneme (Dutcher, 2001; Fuller *et al.*, 1988; Nielsen *et al.*, 2001); therefore, certain specific amino acid sequences of axonemal tubulin appear to be important in determining the specificity of axonemal microtubules. In particular, in *Drosophila* the modification or the absence of the so-called axonemal motif, a short amino acid sequence localized in the carboxyterminal tail of $\beta_2$ tubulin and conserved in all known axonemal β tubulins, is critical for the assembly of a 9+9+2 axoneme because mutants carrying an altered motif express a 9+9+0 axoneme. A similar phenotype, the absence of the central pair, has been reported in *Tetrahymena* mutants exhibiting alterations in the β-tubulin polyglycylation domain (Thazhath *et al.*, 2002); interestingly, these modification sites are also located in the protein carboxyterminal tail and partially overlap with the axonemal sequence motif. Thus, the occurrence of both a specific β-tubulin amino acid sequence and of posttranslational processes is required for the integrity of the axoneme and the assembly of the central pair microtubules, which appears to be regulated independently of the nine outer microtubular doublets in the sperm flagellum.

Expression of glutamylated and glycylated tubulin isoforms has been analyzed in a group of unconventional insect axonemes, including different models all of which are characterized by the absence of the central pair microtubules (Mencarelli *et al.*, 2005). This study has evidenced that all the modified patterns share a strong decrease in both tubulin glycylation and glutamylation levels or even the total absence of both polymodifications (Fig. 9A, B). Because these models include species belonging to different dipteran families (Bibionidae, Cecidomyiidae and Culicidae) and to the primitive paleopteran order Ephemeroptera, this common feature is unlikely to be resulting from an evolutionary relationship. These data suggest that a correlation exists in insects between the reduced expression of tubulin polymodifications and the absence of the central pair. It is interesting to note that this strong decrease in glycylation and glutamylation levels apparently neither affect sperm viability nor its capability to beat; so these spermatozoa must have undergone adaptive coevolutionary events to compensate for structural modification of the axoneme.

The occurrence of an unorthodox axonemal architecture seems to be correlated not only to a decrease in the level of one or both polymodifications, but also to an altered distribution of the modified isoforms along the axoneme (Mencarelli *et al.*, 2004). The highly aberrant axoneme of the cecidomyiid *Asphondylia* shows an extremely low level of glycylation but still contains glutamylated isoforms (Fig. 9B); these, however, do not show the differential proximo-distal localization that has been constantly found in the 9+2 axonemes of other species (Fouquet *et al.*, 1996; Huitorel, 2002; Kann *et al.*, 2003; Lechtreck and Geimer, 2000; Prigent *et al.*, 1996) but are instead uniformly distributed along the axoneme. In this species, the absence of a gradient distribution is concomitant with the lack of inner dynein arms and the occurrence of a very peculiar kind of motility (see later). Thus, it is emerging that the analysis of unconventional insect sperm axonemes may provide interesting contributions to the comprehension of both the assembly and functioning of the 9+2 axoneme and the role that posttranslational tubulin modifications may play in these processes.

## B. Giant Axonemes of Cecidomids

The sperm axoneme expressed in the cecidomyiid supertribes Asphondyliidi and Cecidomyiidi is an almost unique model not only for its totally aberrant architecture (Fig. 6), but also for the very peculiar kind of motility. In fact, it represents the only known example of a motile axoneme whose function is based on the activity of the outer dynein arms only, the inner arms being absent.

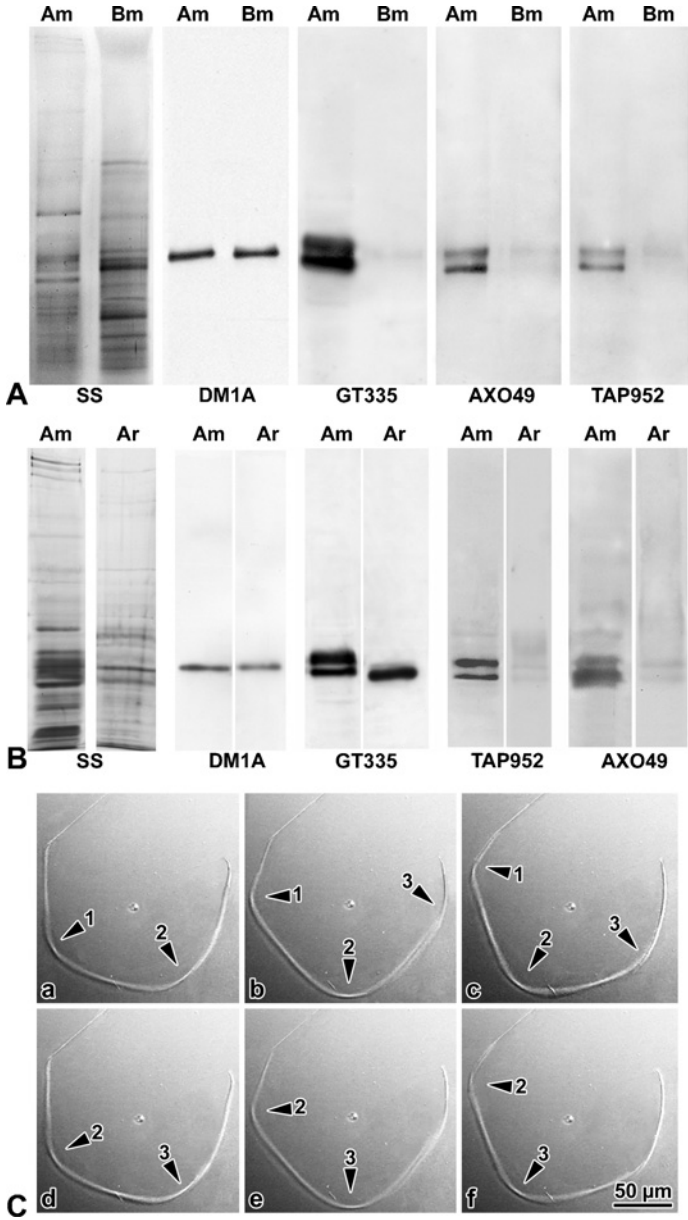


FIG. 9 (A, B) Immunoblot analysis of the tubulin glutamylated and glycylated isoform content expressed in unconventional axonemes. *Apis mellifera* has been here used as a reference species for the 9+9+2 axonemal model. Two modified axonemal models are reported here, the 9+9+0 axoneme of *Bibio marci* (A) and the aberrant axoneme of *Asphondylia ruebsaameni* (B). In each experiment, equal amounts of axonemal tubulin from *Apis* (Am) and *Bibio* (Bm) or *Asphondylia*

Current information on the molecular and functional aspects of the 9+2 axonemal beating has shown that it is based on the spatial and temporal coordination of multiple dynein isoforms (Asai and Wilkes, 2004; Kamiya, 2002; Porter and Sale, 2000). The inner and the outer dynein arms possess different structures, consist of different sets of subunits, and contribute differentially to axonemal motility. The outer arm exhibits a uniform composition along the axoneme, comprising two distinct heavy chains (three in protists), while the inner arm has been shown to comprise different isoforms with specific molecular composition, at least some of which are differentially distributed in the proximal, medial, or distal regions of the axoneme (Gardner *et al.*, 1994; Piperno and Ramanis, 1991). Such complexity seems to be required for axonemal function, because it has been detected in all organisms analyzed until now, including *D. melanogaster* (Rassmusson *et al.*, 1994). *Chlamydomonas* mutants lacking the whole set of outer arms still move with an almost regular waveform, though more slowly; on the contrary, the occurrence of large defects in the inner arms results in mutants that are unable to beat (Kamiya, 1988; Kurimoto and Kamiya, 1991; Mitchell and Rosenbaum, 1985). On this basis, the inner dynein arms are considered to be both necessary and sufficient to generate motility, whereas the outer dynein arms are thought to add just further power to the flagellar beat. Dynein activity is regulated and coordinated by a complex signal transduction pathway involving the central pair, the radial spokes, and a cluster of polypeptides (the so-called dynein-regulatory complex), which is closely associated to the inner dynein arms (Gardner *et al.*, 1994). Thus, inner dynein arms appear to have an essential role in the conversion of dynein-driven microtubule sliding into a regular waveform.

Asphondyliid and cecidomyiid sperm axonemes lack all the structures that are implicated in waveform generation and, indeed, these spermatozoa are unable to undergo any kind of progressive movement *in vitro*; outer dynein arms are clearly functional and able to support microtubule sliding but, after release from the male or female genital tracts, they simply straighten out and stay immotile, thus confirming a defect in the mechanism that converts axoneme sliding into bending (Lupetti *et al.*, 1998; Mencarelli *et al.*, 2001). However, both *Asphondylia* and *Monarthropalpus* spermatozoa exhibit a whirling movement when observed *in vivo*, within the male deferent duct or

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(As) have been loaded onto the gel, as can be judged from the immunoreaction obtained using an anti-α-tubulin antibody (DM1A). The monoclonal antibodies here employed include the antiglutamylated tubulin antibody GT335 and two antibodies, AXO 49 and TAP 952, which are specific, respectively, for polyglycylated and monoglycylated tubulin molecules. (From Mencarelli *et al.*, 2004, 2005). (C) Pattern of motility exhibited *in vitro* by the sperm tail of *Asphondylia ruebsaameni* when subjected to mechanical constraints, which in this case are provided by the attachment of both sperm ends onto the glass. Arrows indicate the torsion wave traveling along the axoneme. (From Mencarelli *et al.*, 2001).

the female spermatheca, where sperm are folded into several turns and are involved in a continuous train of bends. This kind of motility is therefore linked to a particular condition of the sperm tail (i.e., that of being folded), because it can be observed also *in vitro* on both native and reactivated flagella, provided that they are bent by some mechanical constraint (Fig. 9C).

These observations have suggested that in these axonemes the activity of dynein arms might be controlled by external mechanical stimuli. The occurrence of a regulatory mechanism responding to changes in the mechanical state of the axoneme, for example, curvature or interdoublet length, has been proposed to act also in the 9+2 axoneme along with the chemical control involving the central pair/radial spokes complex (Hayashibe *et al.,* 1997). Thus, in the sperm flagella of these insect species only one of the two control systems would have been conserved.

This peculiar kind of motility would have become feasible during the evolution of gall-midge flies for a concomitant modification of the female reproductive apparatus, that is, the presence of a very short spermathecal duct. Due to this anatomical feature, spermatozoa are not required to move any long distance and can contact the egg as soon as, descending through the oviduct, this latter faces the opening of the spermatheca.

The available information has also evidenced a difference in the molecular composition of outer dynein arms in the two species, *Asphondylia* and *Monarthropalpus*. In fact, while in the former the dynein molecule possesses the typical metazoan structure, that is, it comprises two different heavy chains, in the latter dynein has been shown to be a homodimer of a single heavy chain (Lupetti *et al.*, 1998; Mencarelli *et al.*, 2001). These data are interesting in light of the fact that all outer dynein molecules analyzed until now have been shown to comprise different heavy chains thought to play different functional roles. In the genome of *Monarthropalpus*, however, an additional sequence, closely related to the expressed dynein heavy chain sequence, has been found and proposed to be a pseudogene (Lupetti *et al.*, 1998). Comparing data from the two species, it has been therefore speculated that originally in both of them, two dynein heavy chain genes were expressed but that an incorrect excision of a intron from one gene led to its loss of function in *Monarthropalpus*.

Thus, the available molecular data indicate that an evolutionary trend toward a progressive simplification of the dynein heavy chain complement has occurred in the cecidomyiid family. The earlier loss of all the structures involved in the chemical regulation and coordination of dynein activity would have allowed the accumulation of mutations in the dynein heavy chain genes, leading to loss of gene function. It appears odd that the increasing structural complexity of cecidomyiid axoneme has been concomitant with a progressive functional and molecular simplification of the motility process and of dynein arms.

### C. The 9+9+0 Axoneme of Ephemeroptera

Sperm axonemes of Ephemeroptera are characterized (as also previously mentioned) by several interesting and peculiar ultrastructural features. Nine accessory microtubules are visible surrounding a 9+0 axoneme. Their tubular wall is formed by 13 tubulin protofilaments, a feature common for cytoplasmic microtubules but very peculiar for accessory tubules in insect sperm axonemes. The intertubular material is scarce; a central sheath devoid of the central pair of singlet microtubules and with a diameter of about 60 nm is visible in the central axis of the axoneme (Fig. 7D). No outer dynein arm complexes are visible on the nine microtubular doublets and the inner arm complexes are characterized by a peculiar structure. In cross-sectioned sperm axonemes, they appear like a thin hook inward-oriented structure sometimes with a thicker projection (Fig. 7D). These dot-like projections were previously interpreted as thin filaments between microtubular doublets (Baccetti *et al.*, 1966). When ephemeropteran spermatozoa are prepared by the Quick-Freeze, Deep-Etch (QF-DE) procedure and the fracture exposes the surface of A subfiber of microtubular doublets, rows of compact structures corresponding to inner dynein arm complexes are visible (paper in preparation). Their aspect is, however, different from that commonly visualized by QF-DE for other inner dynein arms in axonemes from other invertebrates. Each arm appears smaller than conventional ones and with a more compact aspect. In this respect, it is interesting that a preliminary study on the dynein heavy chain complement of the inner dynein arm of a ephemeropteran species has revealed that it consists of a single isoform, differently from what has been reported for inner dynein arms in all the species analyzed until now (Asai and Wilkes, 2004). Thus, in this case the loss of structures involved in the control of flagellar motility seems to have led also to an oversimplification of the motor protein complexes involved in motility generation.

## IV. Axoneme Variations and Evolution in Different Taxa

It has been mentioned that a tendency of the axoneme to degenerate and finally to disappear has been repeatedly observed in several hexapod groups, once a first modification has occurred in its basic structure. We reconsider here some of these groups with the aim of showing how axonemal structure can provide valuable information for phylogenetic studies.

In several families, the motile flagellar axoneme of Protura consists of a variable number of microtubular doublets (12+0; 13+0; 14+0; 16+0) (Dallai *et al.*, 1992; Yin *et al.*, 1989), but in some members of the family

Hesperentomidae, as well as in Fujentomidae, Sinentomidae, and Eosentomidae, the sperm are aflagellate and immotile. Thus, the proturan basal families are still provided with motile spermatozoa, while more advanced ones have lost their sperm flagellum. The occurrence of aflagellate sperm in the latter groups was expected given the unusual axoneme pattern expressed in the basal species (Dallai *et al.*, 1992). On the contrary, Collembola, considered to be the sister group of Protura, do not show such axonemal variations and exhibit a quite uniform sperm morphology; the only variations concern the length of the sperm flagellum, the extracellular material constituting the central region of the rolled-up sperm, and the appearance of a cylindrical "peduncle" adhering to the acrosome and protruding from the cell as a long, flexible feature (Dallai *et al.*, 2004b).

These results have posed some questions to the otherwise accepted relationship between Protura and Collembola. Interestingly, proturans share their unusual axonemal pattern with a group of chelicerates, the Pantopoda. Both groups exhibit a common and peculiar motile sperm axoneme, consisting of a crown of doublets devoid of outer arms and lacking central tubules. Moreover, spermatozoa in both groups have several mitochondria, a feature that is unusual for a regular insect sperm (Dallai *et al.*, 1992; van Deurs, 1974), and have a single centriole, which is unusual for a chelicerate. The hypothesis of a relationship between Protura and Pantopoda was formerly suggested by Yin (1984). Berlese (1910) considered Protura as a taxon closely related to Myriapoda, the Myrientomata, and recent molecular data have indicated a relationship between Myriapoda and Chelicerata (Cook *et al.*, 2001; Friedrich and Tautz, 1995; Hwang *et al.*, 2001; Negrisolo *et al.*, 2004), a suggestion that would have been widely considered heretical only a few years ago. Thus, a relationship between Protura and Pantopoda is not far from the truth: Protura can be considered as aberrant insects, and Pantopoda are retained aberrant Chelicerata (Dunlop and Selden, 1998; Vilpoux and Waloszek, 2003).

In Ephemeroptera, the ground plan of sperm axoneme consists of a 9+9+0 structure, with microtubular doublets endowed with only inner dynein arms. As in Protura, the presence of species producing aflagellated sperm was expected, due to the loss of some axonemal elements (central pair and outer dynein arms) in the sperm flagella of the basal groups. The family Leptophloebidae is, in fact, provided with an ovoidal aflagellate immotile sperm (Dallai and Afzelius, 1990, 1999; Gaino and Mazzini, 1990, 1991). Ephemeroptera, along with Odonata, constitute the most basal pterygotan taxa, the Paleoptera. Most phylogenetic analyses of hexapod relationships have suggested paraphyly of these two orders, Odonata being the sister group to Neoptera (Kristensen, 1989; Wheeler *et al.*, 2001; Whiting *et al.*, 1997). Although Ephemeroptera exhibit several sperm autapomorphies, Odonata have a quite conventional and uniform model of pterygotan sperm axoneme,

with a 9+9+2 axoneme pattern. From a spermatological point of view, therefore, Ephemeroptera and Odonata appear to belong to widely different lineages.

Trichoptera are the sister group of Lepidoptera and molecular as well as karyological studies support a close relationship between the two taxa (Wheeler *et al.*, 2001; White, 1977; Whiting, 2002). Trichoptera, Integripalpia have a 9+9+2 flagellar axoneme with the outer dynein arms missing (Dallai and Afzelius, 1990, 1994). On the contrary, Trichoptera Annulipalpia exhibit several aberrant flagellar axonemes, which are always devoid of dynein arms and therefore immotile (Dallai *et al.*, 1995c). In the family Hydroptilidae, sperm are aflagellated (Dallai and Afzelius, 1995). Lepidoptera, on the contrary, exhibit a conventional 9+9+2 axoneme. As mentioned elsewhere in this review, the taxon is characterized by the presence of two different types of spermatozoa: the apyrenic (anucleated, nonfertilizing) and the eupyrenic (fertilizing) sperm (Dallai and Afzelius, 1990; Friedländer *et al.*, 2005; Jamieson *et al.*, 1999).

Finally, in the nematoceran Diptera a large variety of sperm axonemes has been described in the different families, from conventional to aberrant models (Jamieson *et al.*, 1999). Thus, Bibionidae and Culicidae exhibit a 9+9+0 (sometimes called 9+9+"1") and Simulidae a 9+9+3 axoneme. Sciaridae and Cecidomyiidae, as we have already described, show the highest axonemal variability so far described in a taxon (Dallai *et al.*, 1996b).When considering the axonemal model occurring in brachyceran Diptera, it appears evident that variations occurring in the whole group concern only morphological details, such as the extension and the different structure of the intertubular material, but the general axoneme pattern results unchanged. As reported earlier, the sperm length can be highly variable in different *Drosophila* species, but this variation does not modify the general structure of the sperm tail. Thus, it appears reasonable to infer that the differentiation of the brachyceran axoneme was the result of a selective process among the several axonemal models of nematocerans, leading to the acquisition of the supposedly most efficient axonemal organization. The presence of axonemes of the brachyceran type (13 protofilaments in the accessory tubules) in the nematoceran Tipulidae and in their related families strongly support this hypothesis (Dallai and Afzelius, 1990; Jamieson *et al.*, 1999).

Questions obviously arising from the preceding analysis: why do Trichoptera show so many sperm models, while Lepidoptera have a rather uniform sperm axoneme? Similarly, why did nematoceran Diptera, but not brachycerans, differentiate so many types of axonemes? In other words, Trichoptera exhibit a high degree of variability, compared to Lepidoptera, as do Diptera Nematocera compared to Brachycera. A convincing answer to this question is still a matter of discussion and we can only hypothesize that the axoneme variations of Trichoptera and Diptera Nematocera are linked to the basal

position of the species in which they occur, whereas the higher taxa (i.e., Lepidoptera and Diptera Brachycera, respectively) express a more evolutionarily stable axoneme as the result of a selective pressure exerted on the different axonemal models expressed in the basal groups. Interestingly, it has been recently shown that axonemal $\beta_2$ tubulin has been completely conserved in species of *Drosophila* and *Hirtodrosophila*, thus suggesting that in these insects special constraints have occurred in the evolution of axonemal tubulin (Nielsen *et al.*, 2006).

## V. Perspectives

### A. Recent Methodological Developments for the Study of Sperm Ultrastructure

The most significant advancements in comparative and functional cell morphology have been achieved by the combined use of the recently developed methodologies applied to carefully identified and selected cell models. Major advancements in structural spermatology are not an exception to the preceding statement. Improvements in fixation and tissue staining procedures, for example, were fundamental for the identification of many new fine structural features in insect sperm axonemes. A significant contribution in this context was the introduction of a fixation protocol making use of tannic acid for biological samples to be studied by transmission electron microscopy (TEM) (Mizuhira and Futaesaku, 1974; Afzelius, 1988). This protocol was later modified by Dallai and Afzelius (1990) making it very efficient for visualization of cytoskeletal components in insect sperm cells. Most of the results discussed in this review were made possible by the invention and subsequent modification of this technique. Prolonged incubation of isolated sperm cell in buffered solution containing a mixture of glutaraldehyde and tannic acid followed by careful rinses in buffer and incubation in uranyl acetate, turned out to induce the selective precipitation of heavy metal on the cytoskeletal components as well as on the surface glycocalyx of sperm cells. It was consequently possible, for example, to resolve, in cross-sections of insect sperm axonemes, the single tubulin protofilaments present in the wall of both microtubular doublets and accessory tubules. We have discussed in Section B2 the potential phylogenetic and functional implications of such structural features.

The introduction of rapid freezing followed by freeze-fracture, deep-etch, and rotary metal replication, QF-DE, by Heuser (1981) represents another methodological improvement of broad impact for major progresses in the field of cell ultrastructure and function. The efficacy of preserving and visualizing the fine structural details of axonemal complexes by rapid

freezing followed by cryofracture and metal replication was established years ago (Burgess *et al.*, 1991; Goodenough and Heuser 1982, 1984). This technology has been in use in our electron microscopy lab since the early 1990s and we have subsequently used it with great advantage for high-resolution studies on the functional morphology of insect sperm axonemes.

With such purpose we have extensively studied by QF-DE the giant sperm axonemes of the two cecydomyiid dipterans *Asphondylia reubsaameni* and *Monarthropalpus flavus*. (Lupetti *et al.*, 1998, 2005; Mencarelli *et al.*, 2001). The peculiar structural model of these motile axonemes has allowed a detailed analysis of the fine structure of outer dynein arms (ODAs). These axonemes are, in fact, devoid of a series of structures, that is, the central complex, radial spokes, inner dynein arms, and accessory tubules, that can interfere with the visualization of *in situ* ODAs, when these are studied by QF-DE of 9+2 axonemes. Furthermore, the microtubular laminae composing these axoneme models are planar enough to show many dynein molecules intercalated among parallel microtubular rows when fracturing occurs nearly parallel to the longitudinal axis of the axoneme (Fig. 10A). Such views of metal-replicated molecules represent a very convenient starting material to perform computer-aided analyses finalized to further increase the final resolution of images.

There are several strategies for image analysis: a first simple approach for noise filtration that has been applied to fields like the one shown in Figure 10A consisted in one-dimensional Fourier filtering using the 24-nm repeat of the arm as basic periodicity (Fig. 10B). Such processing allowed to better visualize the general pattern of organization shared by all ODAs. There is, however, an even more effective strategy to increase resolution and obtain a three-dimensional (3D) model of the molecular structures present in whole axonemes. This protocol is based on taking a series of micrographs as the specimen is tilted around one or two axes (Baumeister *et al.,* 1999). Electron tomography has provided new opportunities for describing the structure of subcellular assemblies and has become a powerful tool for structural analysis of molecular complexes and organelles *in situ* (Baumeister, 2005; Subramaniam and Milne, 2004). The most common material for analyzing dyneins *in situ* consisted of 100–200 nm thick sections from plastic embedded samples (O'Toole *et al.*, 2003).

We have recently demonstrated the suitability of metal replicas for tomographic reconstruction of *in situ* ODA complexes (Lupetti *et al.*, 2005). The 3D model obtained after image analyses on micrographs collected by single tilt axis (Fig. 10C) allowed to identify, in each dynein arm, two head domains that are almost parallel and are obliquely oriented with respect to the longitudinal axis of microtubules. Each head domain consists of a series of globular subdomains that are positioned on the same plane. A stalk originates as a conical region from the proximal head and ends with a globular

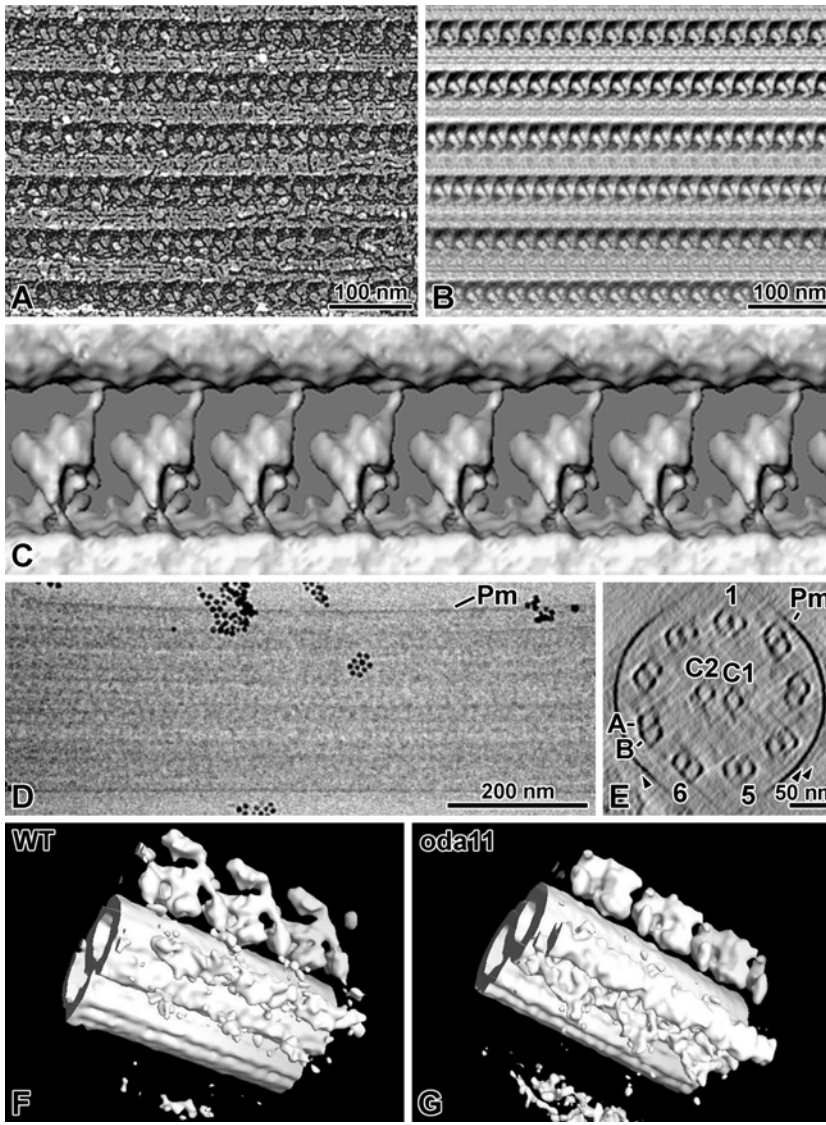


FIG. 10 (A) Replica of a demembranated rapidly frozen, cryofractured sperm axoneme of the gall-midge fly *Monarthropalpus flavus*. This axoneme was fractured nearly parallel to the longitudinal axis of the sperm. In this condition microtubular laminae appear almost planar and a great number of dynein arms can be visualized intercalated among parallel microtubular rows. (B) One-dimensional Fourier filtering using the 24-nm repeat of the arm as basic periodicity is shown in A. Individual arms are better visible after this simple strategy of filtration. (C) Three-dimensional (3D) model of *in situ* outer dynein arms (ODAs) obtained by electron tomography applied to series of tilted images collected from metal replicas of rapidly frozen, cryofractured,

domain that contacts the B tubule. A stem region, located below the two head domains, presents two distinct points of anchorage to the surface of the A tubule. Contrary to what has been observed in isolated dynein molecules adsorbed to flat surfaces (Burgess *et al.*, 2003, 2004; Gee *et al.*, 1997; Goodenough and Heuser, 1984), the stalk and the stem domains are not in the same plane as the head. The visualization of the aforementioned features allowed significant progress toward the understanding of functionality for this important and ubiquitous molecular motor. However, the resolution obtained for the described 3D model turned out to be limited by the amount of information that could be gathered by collecting images by single tilt axis and with the limited tilting angle allowed by the goniometric stage used during observations. The preliminary results obtained when using a higher-voltage TEM equipped with a more-efficient tilting stage and collecting images with a double-axis geometry demonstrated significant improvements in both resolution and isotropy of the 3D model. Further improvements will then be reasonably possible by the use and development of more efficient strategies for image analysis and 3D modeling such as, better refinement in the alignment of projections and the averaging of 3D models of single molecules by 3D cross-correlation. Metal replicas obtained by QF-DE represent a unique source for structural studies, even at molecular level, given the amount of intrinsic resolution they have and their very high stability even under high electron doses such as those necessary for double-tilt image collection. In addition, the images are characterized by high contrast that make easier the preliminary survey for identification of optimal areas in the replica where good freezing occurred and, consequently, the preservation of structural features is optimal. Coupling these technical features with the enormous source of axonemal models peculiar of insect spermatozoa will allow the performance in the near future of interesting comparative and functional studies.

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and metal replicated sperm cells; this model is an average of the reconstructed volume along each individual doublet using the 24-nm repeat periodicity of dynein arms along microtubules. See the text for further comments on this figure. (From Lupetti *et al.*, 2005). (D) Transmission electron micrograph of an intact, frozen-hydrated flagellum from sea urchin spermatozoon. In unstained frozen-hydrated specimens, proteins appear dark, because their scattering of electrons is higher than the surrounding solid water. Clusters of black spheres are 10-nm colloidal gold particles that are used as fiducial markers for alignment of the tilt series. (E) Projection through ~80 nm of the 3D reconstruction obtained from a tomogram of flagella like the one in D. The slice of tomogram is oriented perpendicular to the flagellar axis, showing the flagellum in cross-section; note the ring of microtubular doublets (1, 5, 6), the central singlet microtubules (C1 and C2), the plasma membrane (Pm), and the extracellular material at the plasma membrane (arrowheads) (From Nicastro *et al.*, 2005). (F, G: Courtesy of Dr. Takashi Ishikawa, Dept. of Molecular Biology and Biophysics, ETH, Zurich). Surface-rendered representation of the ODAs from *Chlamydomonas* flagella. Representations are boxed out from electron cryotomograms, aligned, and averaged. (F) ODAs from wild-type cells. (G) ODA11 mutant, which lacks the ODA alpha heavy chain.

## B. New Opportunities for the Molecular Analysis of Hexapod Axonemes

In addition to the previously discussed strategies, another series of protocols have been developed for the observation of cells in frozen hydrated conditions by the use of electron microscopes equipped with sample stages designed to maintain the samples at very low temperatures during observation and image collection. This technology coupled with electron tomography has been used to analyze frozen hydrated axonemes (McEwen *et al.*, 2002; Nicastro *et al.*, 2005). In particular, Nicastro *et al.* used cryo-electron tomography to investigate the 3D macromolecular organization of intact, frozen hydrated sea urchin sperm flagella (Fig. 10D, E). Authors of this paper were able to visualize the heptameric rings of the motor domains in ODA complex, and the reconstructed model they have obtained indicates that rings lie parallel to the plane of the axes of neighboring flagellar microtubules. The same authors were also able to show that both the material associated with the central pair of microtubules and the radial spokes displayed in this model of axonemes a plane of symmetry that helps to explain their planar beat pattern.

More recently T. Ishikawa, was able to visualize by cryo electron tomography the molecular structure of the ODA complex in demembranated axonemes from the green alga *Chlamydomonas reinhardtii* (pers. communication). The preliminary 3D model he has obtained for ODAs *in situ* on microtubular doublets clearly shows three domains stacked atop each other and oriented parallel to the major axis of microtubular doublet. These domains are linked to each other and to the surface of the A tubule by an oblique rodlike region (Fig. 10F). The same researcher also obtained, by cryo electron tomography, 3D models from a mutant of *Chlamydomonas* defective for one of the three ATPase heavy chain domains normally present and identified in flagella from wild-type algae (Fig. 10G). The comparison between the two 3D reconstructions from *in situ* molecules evidences the lack of the distal heavy chain domain in the mutant, thus confirming the location of this domain in axonemal ODAs from wild-type axonemes. Given these first preliminary evidences obtained by cryo electron tomography of axonemes from sea urchin spermatozoa and from *Chlamydomonas* cilia, it is very reasonable to foresee a great future in the contribution that this newly developed technology will be able to perform in the field of functional biology of ciliary axonemes.

The vast modulation of axonemal models displayed by insects represents an outstanding source of "natural mutants" with an overwhelming potential if the proper models are correctly studied with the most current technologies for ultrastructural studies. In this context we are currently extending our analyses by tomography on replicas obtained by QF-DE from giant axonemes

of cecidomyiid dipterans aiming to implement the final resolution of our 3D models by more efficient strategies for image collection and also by refining the protocols for alignment, averaging, and 3D rendering of our data sets. It would certainly be very useful and promising extending these ultrastructural investigations on giant axonemes and other axonemal models by cryo electron tomography.

Particularly important in order to take full advantage of cryo electron tomography would be identification and selection of those insect axonemal models most suitable for such an ultrastructural approach. Namely the simplified models lacking most of the intertubular material and/or missing other axonemal components such as the central complex and radial spokes. Also interesting would be studying axonemes with inner dynein arm complexes only. Such choice seems indicated by the necessity to start studying simple axonemes that will be easier to reconstruct at a molecular level starting from single tilt axis series of images.

**Note added in Proof:**

After submission of this review two new interesting papers have been published using cryoelectron microscopy and advanced image analysis strategies for the elucidation of new insights in the molecular structure of cilia and flagella. Sui and Downing (2006) obtained a three-dimensional map of intact microtubule doublets with a resolution of 3nm that allowed localization of particular non tubulin proteins within the doublets and the structural features of the doublets that define their mechanical properties. In addition Authors proposed new insights on how tubulin protofilaments and accessory protein interact to form doublets.

One month after the publication of the above paper, Nicastro *et al.*, (2006) presented the data set they obtained from a comprehensive study on the molecular architecture of axonemes from Chlamydomonas cilia and sea urchin spermatozoa they performed by cryoelectron tomography and image processing. Their results suggest a model for the way dynein generates force to slide microtubules and also revealed two dynein linkers that may provide wiring to coordinate motor action. Also in this study periodic densities inside the doublet microtubules were observed and a correlation of these structures with doublet stability was proposed by the Authors of this paper.

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# Hsp70 Chaperone as a Survival Factor in Cell Pathology

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Heat shock protein Hsp70 is implicated in the mechanism of cell reaction to a variety of cytotoxic factors. The protective function of Hsp70 is related to its ability to promote folding of nascent polypeptides and to remove denatured proteins. Many types of cancer cells contain high amounts of Hsp70, whose protective capacity may pose a problem for therapy in oncology. Hsp70 was shown to be expressed on the surface of cancer cells and to participate in the presentation of tumor antigens to immune cells. Therefore, the chaperone activity of Hsp70 is an important factor that should be taken into consideration in cancer therapy. The protective role of Hsp70 is also evident in neuropathology. Many neurodegenerative processes are associated with the accumulation of insoluble aggregates of misfolded proteins in neural cells. These aggregates hamper intracellular transport, inhibit metabolism, and activate apoptosis through diverse pathways. The increase of Hsp70 content results in the reduction of aggregate size and number and ultimately enhances cell viability.

**KEY WORDS:** Chaperone, Apoptosis, Cancer, Insoluble protein aggregates, Cell pathology. 

## I. Introduction

Every living cell is able to respond to overheating, hypoxia, heavy metals, and other stressful factors by triggering a variety of molecular protective systems. Nasonov, Aleksandrov, and other researchers postulated that the resistance of cells to harmful conditions could result from the ability of the protein molecule to recover its structure after the action of a given damaging factor.



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This protein feature was named *conformational flexibility* (Alexandrov, 1985). Cells use many defense systems, for example, polyols and trehalose compounds protect tissues from osmotic stress, superoxide dismutase and glutathione scavenge free radicals, and proteins of Bcl/Bag/Bax families regulate apoptotic cell death (Feder and Hofmann, 1999).

The advances in the field of stress and adaptation biology are based largely on the discovery of heat shock or stress proteins (Hsps). The *hsp70* gene was one of the first genes cloned (Holmgren *et al.*, 1979). The gene's 5′-untranscribed promoter region contains a heat shock element (HSE), a sequence of 14 nucleotides that can be bound by a specific heat shock transcription factor (HSF). Because of its properties, in the early 1990s Hsp70 was named *chaperone*, beginning the era of chaperones (and chaperonines). Today chaperones are defined as proteins that interact with misfolded or newly synthesized polypeptides to refold or transport them to other cell compartments. They also help cells cope with irreparable protein molecules. To understand the significance of the discovery of heat shock proteins in the development of modern views on the nature of the response of cells to stressful factors, several facts should be emphasized. First, Hsps are found in all cells and organisms studied to date. The expression of these proteins is a common feature of the reaction of living systems to harmful factors. Second, not only heat but also many other cytotoxic factors are able to induce Hsps, which suggests the universal nature of this mechanism of stress response. Third, Hsps also function in cells in normal conditions, and their levels can be affected by agents stimulating processes such as differentiation or proliferation.

## II. Heat Shock Proteins, Their Inducers, and Classification

The expression of Hsps can be induced by many factors, which fall into three categories (Fig. 1). The first group includes agents that are able to damage polypeptide structure directly. It includes heat shock (for human cells $T > 41^{\circ}C$), oxidative stress (Papp *et al.*, 2003), organic solvents (Mizushima *et al.*, 1993), osmolytes, ethanol, radiation (Ibuki *et al.*, 1998), amino acid analogs, inhibitors of proteasomes or phosphatases (ocadaic acid), and heavy metals (Efremova *et al.*, 2002). The factors from the second group do not directly influence protein structure. These are traumas, particularly brain injuries, amphetamine and some of its derivatives, viruses, including SV40, polyoma, and vaccinia viruses (Sedger and Ruby, 1994), magnetic fields (Goodman and Blank, 2002), some poisons (Barque *et al.*, 1996), and immobilization stress (Filipovic *et al.*, 2005). The third group includes inhibitors or inducers

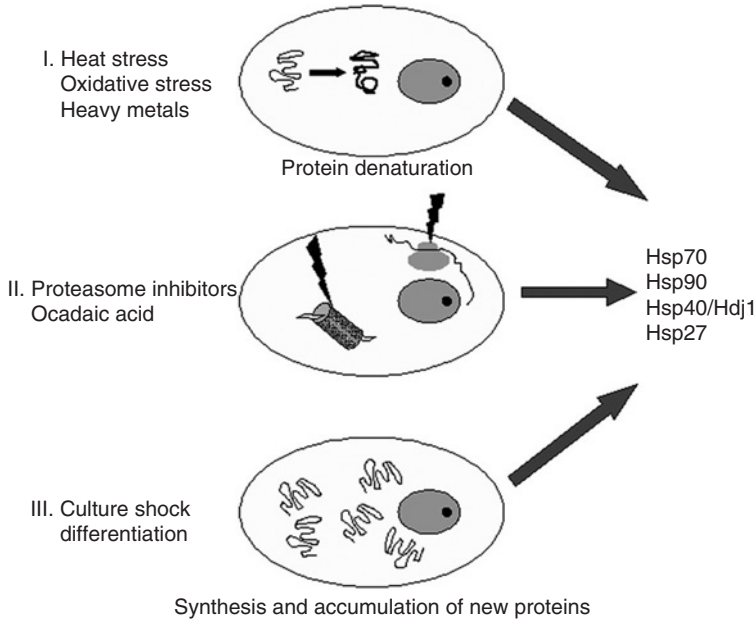


FIG. 1 Hsp-inducing factors. Hsp-inducing factors may be divided into three groups. The first includes agents that can directly influence the structure of cellular polypeptides; heat stress, free radicals, and heavy metals were all shown to denature enzymes in living cells. The second group contains substances that strongly affect the function of protein synthesis and protein modification; their damage may lead to the accumulation of abnormal, misfolded proteins. Finally, the third group does not include stressful factors but rather factors that are able to induce strong changes in cell physiology; these changes tend to adapt systems of protein homeostasis to a newer functional mode. For instance, when myoblasts are induced to differentiate, they should refashion their syntheses to produce muscle types of myosin, actin, and others. These events cannot be retained without the attention of chaperones, so their synthesis is necessary.

of differentiation or proliferation, such as *N*-methylformamide, phorbol ester, prostaglandin, insulin, and growth hormones (Ting *et al.*, 1989; Ito *et al.*, 1995). Ananthan and colleagues suggested that the appearance of damaged proteins or their proteolytic fragments in cells is a prerequisite for the expression of Hsps (Ananthan *et al.*, 1986). In accord with these views, it is not surprising that factors that disturb polypeptide structure, either directly or by suppressing synthetic and posttranslational modification machineries, e.g., proteasome inhibitors, can induce Hsp expression (Fig. 1). The agents in the second group seem to act in a similar manner, for instance, through the inhibition of protein synthesis or invasion of new unknown proteins into a cell, as it is in the case of virus infection. Factors in the third group act by

making cells enter another metabolic state that requires significant changes in protein synthesis, their modifications, and degradation during cell cycle progression or differentiation.

This reasoning may be extended in two ways. On the one hand, Hsps are necessary for myriad reactions occurring at the protein level after certain treatment and, as such, are important for cell life in normal conditions. On the other hand, the level of expression of Hsps can serve as a marker for changes in a given cell or organism, or in their environment. This is why many groups have been trying to develop an assay for the quantification of Hsps since the end of the 1980s. In 2000, the Canadian biotech company StressGen started the production of immunodiagnostic kits for the detection of a variety of stress proteins for biomedical and ecological research. We also contributed to this field by creating the assay for Hsp70 quantification in biological samples, in which Hsp70 is trapped by its ligand, adenosine triphosphate, and immobilized on a solid support (Novoselov *et al.*, 2004).

Generally, the biosynthesis of Hsps is regulated by specific transcription factors (HSFs) that recognize so-called heat shock elements (5′-CNNGAANNTTCNNG-3′, HSE). Upon binding to HSE, HSF summons the RNA polymerase II complex to the appropriate hsp gene. Four HSFs in mammalian cells (Pirkkala *et al.*, 2001) and 21 in *Arabidopsis* (Baniwal *et al.*, 2004) were found to regulate the expression of hsp genes. Although widely studied, the process of HSF activation remains poorly understood. It was established that mammalian HSFs undergo trimerization before binding to HSE. The process of activation and DNA binding of HSF1 is also controlled by its phosphorylation (Holmberg *et al.*, 2001). In addition, Satyal and colleagues identified a small nuclear HSBP1 protein that strongly binds to HSF1 monomers suppressing HSF1 activation (Satyal *et al.*, 1998). Finally, SUMOilation of HSF1 at lysine-298 was found to enhance the transcriptional activity of HSF1 (Hong *et al.*, 2001). The connection between inducers of heat shock response and the activation of HSF is still obscure. Guo and colleagues demonstrated that the accumulation of polypeptides, damaged by a stressful factor, is recognized by Hsps and leads to the depletion of the latter, which results in the subsequent release of HSFs from the Hsp90 chaperone. After that the activated HSF molecules are able to migrate to the nucleus and bind to the appropriate HSEs (Guo *et al.*, 2001). Overall, it appears that the cellular function of heat shock proteins is entangled with the mechanisms participating in the induction of their synthesis. In some cases they are complementary and, therefore, should be explored together. For instance, many tumor cells constitutively have high levels of Hsp70 and Hsp27 (see Section IV.B). Since both proteins possess protective activity, the expression of the chaperones should be inhibited to kill these cells. One of the ways to do that is to affect HSF-1 activity, and this approach has already been demonstrated to be effective (Yin *et al.*, 2005).

It was already mentioned that Hsps could be divided into several groups uniting proteins with similar characteristics: Hsp110, Hsp100, Hsp90, Hsp70, Hsp47, Hsp40, and Hsp27. Each family includes several members with a high degree of amino acid sequence homology. Major groups of Hsps will be discussed below in relation to their role in cancer and proteotoxic diseases.

## A. Hsp90

The proteins of this family are usually from heterodimers composed of subunits with a high degree of homology, Hsp90α and Hsp90β. The dimerization of Hsp90 is dependent on its phosphorylation. The existence of homodimers is suggested by the finding of a 6-fold excess of the α- over the β-subunit in pig brain cells (Garnier *et al.*, 2001). The dimerization occurs through the pair of specific sequences located at both N-terminal and C-terminal parts of the molecule, and this feature is essential for many activities of the protein (Allan *et al.*, 2006; Chadli *et al.*, 2000). Sites responsible for ATPase and chaperone activities of Hsp90 are located at N- and C-terminal parts of the molecule, respectively (Neckers and Ivy, 2003). The function of Hsp90 requires a full-length protein molecule and the interaction between its domains (Johnson and Craig, 2000; Weikl *et al.*, 2000). These data are supported by Minami and colleagues (2001), who showed that both the N- and C-terminal chaperone sites contribute to Hsp90 function as a holder chaperone, however, in a significantly distinct manner. We will describe two important functions of Hsp90 in more detail below.

First, Hsp90 was found to be one of the major components of steroid receptors. In the unliganded state the steroid receptor is associated with the multiprotein chaperone machinery ruled by Hsp90. Along with Hsp90 and the receptor itself, several other proteins are found in these complexes, namely Hsp70/Hsc70, p23, Hdj1/Hsp40, Hop (heat shock organizing protein), Hip (Hsp70-interacting protein), p23, immunophilins FKBP52 and Cyp40, and some other proteins (Pratt *et al.*, 2004). Hsp90 binding to immunophilins connects the receptor complex to the protein-translocation machinery. In an ATP-dependent manner the Hsp90–Hsp70-associated complex opens a special domain on a receptor molecule, which is necessary for steroid hormone accession. Upon the binding of the hormone, chaperone machinery helps to translocate the receptor to the nucleus along a tubulin transport system. At the final point, the chaperone complex promotes the release of the receptor from the transcriptional complex and terminates the cycle of transcriptional activation (Pratt *et al.*, 2004).

The role of Hsp90 in the regulation of steroid receptor activity was recently linked to the control of stress response by HSF1. Li and Sanchez (2005) hypothesized that the chaperone machinery governed by Hsp90 alternatively

binds either the glucocorticoid receptor or HSF1. This interaction involves Hsp70 and is needed to rearrange a cell/tissue homeostasis after stress. The idea that stress response and poststress recovery are regulated by a common mechanism can be ruled out on the basis of work by Guo and colleagues (2001) and by Bharadwaj and colleagues (1999). Of note, both groups observed an HSF1–Hsp90 complex incorporating other chaperone-assisting proteins, i.e., Hip, Hop, p23, and immunophilins.

Another well-established function of Hsp90 relates to its ability to stabilize so-called client proteins. Many of these proteins were found to promote cell proliferation as well as the acquisition of tumor phenotype by cells. Among the most abundant client proteins are steroid receptors, several transcription factors, serine/threonine, and tyrosine kinases (Riggs *et al.*, 2004). Client proteins are stabilized by ATP-dependent binding to specific chaperone sites on Hsp90. In the 1970s, a factor possessing high antineoplastic activity was discovered that was shown to inhibit specifically the chaperone activity of Hsp90. This factor, called geldanamycin, binds with high affinity the pocket-like structure in the ATPase domain of the Hsp90 molecule and competes with ATP. This binding reduces the chaperone activity of Hsp90 and results in the release of bound client proteins (Whitesell *et al.*, 1994). Similar activity was shown for benzoquinone ansamycin. Since the end of the 1990s, many compounds with different Hsp90-binding capacities have been synthesized. Of these, 17-allylaminogeldanamycin (17-AAG) and 17-dimethylaminoethylaminogeldanamycin (17-DMA) have exhibited anti-tumor activity in oncological trials (Sausville, 2004). All these Hsp90-binding substances, including radicicol and herbimycin, have been demonstrated to destabilize Hsp90's client proteins, such as v-Src, Bcr-Abl, Raf-1, ErbB2, and some growth factor receptors. Factors that inhibit the chaperone activity of Hsp90 induce ubiquitination and proteasomal degradation of numerous oncoproteins and can cause growth arrest, differentiation, apoptosis, or the prevention of apoptosis.

## B. Small Heat Shock Proteins

This family includes Hsp27 and α-crystallin, the protein of eye lens. In addition to common stress factors, the expression of Hsp27 can be induced by substances known to reduce the level of glutathione in a cell, such as diethyl maleate and buthionine sulfoximine (Ito *et al.*, 1998). In normal conditions, Hsp27 forms oligomers with a molecular mass of about 700 kDa, whereas stress factors strongly reduce the size of these complexes. Hsp27 is a phosphoprotein, and mitogen-activated protein kinase (MAPK)- and protein kinase C (PKC)-mediated phosphorylation was found to enhance the protective activity of this protein in heart muscle cells (Arnaud *et al.*, 2004).

Two phosphorylation sites, Ser-78 and Ser-82, were found to play important roles in the structure and function of Hsp27. For instance, the phosphorylation of Hsp27 is important for the preservation of the structure of actin cytoskeleton in cells affected by oxidative stress (Nguyen *et al.*, 2004). Hsp27 also plays an important role in apoptosis by preventing the release of proapoptotic cytochrome *c* and Bid factors from mitochondria (Paul *et al.*, 2002). This study has also shown that Hsp27 protects actin filaments from the depolymerization caused by cytochalasin. Both processes were shown to be linked to each other in some cellular models of apoptosis (Paul *et al.*, 2002). Finally, Hsp27 was shown to be one of the molecules involved in disorders caused by aggresomes, the aggregates made of proteins containing expanded polyglutamine (polyQ) tracts. Wyttenbach and colleagues (2002) reported that Hsp27 suppressed polyQ-mediated cytotoxicity in the cell model of Huntington disease. Using this model they showed that the expression of long polyglutamine chains and the subsequent formation of aggresomes were accompanied by an elevation of reactive oxygen species (ROS). The overexpression of Hsp27 reduced the concentration of ROS and the number of apoptotic cells (Wyttenbach *et al.*, 2002). The protective role of Hsp27 in polyglutamine-mediated neurodegenerative diseases was also demonstrated by Chang and colleagues (2005), who revealed the link between Hsp27 protein content and tolerance to the toxicity of aggregates made of mutant ataxin-3. Thus, Hsp27 can protect cells modeling polyglutamine expansion diseases by struggling against the elevated level of ROS.

## C. Hsp70 Family

Hsp70 is a class of proteins sharing a similar structure and function. Several members of the Hsp70 family can be present in a single mammalian cell: inducible Hsp70 (found mainly in human cells), constitutively expressed (cognate) Hsc70, mitochondrial Mtp70, and glucose-regulated Grp78/BiP (immunoglobulin heavy chain-binding protein), which is located in the endoplasmic reticulum. The cross-homology of Hsp70 family members is about 50% and it reaches 80% for the ATPase domain.

The molecule of mammalian Hsp70 consists of two parts: a highly conserved N-terminal domain containing an ATPase/ATP-binding site and a C-terminal peptide-binding domain (Fig. 2). The structure of the Hsp70 ATP-binding domain is similar to that of actin and hexokinase. Due to their high ATP-binding capacity ($K_d \sim 10$ n$M$), proteins of the Hsp70 family can be purified in a single chromatographic step using ATP-agarose (Welch and Feramisco, 1985). In general, the structure of the Hsp70 molecule resembles that of MHC class I and II antigens. This similarity prompted

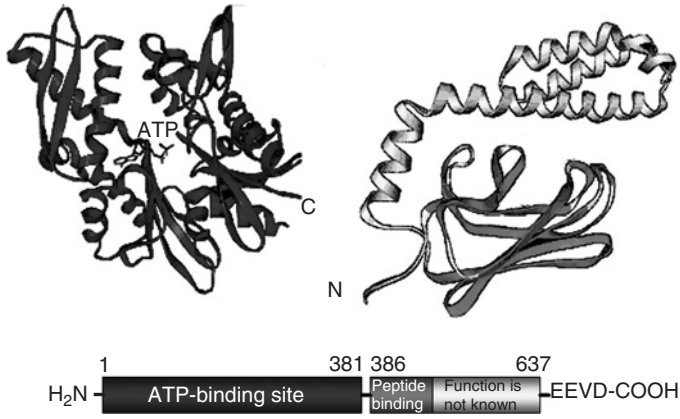


FIG. 2 Molecular structure of Hsp70. The Hsp70 molecule consists of two parts: a conservative N-terminal domain including an ATPase pocket and a C-terminal peptide-binding domain with a strongly conservative EEVD sequence on the very end of the molecule. On both domains the sites are located so as to facilitate the interaction with cochaperones and other proteins; for instance, Bag-1 and Hip cochaperones recognize some parts of the ATPase pocket, whereas J-domain-containing proteins are known to bind another sequence in the pocket and the EEVD motif as well.

some researchers to hypothesize that bacterial Hsp70, DnaK, is an ancestor of both classes of molecules (Flajnik *et al.*, 1991).

In addition to sites responsible for nuclear localization and binding of Hdj, Bag-1, and other cochaperones, Hsp70 may have other functional motifs. We and other researchers have shown that Hsp70 is able to cross the plasma membrane of certain cells, a feature found in five to eight other proteins called permeases (Derossi *et al.*, 1998; Fujihara and Nadler, 1999; Guzhova *et al.*, 1998). The identification of amino acids involved in this process can greatly advance the use of Hsp70 in biomedical research.

## III. Stress Proteins Are Molecular Chaperones

The fate of a protein molecule in a cell depends largely on its size. Small single-domain proteins rapidly hide their hydrophobic parts and fold within microseconds after the termination of translation. For a chain of 300 amino acids, the process of folding takes about 1 min, during which time the immature part of the newly synthesized polypeptide remains exposed to the protein environment. Larger, especially multidomain protein molecules take much longer to fold. During this process, hydrophobic regions are exposed to

the environment, which can lead to either self-aggregation or the formation of heterogeneous aggregates. In conditions of protein "crowding," characteristic of cells, the aggregation can go much more quickly despite the presence of chaperones that are supposed to enhance the correct assembly of polypeptides. Since a number of diseases are found to be associated with the loss of protein function due to polypeptide misfolding, the molecular basis of chaperone activity is of primary interest.

One of the most abundant cellular chaperones, Hsp70, can recognize and bind to three types of polypeptides: (1) polypeptides newly synthesized/nascent or damaged by stress, (2) mature proteins that undergo certain modifications and subsequently change their conformation, and (3) cochaperones and other proteins that are recognized by specific sequences within the Hsp70 molecule. The first and the second groups of proteins probably share a common motif on their molecules that can be recognized by Hsp70. Such a site has been found to contain hydrophobic residues as well as a leucine–isoleucine moiety in its center (Hartl, 1996). These sequences are located at approximately every 40 residues on a target (substrate) polypeptide and are recognized by the chaperones with affinity ranging from 5 n*M* to 5 μ*M* (Bukau and Horwich, 1998). To date, after screening of thousands of such peptides, two of them, NIVRKKK and FYQLALT, were shown to have affinities to Hsp70/Hsc70 below 5 μ*M* (Takenaka *et al.*, 1995; Thulasiraman *et al.*, 2002). The structure of both peptides satisfies the above-mentioned requirements, namely, the occurrence of a central hydrophobic part and leucine/isoleucine residues. These peptides became important tools for further studies concerning molecular mechanisms underlying the actions of the chaperones.

## A. Hsp70: Structure–Function Relationship in Chaperone Act

The structural and functional features of Hsp70 are best understood for its bacterial homolog DnaK. Below we present data that are pertinent to both prokaryotic and eukaryotic Hsp70s (Hartl and Hayer-Hartl, 2002). The C-terminal domain of Hsp70 consists of a β-sandwich subdomain with a peptide-binding pocket and a latch-like structure composed of α-helices (Zhu *et al.*, 1996). Target peptides are bound by Hsp70 through hydrophobic side chain reactions and hydrogen bonds, which are formed with a peptide backbone (Zhu *et al.*, 1996). Rapid peptide binding occurs when Hsp70 is in an ATP-bound form and the α-helical latch is open (Fig. 2). The ATP-bound form is thought to be a transient intermediate. In this conformation, Hsp70 can either release the bound polypeptide or bind it more strongly, if the polypeptide needs to be corrected. Stable holding of a peptide presumes

the closing of the latch, an act that requires the hydrolysis of ATP to ADP and the participation of a specific activator of Hsp70 ATPase. In archea, the protein responsible for the activation of Hsp70 ATPase and, correspondingly, for the cycling of the chaperone between ATP/ADP-bound forms is DnaJ. In mammalian cells, an extensive family of proteins with such properties exists; Hdj1/Hsp40 and Hdj2 cochaperones are the best known among them (see the next section for details). Another protein assistant of DnaK is GrpE, which finishes the chaperone cycle by replacing ADP with ATP (Hartl and Hayer-Hartl, 2002). The rebinding of ATP results in the dissociation of a substrate peptide. While eukaryotic Hsp70s are dependent on DnaJ-like proteins in their chaperone cycle, their functional linkage to GrpE-like nucleotide exchanging factors seems to be dispensable. However, the Bag-1 protein shares limited homology with GrpE, suggesting a specific role of the former in mammalian cells (see the next section for details). The released polypeptide may follow several different routes. It may (1) adopt a correct conformation, (2) pass another circle of folding by Hsp70-based machinery, (3) be transferred to an alternative chaperone system based on the Hsp90 or Tric chaperonine, and (4) be digested by proteasomes or other proteolytic systems.

DnaK and eukaryotic Hsp70 chaperones usually work with two major targets: newly synthesized polypeptides and ones damaged by a certain stressor. Under normal conditions, the first function prevails. In *Escherichia coli* the number of DnaK molecules was found to be higher than that of ribosomes (Teter *et al.*, 1999). This means that all nascent peptide chains that pass through transcriptional quality control (a total of ~15–40% of *E. coli* proteins) are assumed to be subject to the action of the DnaK–DnaJ–GrpE system. Not as much is known about the function of this chaperone mechanism in stress conditions. In eukaryotic cells, Hsp70/Hsc70 also binds to a significant portion of newly synthesized polypeptides (15–20% of the total amount), including multidomain proteins with a molecular mass over 50 kDa (Thulasiraman *et al.*, 1999). It has been established that Hsp70 performs short-time cycles of trap and release of small polypeptides, while such a cycle for longer molecules takes more time. It is thought that this can prevent multidomain proteins from intramolecular misfolding and from the subsequent formation of aggregates.

## B. Role of Cochaperones

Most chaperone mechanisms in eukaryotic cells are based on Hsp70/Hsc70. There are various proteins assisting them in different cell compartments. We have already mentioned two DnaJ-like proteins, Hdj1/Hsp40 and Hdj2. To date this protein family consists of over 50 members including Hdj1–4,

DnaJ-like proteins inhabiting various cell compartments, as well as several bacterial and yeast homologs. The transcription of typical representatives Hdj1 and Hdj2 is initiated by the activation of HSF1, and hyperthermia causes their transport to the nucleus (Hattori *et al.*, 1992). All these proteins have an N-terminal so-called J-domain, which consists of approximately 70 amino acids forming two antiparallel α-helices connected by a highly conserved HPD sequence (Hennessy *et al.*, 2005). There are three types of Hsp40-like proteins, which can be distinguished by the presence of certain sequence motifs. DnaJ-like polypeptides related to type I are characterized by clusters of cysteins forming tertiary structures with the use of two zinc ions (Linke *et al.*, 2003). Type II proteins have G/F-rich regions of 30–40 amino acids in length that contain 40% glycine (G) and 15% phenylalanine (F) residues. Hsj1α/β and Hdj1/Hsp40 represent type II DnaJ-like proteins in mammalian cells. Type III proteins possess only a J-domain and no other specific motifs. Representatives of this type in animal cells include p58IPK kinase, mitochondrial protein Mtj1, protein of clathrin vesicles auxilin, and cysteine string protein (CSP) located at the plasma membrane, among others (Cheetham and Caplan, 1998).

DnaJ-like proteins of the J and, partially, G/F domains are responsible for binding to Hsp70 proteins. However, the corresponding sequence on the latter is still unidentified (Hennessy *et al.*, 2005). It has been suggested that one of the possible sites for the interaction with Hdj1 lies within the ATP-binding domain (Landry *et al.*, 2003). Another possible site is probably located at the α-helical latch-like part of Hsp70, while yet another one is represented by four C-terminal amino acids, EEVD (Hennessy *et al.*, 2005). It is thought that the interaction between Hsp70 and DnaJ-like proteins occurs via different sites and depends on the conformational state of both molecules and the stage of the chaperone cycle.

A single mammalian cell may contain 5–10 different DnaJ-like proteins, which play distinct roles in cell physiology. J-domain-containing proteins are able to bind denatured substrates (Rudiger *et al.*, 2001). This binding prevents aggregation of target polypeptides, although no recovery of their correct structure was observed (Johnson and Craig, 2001). Both Hdj1 and Hdj2 lack substrate-binding activity but were found to stimulate Hsp70 ATPase (Nagata *et al.*, 1998). The difference between the two proteins at a cellular level is that only Hdj2 possesses membrane-trophic activity and participates in the transport of proteins into mitochondria (Terada *et al.*, 1997). Hdj2 is involved in the biogenesis of the cystic fibrosis transmembrane conductance regulator (Meacham *et al.*, 1999), whereas Hsj1 together with Hsc70 and auxilin take part in endo- and exocytosis processes (Cheetham and Caplan, 1998; Gruschus *et al.*, 2004).

Another family of Hsp70 chaperone regulators includes proteins belonging to the Bag-1 family. Bag stands for Bcl-2-associated athanogene, which

emphasizes the relationship of this protein to programmed cell death (Takayama *et al.*, 1995). Bag-1 was initially identified as a Bcl-2-binding protein. This pair is a potent protector of cells from apoptosis. In human cells, the Bag-1 family consists of p29, Bag-1S, Bag-1M, and Bag-1L proteins with molecular masses of 29, 36, 46, and 57 kDa, respectively. An alternate name for Bag-1M is RAP-46, the receptor-associated protein (Zeiner and Gehring, 1995). Similar to the family of J-domain-containing proteins, a large group of Bag proteins was identified: Bag-2, Bag-3 (CAIR-1/Bis), Bag-4 (SODD), Bag-5, and Bag-6 (BAT3/Scythe).

All Bag-1 proteins have a specific sequence of ~100 amino acids (Bag domain) at the C-terminal part of the protein, which is composed of three α-helices. Ubiquitin-like domains are also identified in the middle and at the N-terminal parts of these proteins. The Bag domain is necessary for the binding to Hsp70/Hsc70, while ubiquitin-like domains of Bag-1 can interact with proteasomes (Demand *et al.*, 2001; Takayama *et al.*, 1999). The binding site for Bag-1 was found within the ATPase domain of Hsp70/Hsc70. It was also shown that the interaction of Hsp70 with Bag-1 does not depend on the presence of ATP (Zeiner *et al.*, 1997). The binding of Bag-1 to Hsp70/Hsc70 results in the acceleration of steady-state ATPase activity of the chaperone (Alberti *et al.*, 2003). This stimulation of ATPase activity is also critically dependent on the presence of Hdj1. Thus, Bag-1 stimulates ADP release from Hsp70/Hsc70 after Hdj-activated conversion of the chaperone into the ADP-bound form. The transition to the ATP-bound form induced by Bag-1 causes a dramatic change in the chaperone conformation that is transferred from the ATPase domain to the peptide-binding domain and results in the release of substrate polypeptide (Lüders *et al.*, 2000b; Sondermann *et al.*, 2001). Upon acquisition of ATP, Hsp70/Hsc70 is ready for a new chaperone cycle.

Bag-1 family members participate in a variety of cellular processes including differentiation (Kermer *et al.*, 2002), regulation of transcription (Schmidt *et al.*, 2003; Zeiner *et al.*, 1999), and stress signaling (Song *et al.*, 2001). Song *et al.* (2001) indicated that Bag-1 binds Raf-1 protein kinase, stimulates its activity, and promotes cell proliferation. Bag-1 sites for the binding of Raf-1 and Hsc70 overlap. The very important conclusion is that under normal conditions Bag-1 promotes cell growth, whereas during acute stress Hsp70 accumulation inhibits cell proliferation through binding to Bag-1.

In addition to DnaJ-like proteins and Bag-1, chaperone machineries governed by Hsp70 include several other proteins. One of these is Hip (Hsp70 interacting protein), a protein consisting of several domains that allow it to self-oligomerize and bind to denatured substrates preventing their aggregation (Velten *et al.*, 2002). Similar to other cochaperones, Hip contains TPR, a tetratricopeptide sequence that is needed for the binding to Hsp70 and Hsp90 chaperones. Hip was implicated in the chaperone cycle in a manner opposed to that of Bag-1: it maintains an Hsp70–ADP–substrate complex.

Together with the ability to prevent aggregation of abnormal proteins, this function was shown to accelerate the refolding of denatured luciferase both *in vivo* and *in vitro* (Nollen *et al.*, 2001). In this process it was suggested that Hip utilizes multiple sites on the Hsp70 molecule including the one needed for the interaction with Bag-1.

Hop (p60, Hsp70–Hsp90-organizing protein) was also found to associate with Hsp70. This protein has several TPR domains, which include three tandem TPRs combined with a neighbor α-helix. It forms the groove that is able to capture specific C-terminal sequences of Hsp70 and Hsp90 (Brinker *et al.*, 2002). TPR domains allow Hop to participate in the assembly of steroid receptors (Pratt *et al.*, 2004; see also Section II.A for details concerning Hsp90). Basically, this process can proceed without Hop, although it takes a much longer time (Morishima *et al.*, 2000). At first, the inactive receptor must be retained by Hsp70 in ADP form in an Hdj1/Hsp40-dependent manner. After that Hop (assisted by p23) changes the conformation of Hsp70 and Hsp90 and transfers the receptors onto the latter chaperone. This step results in the opening of the steroid-binding domain on the receptor molecule (Pratt and Toft, 2003).

One particular cochaperone, HSBP1 (Hsc70-binding protein 1), is also worth mentioning in relation to Hsp70 function. This 40-kDa protein binds the ATPase domain of Hsp70/Hsc70 making it inaccessible to ATP (Raynes and Guerriero, 1998). The inhibition of ATPase activity may be reversed by Hdj1 and Tpr1 adaptor proteins (Oh and Song, 2003). Thus, Hsp70/Hsc 70-based chaperone mechanisms work in a cyclic manner with numerous cofactors involved in this activity at a certain stage. Some factors, such as DnaJ-like proteins, serve to enhance chaperone activity, while others accelerate the dissociation of a target polypeptide from the chaperone molecule. In addition to the "primary quality control" of cellular polypeptides, Hsp70/Hsc70 takes part in the elimination of useless polypeptides.

## C. Hsp70 Family Proteins in Regulation of Proteolysis

Proteolytic machineries have been extensively studied in the past 20 years. Studies not only focused on the mechanism of their action, but also aimed to depict the rules of their function, that is, how they recognize the targets and what is needed for a given protein to become a target. Exciting progress in this field is associated with the discovery of the ubiquitin- and SUMO-based protein-modifying systems. Ubiquitin is a small protein that is expressed in all known eukaryotic cells and marks proteins that should be cleaved or fully degraded by specific heterooligomeric protein machineries called 26S proteasomes. The ubiquitinylation of a certain polypeptide and its exposure to a proteasome are mediated by the hierarchy of special proteins,

ubiquitin-conjugating enzymes, and ubiquitin ligases. The ubiquitin-binding domain in Bag-1 does not serve as a cleavage site or as a ubiquitin transmission point. The data of Lüders and others show that this domain is necessary for the binding of Bag-1 to the proteasome (Lüders *et al.*, 2000a). It seems significant that Bag-1 can be simultaneously bound to the proteasome and to Hsc70. This implies that Bag-1 may transfer an irreparable polypeptide from Hsp70 to the proteasome for degradation. It is possible that Bag-1 assists the sorting of protein substrates to the proteasome proteolytic machinery (Hartmann-Petersen *et al.*, 2003). This hypothesis was corroborated by the finding that another protein in the complex is CHIP (carboxyl terminus of Hsc70 interacting protein). The CHIP molecule also contains TRP motifs needed to bind C-terminal sites of Hsc70 and Hsp90 molecules (Alberti *et al.*, 2003; Ballinger *et al.*, 1999). Overexpression of CHIP was found to inhibit chaperone activity of Hsp70/Hsc70, probably at the phase of chaperone cycle initiation by Hdj-like proteins. The C-terminus of CHIP also contains a U-box, structurally related to similar domains found in ubiquitin ligases. Indeed, CHIP is able to link ubiquitin to substrate molecules presented by Hsp70 and Hsp90 in cooperation with the ubiquitin-conjugating enzymes of the Ubc4/5 family (Alberti *et al.*, 2003; Jackson *et al.*, 2000). Both CHIP and Bag-1 are able to interact with Hsc70 simultaneously. All three proteins form the ternary complex that presumably works in the following manner. Bag-1 induces the release of a substrate polypeptide from the Hsp70 molecule; this process is mediated by ADP–ATP exchange, as it does in the chaperone cycle (see the previous section). Then, with the help of CHIP, it facilitates the transfer of a substrate to the core of the proteasome. The role of CHIP in this complex is critical, because it catalyzes the ubiquitin attachment to the substrate molecule released by Bag-1 from Hsp70.

To summarize the data presented in the two previous sections, the following seems conceivable. The key chaperone Hsp70 performs cycles of binding release of a substrate polypeptide with the assistance of Hdj1 and Bag-1 using ATP as the conformational switch until the moment when the polypeptide is converted into the folded state or until CHIP binds the Hsp70–substrate complex. CHIP retains Hsp70 in an ATP-bound state for the next substrate and/or helps to attach ubiquitin to the substrate and to seize Bag-1, which is needed to finally present the whole assembly to the proteasome (Fig. 3).

## D. The Variety of Chaperone Mechanisms in a Single Cell

Mammalian cells have to perform many chaperone acts continuously even under normal conditions. During stress this activity is greatly increased. Various cellular chaperone mechanisms are engaged in distinct cellular compartments dealing with different substrates. All of these are covered by just a

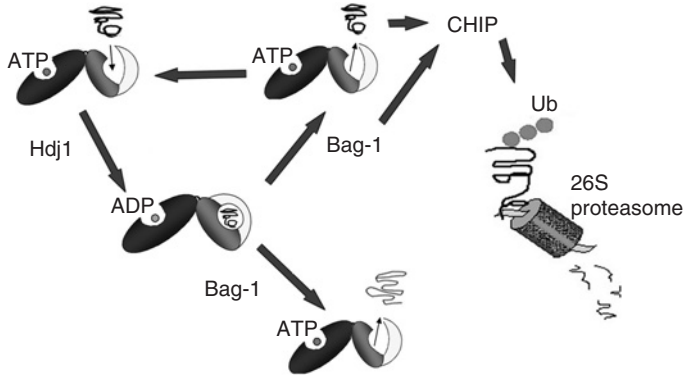


FIG. 3 The chaperone cycle. The cycle of chaperone mechanisms starts from the primary binding of the polypeptide substrate to the Hsp70 chaperone that is in an ATP-bound form. Being in this intermediate state Hsp70 can alternatively release the polypeptide or through ATP hydrolysis stimulated by Hdj1 transiently retain it. In the latter case Hsp70 can reside there until ADP is replaced by ATP in a process initiated by Bag-1 class proteins. The cycle is over and Hsp70 returns to its ATP-bound form. The released polypeptide can be further transported to the point of its function, subjected to the next cycle of chaperoning, or exposed to a proteasome for degradation via the mechanism implicating CHIP.

few constituents of the Hsp70 family and by a number of cochaperones described in previous sections. One of the first chaperone machines ever discovered was found to participate in the dissociation of clathrin vesicles. Clathrin triskelions form spherical baskets that are involved in vesicular transport between the endoplasmic reticulum and Golgi. Schlossmann and colleagues were the first to demonstrate that the 70-kDa protein possessing ATPase activity is functionally linked to vesicle-dissociating activity (Schlossman *et al.*, 1984). A year later, another group identified this protein as a constitutive member of the Hsp70 family, Hsc70 (Chappell *et al.*, 1986). As in other chaperone mechanisms, an activator of Hsc70 ATPase activity is necessary to start the cycle of clathrin dissociation. Such an activator was found to be auxilin, belonging to the third group of DnaJ-like proteins (see Section III.B). Auxilin causes a 5-fold increase in Hsc70 ATPase activity and a corresponding increase in the steady-state level of bound ADP with a dissociation constant of about 0.6 $\mu M$ (Jiang *et al.*, 1997). The first round of clathrin uncoating was shown to require the formation of an assembly protein (AP)–clathrin–Hsc70–ADP complex. Then, following the exchange of ADP for ATP, a steady-state AP–clathrin–Hsc70–ATP complex forms, which ties up Hsc70 and prevents further uncoating. This complex exists only during the uncoating process in the presence of APs. In the absence of APs, Hsc70 rapidly dissociates from the clathrin and proceeds to the next step of uncoating (Jiang *et al.*, 2000). Jiang *et al.* (2000) proposed that Hsc70 not

only uncoats clathrin but also prevents it from polymerization in cytosol and primes it to reorganize clathrin-coated pits.

Well-studied chaperone machinery is found in mitochondria (MT). The mechanism of polypeptide delivery to the mitochondrial matrix is among the most interesting discoveries of the past few years. This machinery consists of five proteins: Mtp70 (matrix chaperone sharing a high degree of homology with cytosol Hsp70s), Tim44, Mge1 (nucleotide exchange factor), and Pam16 and Pam18 (two subunits of presequence translocase-associated motor proteins) (Frazier *et al.*, 2004). Pam18 is a J-domain protein that is involved in the initiating step in the Mtp70-driven chaperone cycle, whereas Pam16 interacts with Pam18 and is needed for the association of Pam18 with the presequence translocase and for the formation of the mtHsp70–Tim44 complex (Frazier *et al.*, 2004). Another constituent of the MT-import motor complex was described, Zim17, possessing a zinc finger motif reminiscent of class III J-domain-containing molecules. It was suggested that this protein binds polypeptides to present them to Mtp70 and to prevent the self-association of the matrix Mtp70 chaperones (Burri *et al.*, 2004; Sanjuan Szklarz *et al.*, 2005). Another inhibitor of Mtp70 self-aggregation, Hep1, was recently found. Hep1 binds to the nucleotide-free form of the chaperone, the form particularly prone to aggregation. The aggregation of Mtp70 was prevented by Hep1 (Sichting *et al.*, 2005). Thus, in MT-import motor machinery, as in other chaperone systems, Hsp70 activity is initiated by a DnaJ-like protein and mediated by the nucleotide-binding factor Mge1.

In the early 1990s, many studies aimed to establish a link between Hsp70 class chaperones and the plasma membrane. One such link was found in the complex formed by the cystic fibrosis transmembrane conductance regulator (CFTR). The common cause of cystic fibrosis is the deletion of phenylalanine-508 (F508), which leads to the incorrect processing of the protein molecule in the endoplasmic reticulum. It was found that the processing of CFTR is controlled by Hsc70 in an ATP-/Hdj2-dependent manner, so that the chaperone dissociates from the target before its transportation to the Golgi (Meacham *et al.*, 1999). Recently, data were presented suggesting that Hdj1 rather than Hdj2 serves in the activation of the Hsc70 chaperone (Farinha *et al.*, 2002). It appears that similar to chaperone systems in cytosol, membrane-associated Hsp70/Hsc70 perform a dual function: they prevent incorrect folding of the large CFTR molecule and cope with mutated F508. For the latter process, Hsc70 interacts with CHIP promoting the ubiquitinylation of the target protein (Meacham *et al.*, 2001).

The involvement of Hsp70 class proteins in cellular proteolytic machineries has been the subject of numerous studies in the past decade. The most impressive data were obtained on the link of chaperones to proteasome through Bag-1 and CHIP (see Section III.C). Several earlier studies have demonstrated that Hsc70 can also reside within lysosomes and that this

relocation coincides with the more efficient degradation of cytosolic polypeptides (Cuervo *et al.*, 1997). This was further supported by experiments aimed at inhibiting Hsc70 activity with the aid of an Hsc-70-specific antibody microinjected into the cell. This treatment has led to nearly complete inhibition of the proteolysis of radiolabeled cellular polypeptides (Agarraberes *et al.*, 1997). It was hypothesized that lysosomal hsc73 is required for a certain step in the degradation pathway before protein digestion within lysosomes, most likely for the import of substrate proteins. It was later shown that Hsc70 is located at the cytosolic part of the lysosomal membrane, where it recognizes and binds target polypeptides. Along with Hsc70, the whole set of cochaperones was found, including Hsp90, Hdj1/Hsp40, Hop, Hip, and Bag-1. All of them constituted active parts of the chaperone machinery, because respective specific antibodies blocked the transport of target polypeptides into purified lysosomes (Agarraberes and Dice, 2001).

One of the Hsp70 cochaperones, CHIP, plays the role of a molecular switch between two distinct proteolytic systems, proteasomes and lysosomes. The experiments aimed at dissecting the mechanisms of the degradation of α-synuclein, the major pathogen in Parkinson disease, have demonstrated that CHIP, being associated with Hsp70, can reduce aggregates formed by α-synuclein in a ubiquitin-dependent manner or direct the substrate to lysosomes using a U-box (Shin *et al.*, 2005).

We have addressed only a part of the chaperone system working in a single eukaryotic cell. Molecular chaperones display similar properties and usually work in a dual manner choosing between improving the folding states of cellular polypeptides or removing the ones that cannot be folded. These systems are vital for all processes of cell physiology, both normal and pathological. Examples of chaperone links to various human pathologies will be discussed in the following sections.

## IV. Cellular Function of Hsp70 Class Proteins

### A. Cytoprotective Activity of Hsp70

One of the most remarkable properties of Hsp70 is its protective function as revealed in many studies both *in vitro* and *in vivo*. This property can be easily demonstrated by subjecting cells to mild heating followed by a short period of incubation in normal conditions. After this, cells become thermotolerant, which allows them to resist the action of a wide range of cytotoxic factors (Lindquist, 1986). The specific role of Hsp70 in the development of tolerance is supported by multiple experiments using a variety of different methods. For instance, liposome-mediated delivery of purified Hsp70 into pancreatic

islet cells and mouse myeloma cells was shown to protect these cells from the deleterious effects of interleukin-1β and heat stress, respectively (Lasunskaia *et al.*, 1997; Margulis *et al.*, 1991). Similar data were obtained when the Hsp70 gene was expressed under a strong constitutive promoter in cultured cells or in mice. In most cases Hsp70 gene overexpression resulted in increased resistance to a variety of damaging factors, such as traditional stressors, e.g., heat shock, as well as tumor necrosis factor-α (Jäättelä *et al.*, 1992) and ischemic stress (Plumier *et al.*, 1997). The opposite is also true—the reduction of Hsp70 expression can increase cell sensitivity to stress factors. First, Johnston and Kucey (1988) demonstrated that the delivery of the excess of heat shock element DNA oligonucleotides caused multiple errors in the HSF1 recognition pattern and a subsequent increase in cellular susceptibility to heat shock. Second, the use of antisense RNA revealed that the inhibition of Hsp70 expression in human lymphoma Molt-4 (Wei *et al.*, 1995) and breast carcinoma MCF-7 (Nylandsted *et al.*, 2000) caused massive apoptosis in the cells. Therefore, it can be hypothesized that Hsp70 is important for cancer cells to survive even in normal conditions.

Based on these data, it is clear that Hsp70 constitutes a powerful antistress system in an individual cell, and, as we shall see in the next section, in a whole organism as well. As for healthy cells, it appears to be good to possess a reliable protective mechanism. However, proper functioning of the latter in cancer cells may undermine the treatment of oncological diseases.

## B. Expression of Hsp70 and its Cochaperones in Human Tumors

The elevated level of Hsp70 was found in tumors of different genesis in comparison to normal tissues. Almost all cell lines derived from clinical tumor samples and employed in cell biology experiments contain elevated amounts of this protein. For instance, the increased level of Hsp70 was observed at earlier stages of malignancy of hepatocellular carcinoma, a fact that indicates that Hsp70 content is an early marker of tumor progression (Chuma *et al.*, 2003). A positive correlation between *hsp70* gene expression and malignancy has been observed in cases of bladder cancer (Syrigos *et al.*, 2003), colon cancer (Hwang *et al.*, 2003), endometrial carcinoma (Li *et al.*, 1999; Piura *et al.*, 2002), and uterine cervix carcinoma (Novoselov *et al.*, 2004; Piura *et al.*, 2002), among others. It seems significant that in addition to Hsp70, cochaperones are also on the list of tumor prognosis markers. A high level of Bag-1 expression was found in several breast carcinomas (Turner *et al.*, 2001), in prostate tumors (Froesch *et al.*, 1998), in the large intestine (Hostein *et al.*, 2001), and in leukemia cells (Takayama *et al.*, 1998). Bag-1 was shown to cooperate with cytoskeletal proteins in the

enhanced motility of intestinal carcinoma cells, which led to the enhancement of the metastatic process (Naishiro *et al.*, 1999). The involvement of DnaJ-like proteins in carcinogenesis is less documented. The increased amount of Hdj1 was found in lung (Oka *et al.*, 2001) and stomach (Isomoto *et al.*, 2003) cancers. Enhanced expression of another J-domain protein, Hdj2, was observed in acute lymphoblastoma cells (Chen *et al.*, 2001).

All proteins mentioned above are known to defend cells against numerous cytotoxic factors including those that are able to induce programmed cell death. In the next two sections, we will focus on mechanisms of apoptosis and the role of chaperones in this process.

## C. Proapoptotic and Antiapoptotic Systems: A Contradiction or a Compromise?

Apoptosis is a genetically controlled program of cell destruction that requires specific nucleases and proteases (caspases) and is accompanied by the activation of multiple cell signaling systems (Kerr *et al.*, 1972; Sen, 1992; Thompson, 1995). The morphological pattern of apoptosis is quite different from that observed in necrosis. Apoptosis is usually realized through a chain of events started by an exogenous stimulus and ending in the conversion of a cell into several parts, each surrounded by a separate membrane. The list of apoptotic stimuli includes an excess or depletion of nutrients and growth factors, loss of contact with the basal membrane or other surface (termed *anoikis*), nonreparable DNA damage, inopportune expression of regulators of the cell cycle, viral infection, and drugs used in clinical practice (Ashkenazi and Dixit, 1998; Evan and Littlewood, 1998; Green and Reed, 1998). Cells respond to apoptotic signals by entering a so-called commitment phase, the period within which it remains alive and functionally active; although its death is already predetermined, this phase may last for hours and even days. The final stage is significantly shorter; it lasts 15–60 min and is typically contingent upon morphological and biochemical changes not present in necrotic cells. This phase is highly conservative, i.e., it proceeds in a similar way in all cell types studied to date. During this stage, chromatin in the nucleus becomes condensed, it locates near the nuclear envelope, and later may become fragmented. This process coincides with the reduction of cell volume, compacting of cell organelles, and loss of contact with other cells and the basal membrane followed by the formation of apoptotic bodies. An inflammatory reaction, typical of necrosis in programmed cell death, is absent (Herget and Kock, 1999).

One of the key mechanisms acting in the process of apoptosis is proteolysis. There are several families of proteases, among which the most significant to apoptosis is thought to be caspases (caspase—*cysteine-dpendent*

*aspartate-specific proteases*). Caspases are synthesized in cells as nonactive proenzymes (procaspases or zymogens), which can be activated through the proteolytical dissociation of their N-terminal domain cleaved at a specific site containing an asparaginic acid residue (Fig. 4). As a result, two subunits, large and small, are released and immediately form an active protease complex. Activation of caspases resembles another cascade process, the activation of blood clotting factors (Herget and Kock, 1999).

Depending on their position in the cascade, caspases can be divided into two groups: initiating caspases, acting at the upper levels of the cascade (caspases 2, 8, 9, and 10), and effector caspases (3, 6, 7, and 14). The function of effector caspases is well established, and it is generally accepted that caspases 3 and 7 play a major role in the late stages of apoptosis.

The activation of a ''death receptor'' triggers apoptosis via at least two canonical pathways. The signal from receptors related to tumor necrosis factor-R or Fas superfamilies induces the modification of caspase 8, which in turn triggers the activation of effector caspases 3 and 7 (Fig. 4). In the

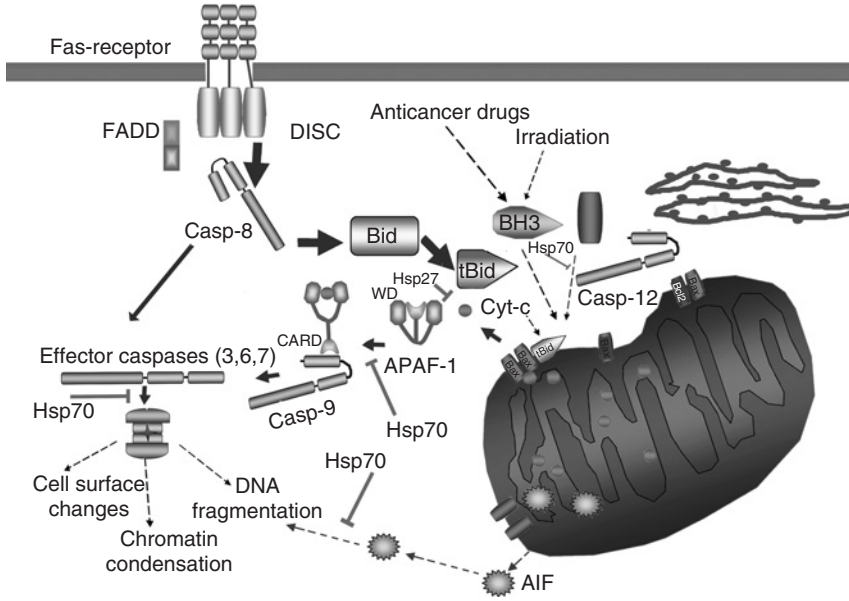


FIG. 4 Molecular pathways of apoptosis. Apoptosis is a rather sophisticated process that could sometimes simultaneously involve several signaling mechanisms. The advantage of one of these pro- and contraapoptotic systems leads to a progressive death of a cell population or to the inhibition of apoptosis. Hsp70 is one of the antiapoptotic proteins and its activity is noticeable at the different stages of the process from the banning of Bax release from mitochondria to binding effector caspases 3 and 7.

second pathway, caspase 8 cleaves a small protein, Bid, followed by the release of its N-terminal part, while the C-terminal domain, tBid, causes the oligomerization of the cytosolic protein Bax. These oligomers create pores in the mitochondrial membrane that promote release of cytochrome *c* to the cytosol (Green and Evan, 2002). Similarly, tBid can trigger the oligomerization of another proapoptotic protein, Bak, which was also shown to cause pore formation and the release of cytochrome *c*.

The leakage of cytochrome *c* is thought to be a key event in the mitochondrial pathway of apoptosis because it leads to the assembly of a high-molecular-weight complex, the apoptosome. Cytochrome *c* binds APAF-1 (apoptotic protease activating factor) possessing three specific domains, N-terminal CARD, Ced-4 (domain of homology to the Ced-4 protein), and WD-40, which is important for protein–protein interactions. Nonactive APAF-1 is monomeric; its CARD domain is hidden by WD-40 repeats. Binding of cytochrome *c* induces a conformational change in the APAF-1 molecule followed by the release of CARD and Ced-4 functional fragments. The latter are necessary for the oligomerization of the protein, which in turn leads to the recruitment of procaspase 9 into an apoptosome complex and its autocatalytic activation (Fig. 4). Active caspase 9 is released from the apoptosome and triggers the cascade of effector caspases. It is noteworthy that a considerable part of the mature caspase 9 molecules usually remains in the apoptosome. Moreover, the enzyme in the complex is approximately 1000-fold more active than the free enzyme. This suggests the importance of APAF-1 as an allosteric regulator of caspase 9 (Adrain and Martin, 2001).

Several other proteins are released from mitochondria, and at least three of them take part in apoptosis, such as AIF (*apoptosis-inducing factor*), which was found to be mitochondrial oxireductase. AIF can be translocated to the nucleus and with the use of some additional molecular mediators can cause chromatin condensation and/or DNA fragmentation (Joza *et al.*, 2002).

Caspases are not the only proteases acting in apoptosis. It was observed that sometimes a cell exhibits all the attributes of apoptosis while caspases are not activated. In this case, cathepsin B may play a major role. This enzyme becomes essential when it is released from lysosomes due to damage provoked by apoptogenic stimuli, such as tumor necrosis factor or the anticancer drug etoposide (Jäättela, 2002). Cathepsin B with extremely high (although not as specific as that of caspases) proteolytic activity is able to destabilize cell functions through the destruction of vital protein and protein structures. The cathepsin-dependent pathway may be an independent proapoptotic signaling system, though it can work in conjunction with the caspase mechanism.

In the process of carcinogenesis, tumor cells develop protective systems, and, therefore, they differ in their ability to enter apoptosis from their normal counterparts. Among the most studied antiapoptotic factors is the

protooncogene Bcl-2. This protein has been discovered in B cell follicular lymphoma. In these cells, because of translocation, the gene coding for this protein is taken under the control of the enhancer of immunoglobulin heavy chain and its high activity leads to the elevated expression of Bcl-2 (Jäättelä, 1999). Bcl-2 was shown to prevent the release of cytochrome *c* from mitochondria by forming heterodimers with its close homolog, Bcl-$X_L$. This inhibition is thought to be due to the binding of the dimers to the potential-dependent ion channel, VDAC, which locks molecules of cytochrome *c* (Huang and Oliff, 2001). In addition, Ruiz-Vela and colleagues (2001) have shown that Bcl-$X_L$ can bind Apaf-1 and hinder the maturation of caspase 9.

Antiapoptotic molecules include heat shock proteins, particularly Hsp27 and Hsp70. Hsp70 has been shown to contribute to the protection of cells from factors inducing apoptosis, DNA damage, serum deprivation, and anticancer drugs. The suppression of Hsp70 in breast cancer cells with antisense RNA caused cell death via apoptosis even in the absence of anticancer drugs or other cytotoxic factors (Nylandsted *et al.*, 2000).

The mechanisms underlying the protective activity of Hsp70 include its chaperone activity. For instance, in cells subjected to heat stress, molecular aggregates were found to incorporate the RNA polymerase II complex TFIIH. The solubility of this enzyme and its activity were also strongly reduced (Dubois *et al.*, 1997). A study evidenced the formation of aggregates consisting of newly synthesized and mature polypeptides in cells affected by heat shock (Kampinga *et al.*, 1997). Cells were transfected with vectors carrying the Hsp70 gene controlled by constitutive or inducible (non-HSF-dependent) promoters as well as with the luciferase gene to follow its activity under conditions of stress. It was concluded that Hsp70 takes part in the reactivation of the enzyme after heat stress and the elevation of the intracellular Hsp70 level precisely correlates with the increase in cell protection (Michels *et al.*, 1997; Nollen *et al.*, 1999). In both studies, heat shock was employed as the damaging factor, and it is not clear whether the same Hsp70 chaperone-based mechanism functions in response to other cytotoxic agents. Today, based on findings accumulated in the past decade, we favor the hypothesis that Hsp70 directly interferes with the signaling pathways in apoptosis, although the chaperone-like nature of this intervention cannot be excluded (Mosser and Morimoto, 2004).

## D. Intervention of Hsp70 into Apoptotic Pathways

The antiapoptotic activity of Hsp70 or the link between the elevation of the level of Hsp70 in human leukemia cells and the reduction of their sensitivity to antitumor chemicals, camptothecin, Adriamycin, and etoposide, was

initially reported in the mid 1990s (Karlseder *et al.*, 1996; Samali and Cotter, 1996). It was also observed that Hsp70 does not always prevent apoptosis: upon the activation of T cell receptors or Fas/Apo-1/CD95, the overexpression of Hsp70 resulted in enhanced cell death in Jurkat (human T-lymphoma) cells (Liossis *et al.*, 1997). Of two cytosolic Hsp70 family members only the expression of the inducible Hsp70 component, mediated by HSF activation, was responsible for the inhibition of the apoptotic process (Buzzard *et al.*, 1998; Schett *et al.*, 1999).

The mechanism of antiapoptotic activity of Hsp70 was the subject of numerous studies, and to date most investigators believe that the activity is due to the ability of the chaperone to interact with molecules participating in the apoptotic signaling cascade at different levels. It was suggested that the main targets of Hsp70 are downstream of mitochondria. However, this was called in question by recent reports. Steel and colleagues (2004) failed to prove that Hsp70 is able to block apoptosis after the release of cytochrome *c*. Instead, they showed that Hsp70 acts upstream of mitochondria. Later these data were confirmed by three other groups. First, it was shown that Hsp70 can inhibit Fas-mediated apoptosis of type II CCRF-CEM cells, but not type I SW480 or CH1 cells (Clemons *et al.*, 2005). In CCRF-CEM cells, Hsp70 inhibited mitochondrial membrane depolarization and cytochrome *c* release; however, it did not change surface Fas expression or processing of caspase-8 and Bid, indicating that Hsp70's target is upstream of the mitochondria. A second group focused on the Bax protein, known to facilitate the release of cytochrome *c* from mitochondria. It was demonstrated that the overexpression of Hsp70 negatively regulated oligomerization and translocation of Bax to mitochondria (Stankiewicz *et al.*, 2005). Also evidenced was Hsp70-mediated inhibition of stress-induced induction of JNK protein kinase, which in turn resulted in the suppression of Bax transport to the mitochondrial membrane. The third group used vectors with the full-length *hsp70* gene as well as its deletion mutants lacking either chaperone (hsp70-EEVD) or ATPase functions (hsp70-ATPase). The overexpression of the full-size protein was found to inhibit Bax activation and reduce AIF (see Section IV.C) release from mitochondria initially caused by a metabolic inhibitor (Ruchalski *et al.*, 2006). In contrast, both mutants lacking either functional domain were unable to prevent AIF release. Although the deletion of EEVD did not inhibit Bax activation, this mutant was coimmunoprecipitated with AIF, whereas the mutant lacking an ATPase domain did not interact with AIF and failed to prevent its intranuclear transport. It was concluded that to elicit the protective activity of Hsp70 the domains responsible for both chaperone and ATPase activities are needed (Ruchalski *et al.*, 2006). In connection with postmitochondrial signaling it is worth mentioning that AIF can be bound by the full-length Hsp70 molecule, and this interaction may be beneficial when multiple targets of Hsp70 are specified (Cande *et al.*, 2002).

At the beginning of this section we cited the recent work of Steel and co-workers who revised two earlier reports on the interaction of Hsp70 with Apaf-1. According to these data, Hsp70 is able to bind to the CARD domain of Apaf-1 as well as prevent the oligomerization of the latter and the consequent activation of caspase 9 (Saleh *et al.*, 2000). Although data from other groups indicate that the oligomerization takes place in the presence of Hsp70, no binding of procaspase 9 to the newly formed complex was found because the CARD domain was already occupied by Hsp70 molecules (Fig. 4) (Beere *et al.*, 2000). Despite some criticism, both reports have shown the possibility of a transient Hsp70–Apaf-1 complex. Most likely Hsp70 acts before and after mitochondria-mediated apoptosis signaling.

It was suggested that Hsp70 might influence the activation of caspases 3 and 7, since their truncated forms can attract the chaperone. We found that the delay in the proteolysis of these enzymes in U-937 leukemia cells subjected to anticancer drugs was associated not only with the inhibition of caspase 9 activity, but also with the trapping of both enzymes by Hsp70. It seems probable that the binding of the chaperone to caspases occurs within the period when their conformation was changing at the first step of proteolytic modification in apoptotic signaling (Komarova *et al.*, 2004).

In addition, Hsp70 was found to prevent the process of cell death even further downstream of caspase 3 and 7 activation. These caspases were shown to cleave enzymes responsible for the formation of typical apoptotic morphology, including phospholipase $A_2$. In certain cell lines the activation of phospholipase $A_2$ is necessary for the last phase of apoptosis. This is true for mouse fibrosarcoma WEHI-S cells, in which Hsp70 was reported to inhibit phospholipase $A_2$ activation and to rescue these cells from tumor necrosis factor-mediated apoptosis. Although the mechanism of that inhibition remains unclear, it can be concluded that Hsp70 is also able to act at the last stage of cell programmed death (Jäättelä, 1993).

Several other molecular targets for Hsp70 are also implicated in apoptosis signaling. First, Hsp70 actively suppresses signal transduction through the cascade of stress-kinases. These proteins, mainly SAPK and JNK, take part in apoptosis and in the initiation of cell proliferation. Mosser and others (1997) showed that Hsp70 prevents JNK activation, and, more recently, Hsp70 was shown to directly interact with JNK and to suppress its binding to the substrate, c-Jun (Mosser *et al.*, 2000). These functions were based on the chaperone activity of Hsp70, which is supported by experiments with its deletion mutants.

In Section IV.C, cathepsin B-based signaling was regarded as an alternative to the "mainstream" apoptotic machinery. This pathway of apoptosis associated with the destruction of lysosomal membranes and the release of cathepsin B may be realized in cells in which Hsp70 is depleted by the use of Hsp70-specific antisense RNA or siRNA. Conversely, the overexpression

of Hsp70 was found to strengthen these membranes and, correspondingly, to reduce the level of cytosolic cathepsin B and the rate of apoptosis in general (Nylandsted *et al.*, 2004).

It was also shown that proteins of the NF-κB complex of transcriptional regulators are targeted by Hsp70. These proteins are known to perform numerous functions in cells by controlling genes possessing a kappa sequence in their promoter regions. These genes, in turn, are able to regulate cell proliferation and apoptosis. The initial steps of NF-κB-dependent transcriptional activation occur in the cytosol. Hsp70, especially when accumulated at a high level, was shown to bind p50, p65, and c-Rel, preventing their translocation into the nucleus (Guzhova and Margulis, 2000; Guzhova *et al.*, 1997).

Overall, the process of apoptosis, including the one that is induced by anticancer drugs, is an extremely complicated tangle of various pro- and antiapoptotic factors. Jäättelä (2002) said that cancer cells have many opportunities to "die decently." Hsp70 can inhibit the process of apoptosis at many points, from creating obstacles to mitochondrial membrane destruction to inhibiting enzymes that are important for the last stages of the process. In all of these actions, Hsp70 employs its chaperone capacity, which is to bind various signaling molecules when they turn into active forms and transiently expose their hydrophobic domains, which are usually hidden. Hsp70 constitutes one of the most efficient systems engaged in the protection of cancer cells from a variety of antitumor drugs. The elevated level of Hsp70 renders resistance to cancer cells. Conversely, some antitumor chemicals or substances with antiapoptotic activity, such as bleomycin, quercetin, and staurosporine, were convincingly shown to reduce the expression of Hsp70.

## E. Hsp70 Expression on the Surface of Tumor Cells

In most cases, tumor cells cannot be recognized by an organism as alien to the body. Inflammatory sites are rarely found in a tumor area before the beginning of massive necrosis, even if the tumor becomes large. However, it became obvious that the immune system may start recognizing cancer cells expressing elevated amounts of Hsp70, and this may be used to benefit a patient.

The pioneering work in this field was initiated by P. Srivastava. In the early 1990s in a series of elegant experiments he showed that Hsp70 could increase the immunogenicity of tumors. He isolated Hsp70 from MethA mouse sarcoma employing several chromatographic procedures without affinity chromatography on ATP-agarose. Electrophoretic analysis of the Hsp70 final preparation showed that it contained no other protein contaminations. This preparation was used to immunize mice, which was followed by the injection of MethA tumor cells. As a result, tumors were not grafted or

regressed soon after the inoculation. The immunization was found to be ineffective against two unrelated sarcomas, CM4 and CM5. Preparations of Hsp70 obtained with the use of affinity chromatography on ATP-agarose did not have any immunogenic effects. Based on these experiments, it was concluded that Hsp70, isolated by the "mild" procedure, is able to capture low-molecular-weight peptides, undetectable by conventional protein analysis, which are able to elicit immunogenic properties (Blachere *et al.*, 1993). It is likely that these peptides acquired antigenic activity only when they were associated with Hsp70 chaperone. In other words, their conformation was modulated by Hsp70 in such a way that they become targets for antigen-presenting cells. Each tumor seems to have its own specific antigenic pattern that may be enhanced by an association with Hsp70.

Additional evidence about tumor antigen presentation by Hsp70 came from experiments of Dr. R. Vile and colleagues. B16 melanoma cells were transfected with the Hsp70 gene and were then inoculated into C57BL mice. Nontransfected cells were injected into another group of animals. Tumors started to grow in both groups of mice. Incredibly, tumors formed by Hsp70-transfected B16 cells were infiltrated by T-lymphocytes, macrophages, and dendritic cells. After the tumors were formed, they were removed and the same animals were inoculated again with nontransfected B16 cells. It was found that new tumors grew only in mice originally primed with nontransfected cells (Melcher *et al.*, 1999).

In the mid 1990s, several reports indicated that Hsp70 could be found on the surface of malignant cells, which was not observed in normal cells (Multhoff *et al.*, 1995). The membrane-associated Hsp70 was found to be a target for natural killers, as was demonstrated for cancer cell cultures and for tumor biopsies from patients (Hantschel *et al.*, 2000). The cells from nearly 75% of head and neck malignancies (Kleinjung *et al.*, 2003) and from 66% of aspirates from the bone marrow of patients with acute myeloid leukemia (Gehrmann *et al.*, 2003) carried Hsp70 on their surface.

The mechanism by which Hsp70 reaches its immune cell-activating effect remains unclear despite a great number of studies on this topic. Hsp70 was located at the plasma membrane of cancer cells, so that its 14-mer peptide TKD (TKDNNLLGRFELSG, aa 450–463) protrudes through the outer surface. This particular peptide was found to activate the cytolytic activity of NK cells, $CD3^{-}CD56^{+}CD94^{+}$. Additionally, TKD was shown to promote chemotaxis of NK cells (Gastpar *et al.*, 2004).

Recent data indicate that Hsp70 or its TKD peptide cannot induce cytotoxic activation of NK cells on its own. However, they do so in many cancer cells in a complex with peptidoglycan recognition protein Tag7. The complex of Tag7 with Hsp70 is relatively stable and presumably released from $CD3^{+}CD8^{+}$ lymhokine-activated killers (Sashchenko *et al.*, 2004). Another possible location for this complex formation is the surface of tumor cells.

Interestingly, surface Hsp70 and not its cognate counterpart was associated with tumor-specific immunity in cancer cells (Ménoret *et al.*, 1995).

In accordance with another hypothesis, the immunomodulatory function of Hsp70 may be related to the chaperone activity that it exerts when it appears at the surface of a tumor cell. In this model Hsp70 exposes tumor-specific peptides to antigen-presenting cells, and the resulting immune response must be directed not against the chaperone itself but against these peptides (Blachere *et al.*, 1993; Pierce, 1994).

Based on the proposed mechanisms there were attempts to create an anticancer vaccine (Fig. 5). They are briefly summarized as follows. Tumor excised from a level II or III oncological patient is used for the isolation of Hsp70 according to a special procedure, which is protected by numerous patents. The final preparation containing, in addition to Hsp70, a peptide library typical of each individual is used to immunize the same patient. According to the data from companies focused on this type of treatment, particularly Antigenics (USA), remission is observed in 70–80% of patients, which is an extremely good result in oncology. In addition to Hsp70, glucose-regulated protein GP96 (TRA, tumor rejection antigen) was also shown to possess extremely high anticancer activity.

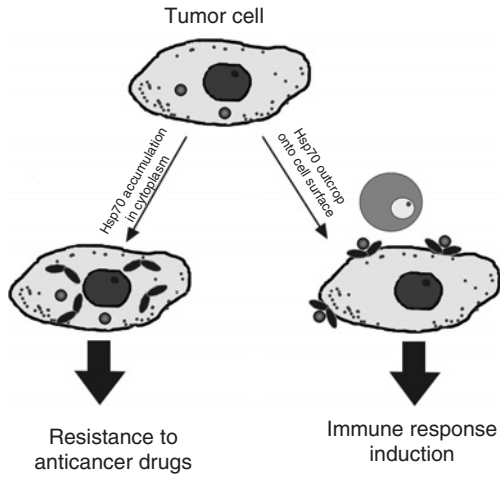


FIG. 5 Destiny of a cancer cell. Many cancer cells were found to express elevated amounts of Hsp70 even under normal conditions and therefore acquire resistance to antitumor therapy. However, overexpression of the protein leads to its appearance on the plasma membrane in a complex with tumor antigens. This phenomenon can be used in anticancer therapy if necrosis can be induced in a tumor with the help of an extreme stressor (strong hyperthermia, for instance) or Hsp70 can be isolated with bound antigen. The immunization with these concentrated peptides presented by the chaperone should induce anticancer immunity in the patient organism.

In conclusion, Hsp70 protects tumor cells from stress factors and from agents killing tumors through the process of apoptosis. This makes anticancer therapy ineffective. However, elevated expression of Hsp70 on the surface of cancer cells can induce an antitumor response in the organism. At later stages of cancer development, the immune system of a patient cannot resist either tumor growth or ordinary infections. Therefore, we suggest using the immunomodulatory property of Hsp70 at earlier stages of cancer when it is most effective.

## V. Function of Cellular Chaperones in Neurodegenerative Diseases

Ever increasing numbers of harmful natural and anthropogenic factors lead to the emergence of diseases that were not previously known. This demands new medicines based on an extensive knowledge of the molecular and cellular basis of a particular pathology. Proteotoxic, or chaperone, diseases are found among the most actively explored in the past 10 years. The term "proteotoxic" defines the nature of these pathologies: they stem from the accumulation of insoluble aggregates made of mutant and/or misfolded proteins inside a cell or in close proximity to the plasma membrane (Burel *et al.*, 1992). These aggregates render cells susceptible to factors that in normal cell life do not exert any toxicity or change cellular physiology. These factors often stimulate the process of apoptosis in affected cells.

As mentioned earlier, a great variety of factors target the structure of cellular polypeptides. First, treatment of a cell with a denaturing agent may often cause the formation of insoluble aggregates, which leads to irremediable damage of a cell. This effect can be associated with the malfunction of protein-synthesizing or protein-modification systems or with a direct detrimental effect of a denaturing factor. Aggregates made of denatured proteins were observed in reticulocytes treated with amino acid analogs (Klemes *et al.*, 1981) or with mercury salts (Asakura *et al.*, 1977). In addition, mutations in a certain gene can strongly affect the conformation of the protein product and may give rise to hereditary pathologies such as Alzheimer's, Parkinson's, and Huntington's disease. Diseases of the third group are caused by the aggregation of seemingly functional proteins such as amyloid and amylin. Irrespective of the origin of the pathology, initial damage of protein structure can lead to loss of function due to a structural defect and the formation of aggregates. The latter happens when a protein tends to accumulate in great amounts and if cellular protective systems cannot dissasemble growing protein complexes.

Unique features of each proteotoxic disease are related to the type of aggregate, the speed with which aggregates are formed, the type of cell hosting the complexes, and to whether aggregates are located inside or outside a cell. For instance, amyloid aggregates, typical for amyloidosis, are accumulated in blood within months. Such complexes disturb the architectonics of adjacent tissues and exert a strong cytotoxic effect on them. The nature of Alzheimer's disease (AD), despite it being extensively studied, has not yet been definitely determined. The key pathogen in AD is the product of amyloid cleaved by β-secretase, Aβ-amyloid. This peptide can aggregate, thus forming oligomers, which in turn are able to adsorb more monomers and form growing insoluble amorphous deposits outside cells (Muchowski and Wacker, 2005). Small diffusable Aβ oligomers and soluble protofibrils composed of oligomer strings are found to be major cytotoxic species in AD and other neurodegenerative diseases (Caughey and Lansbury, 2003). Aβ oligomers localize to multivesicular bodies, which are components of the endocytic-lysosomal pathway. The ability of Aβ to impair primary neurons is thought to be based on the induction of the leakage of lysosomal enzymes into the cytoplasm (Smith *et al.*, 2005). The major known consequence of this cytotoxic effect is massive neuronal death resulting in progressive dementia.

Parkinson's disease (PD) is the second most common neurodegenerative pathology. It is characterized by resting tremor, postural rigidity, and the progressive degeneration of dopaminergic neurons in the substantia nigra. At the molecular level the characteristic feature of PD is Lewy bodies consisting of ubiquitinated proteinaceous inclusions. In addition, two missense mutations in the gene coding α-synuclein are linked to dominantly inherited PD (Polymeropoulos *et al.*, 1997).

One particular group of neurodegenerative diseases includes 10 so-called polyCAG (polyglutamine, polyQ) and several polyalanine pathologies, which originate from the expanded tracts of Glu or Ala residues in various proteins. For instance, Huntington's disease (HD) is caused by the huntingtin gene with glutamine repeats that exceed 35 residues in exon 1 (Squitieri *et al.*, 2001). Oculopharyngeal muscular dystrophy is associated with the expansion of alanines in a polyadenine-binding protein 2 gene exceeding 12 residues (Brais *et al.*, 1998).

The process of the formation of aggregates appears to be independent of the type of disease-related abnormal protein and starts by the formation of β-sheet structures followed by their association using hydrogen bonds in amyloid-like fibrils. The degree of similarity between structures of different pathogenic aggregates is so high that the oligomers of amyloidogenic peptides from Alzheimer's, Parkinson's, polyglutamine, and prion diseases share a common structural feature that can be recognized by a specific antibody raised to the oligomeric form of the Aβ peptide (Kayed *et al.*, 2003). The genesis of cytosolic inclusions is a multistage process that includes nucleation,

i.e., the formation of protofibrils on the cell periphery, which can be further transported toward centrosomes in a microtubule-mediated mode. During this process, inclusion bodies recruit heat shock proteins, ubiquitin-conjugating enzymes, and 26S proteasomes. These assembled structures are called aggresomes. The distinct feature of polyglutamine expansion-dependent diseases is that the aggregates can be strengthened by cross-linking. According to this model, polyglutamine tracts are excised from the corresponding protein by caspases (Tarlac and Storey, 2003) and then are covalently linked to amino groups of a proper lysyl donor. One of the lysyl donors was shown to be glyceraldehyde phosphate dehydrogenase. This process is catalyzed by tissue transglutaminase (Orru *et al.*, 2002).

There is much speculation about the possible mechanisms of aggregate cytotoxicity, and the most established points are as follows. First, the formation of aggresomes in some cases demands the activation of caspases and this may lead to a noncontrollable stimulation of apoptosis signaling. Second, aggregates are known to sequester various cellular proteins necessary for normal cell life, such as proteasomes. This causes the abrogation of their routine proteolytic function. Other proteins, whose activity can be strongly affected by aggresomes, are TATA-box-binding protein (TBP) and cAMP-regulated element-binding proteins (CREB1 and CREM). The former can be sequestered by aggresomes, and the activity of the latter two is inhibited by the formation of inclusion bodies (Sakahira *et al.*, 2002). Suprisingly, sometimes aggresomes may play a positive role in the process of neurodegeneration: the overexpression of α-synuclein and synphilin-1 causing the formation of aggresomes was found to protect transfected cells from apoptosis induced by proteasome inhibitors (Tanaka *et al.*, 2004). It is commonly thought that aggresomes made of abnormal proteins serve to preserve the cytoplasmic ambiance from the influence of cytotoxic oligomers.

To cope with aggregates, cells use several protective systems. For example, small aggregates can be transported with the aid of autophagocytic vesicles to lysosomes for degradation (Fortun *et al.*, 2003). A certain portion of aggregate-prone proteins is eliminated by proteasomes. However, this pathway does not function properly in cells that are already filled by aggregates. Evidence has been obtained indicating that proteasomes are inhibited in such cells (Sherman and Goldberg, 2001). The only system that continues to struggle with protein aggregates is the chaperone mechanism. Its function in cells affected by aggregates is also influenced by several opposing factors.

## A. Expression of Chaperones in Aging

Mutations in potentially pathogenic genes start at birth. However, the first manifestations of an illness can be observed much later. The majority of

proteotoxic diseases affect patients at a rather late age, when disturbances in the function of chaperone systems can be significant. Large amounts of data published within the past few years indicate that aging is accompanied by the failure of cells to express heat shock proteins in response to stress (Soti and Csermely, 2002). Zhang and colleagues (2005) showed that after heat stress liver Hsp70 mRNA and protein levels are reduced in old rats compared to young ones. In addition, it was found that physical exercise led to an increase in Hsp70 levels in muscle, liver, and heart of 6-month-old rats, and had no effect in 24-month-old rats. In the old rats, only a modest Hsp70 induction in muscle was observed (Starnes *et al.*, 2005). The experimental stroke has more severe consequences in old rats: more oxidative DNA and protein damage accompanied by reduced Hsp70 expression in per-infarct cortex (Li *et al.*, 2005). Hsp70 synthesis in lymphocytes of people over 70 years old was 66% lower than in individuals 20–40 years old (Jurivich *et al.*, 2005). However, in experiments involving *Caenorhabditis elegans* and *Drosophila melanogaster*, it was shown that those with high levels of Hsp70 and Hsp22 expression have longer life spans (Morley and Morimoto, 2004). Finally, recent data have indicated that centenarians have the same level of Hsp70 expression in B-lymphocytes as young people (Ambra *et al.*, 2004), and the maintenance of physical activity in aging can preserve the ability to induce Hsp70 in response to physiological stress (Simar *et al.*, 2004). In conclusion, Hsp70 expression regulated by HSFs may be reduced in aged persons, while enhanced levels of protein can correlate with increased longevity of life. Therefore, all increases in Hsp70 content may protect humans, especially older ones, from the deleterious effects of proteotoxic pathogens.

Besides the diminished expression of chaperones in aged persons, there are differences in the levels of these proteins in tissues. As we know, cancer cells usually express higher levels of Hsp70, which makes them resistant to antitumor therapy. However, there are tissues, e.g., special types of neurons, in which Hsp70 expression is low (Sprang and Brown, 1987). Moreover, retinal neurons (Barbe *et al.*, 1988), cerebellum (Clark and Brown, 1985), and hippocampus (Marini *et al.*, 1990), among others, do not synthesize Hsp70 in response to stress. Similar results were obtained when the expression of Hsp70 was explored in cell lines of neuronal origin: human retinoblastoma Y79 (Mathur *et al.*, 1994) and mouse neuroblastoma N18TG2 (Satoh and Kim, 1995) cells were unable to increase the synthesis of Hsp70 in response to heat stress. In our experiments the induction of the differentiation of human neuroblastoma LA-N-5 cells resulted in a strong reduction of Hsp70 content that was correlated with the elevation of cell sensitivity to heat stress, free radicals ($H_2O_2$), and the inducer of apoptosis, staurosporine (Guzhova *et al.*, 2001).

In stress conditions, axons can receive Hsp70 from adjacent glial cells, which are located much closer to the axon than the body of a neuron and the

axoplasm cannot synthesize proteins autonomously (Sheller and Bittner, 1992; Tytell *et al.*, 1986). We have recently presented evidence concerning such a transfer based on experiments with human neuroblastoma and glioblastoma cells in culture (Guzhova *et al.*, 2001). It seems important that glial cells can respond to stress with rapid synthesis of heat shock proteins (Houenou *et al.*, 1996). It is possible that in aged individuals, when symptoms of neurodegenerative disorders are already evident in neurons, both glial cells and neurons are not able to produce Hsp70 and/or the mechanism of the transmission of the protein between cells becomes inefficient.

## B. Up-Regulation of Chaperones Is a Powerful Tool against Cytotoxic Protein Aggregates

As stated, molecular chaperones, particularly belonging to the Hsp70 class, protect cells from a variety of cytotoxic factors including those causing pathogenic processes in human brain. Initially, such conclusions were based on *in vitro* experiments in which cells were transfected with a gene coding certain pathogenic polypeptides. The formation of aggregates and cell viability can be assayed in the following typical experiment. Twenty-four hours after transfection with a construct consisting of a mutant protein gene, e.g., exon 1 of huntingtin (103 glutamines) fused with green fluorescent protein, neuroblastoma cells accumulated the protein as diffuse material located throughout the cytosol. Within the next 12 hours mutant proteins assemble in small insoluble aggregates. Forty-eight hours after transfection large aggregates can be observed, and by this time, nearly 70% of the cell population is dying with features typical of apoptosis (Fig. 6).

In similar experimental settings Hsp70, Hdj1/Hsp40, and Hdj2 were found to be sequestered by growing aggregates of polyglutamine chains (Jana *et al.*, 2000; Stenoien *et al.*, 1999; Suhr *et al.*, 2001). In the cellular model of Huntington disease, the overexpression of both Hsc70 and Hdj1 caused the suppression of aggregate formation. Interestingly, J-domain alone or ATPase domain deletion of Hsc70 was almost as potent as full-length proteins in the suppression of aggregate formation (Jana *et al.*, 2000). Similar data were obtained by Bailey and colleagues in the model of spinal and bulbar muscular atrophy, which is linked to the expanded polyglutamine chain in the androgen receptor. In addition to the protective effect of the overexpression of pairs Hsp70-Hdj1 and Hsp70-Hdj2, transfection with these genes not only increased the solubility of the mutant receptor but also enhanced the function of the proteasome machinery (Bailey *et al.*, 2002). In another study, Zhou and colleagues (2001) showed that Hsp70 or Hdj1/Hsp40, expressed in cells transfected with mutant huntingtin gene, effectively inhibited cytotoxicity caused by aggresomes. Both chaperones suppressed

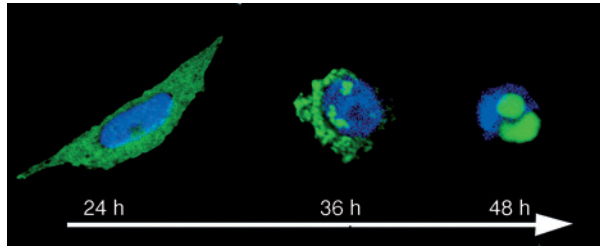


FIG. 6 Formation of aggresomes in a cell model of Huntington disease. In studies on neural pathologies it is common to use *in vitro* models containing cells transfected with a disease protein. SK-N-SH human neuroblastoma cells 24 hours after transfection with 103 CAG (coding glutamine) repeats fused with green fluorescent protein contain luminous material throughout the cytosol that 12 hours later is converted in small aggregates. The latter grow in large aggregates 48 hours after transfection, and at this time more than 70% of the cell population is committed to apoptosis.

aggresome formation and enhanced the activity of proteasomes. A similar effect of the suppression of polyglutamine tract aggregation was found for Hdj1 in spinocerebellar ataxia. Ataxin-3, the disease protein in spinocerebellar ataxia type 3/Machado-Joseph disease, was expressed and resulted in aggregate formation. Hdj1, but not Hdj2, was shown to protect transfected cells against aggresome-mediated toxicity (Chai *et al.*, 1999). In all of these studies, cell models of polyglutamine diseases were employed. The question arises whether the antiaggregate activity of chaperones can be observed in a whole brain. The results of experiments on transgenic *Drosophila* flies expressing ataxin-3 with a 78-polyglutamine tract showed that Hsp70 can fully restore the structure of the external eye. The structure of the internal eye was also restored. In the same study, similar to Hsp70, Hdj1 (but not Hdj2) was able to recover eye structure. There was no reduction of aggregate size or amount in eyes of flies overexpressing Hsp70 and Hdj1. However, the solubility of ataxin-1 was much higher than in nonexpressing chaperones flies, which was linked to the less degenerated neural tissue (Bonini, 2002; Chan *et al.*, 2000). In one of the studies, a transgenic mouse expressing androgen receptor with a 97-glutamine chain (pathogen of spinal bulbar muscular atrophy, SBMA) was cross-bred with a mouse expressing human Hsp70. The hybrid was found to possess much higher motor activity than that carried only by the gene responsible for the disease. At the cellular level, the number of nuclear inclusion bodies was significantly reduced in double-transgenic mice (Adachi *et al.*, 2003). The reduction of the monomeric form of the mutant androgen receptor was also observed, suggesting that chaperone expression enhanced proteolytic degradation of the mutant protein. Recently published data confirm the principal role of the Hsp70 chaperone in the amelioration of the phenotype of polyglutamine-dependent diseases.

Its reduction in mice expressing mutant huntingtin caused the progression of the disease, whereas the pharmacological induction of chaperones with the use of radicicol or geldanamycin increased the solubility of the aggregates (Hay *et al.*, 2004).

*In vitro* and *in vivo* models of Parkinson's disease were also employed to prove the protective function of chaperones in this disorder. One study using *Drosophila* overexpressing α-synuclein demonstrated that Hsp70 was effective in the protection of dopaminergic cells against aggregate cytotoxicity. However, the number and morphology of α-synuclein inclusions in the transgenic *Drosophila* brain did not change. It is important that the expression of Hsc4, a *Drosophila* analog of Hsp70 bearing an amino acid substitution in the ATPase domain and, therefore, inactive, was found to accelerate neuronal loss (Auluck *et al.*, 2002). Another study, using both *in vitro* and *in vivo* PD models, showed that Hsp70 certainly protects brain cells of α-synuclein transgenic mice and human neuroglioma H4 cells against disease protein toxicity. However, contrary to data of Auluck and co-authors, evidence indicated that Hsp70 was able to reduce the amount of misfolded/aggregated α-synuclein both *in vivo* and *in vitro*, suggesting that the chaperone function is crucial for the protection of aggregate-mediated cytotoxicity (Klucken *et al.*, 2004).

Although the data on a positive chaperone role in proteotoxic diseases are convincing, one of the major parts of the puzzle is missing: the mechanism used by chaperones to achieve the protective effect. In this regard, a long list of possibilities can be presented and some of them have been discussed in recent reviews (Meriin and Sherman, 2005; Muchowski and Wacker, 2005). First, since chaperones were found to form dynamic complexes with aggresomes (Kim *et al.*, 2002), they may sequester these from the cytosol and, thus, reduce the cytotoxicity associated with mutant proteins. Second, Hsp70, either alone or in tandem with Hdj1, may prevent the formation of large aggresomes by taking away smaller aggregates or monomers early in the process. Third, Hsp70 is known to bind specific proteins participating in the transduction of the apoptosis signal. It is possible that the chaperone provides efficient protection in part by downregulating stress-inducible kinases, which are known to be active in PD and polyglutamine pathologies (Meriin and Sherman, 2005).

Experiments in gene transfection have provided reliable data regarding the protective role of chaperones in neurodegenerative diseases. However, the use of gene therapy in the treatment of such pathologies currently is not feasible. In this regard, the use of preparations of purified Hsp70 as a potential molecular medicine looks more promising. As mentioned earlier, the story of "exogenous" Hsp70 started in the 1980s with the pioneering work of Tytell *et al.* (1986), who found that Hsp70 was able to migrate from the glia to the giant axon of the squid. Later the effect of Hsp70 transition

from glia to neuron was proved using shrimp neural tissue (Sheller *et al.*, 1998). These data prompted us to explore the possibility of such a transition and its consequences on cultures of human glioblastoma T98 and neuroblastoma LA-N-5 cells. First, we found that Hsp70 can be exported by T98 cells and can be internalized by neuroblastoma cells. Second, and more important, we have shown that LA-N-5 cells, which acquired Hsp70 from the extracellular matrix, also received this protection, e.g., resistance to several cytotoxic factors, including thermal shock, hydrogen peroxide, and staurosporine (Guzhova *et al.*, 2001). Kelty and colleagues (2002) reported that along with moderate heat shock treatment, which induced Hsp70 accumulation, purified Hsp70 rendered neurons in surviving slices of brain resistant to the stress-induced release of neurotransmitters. In other words, both treatments were able to preserve the normal physiological function of neurons. We have also employed this methodology in studies regarding pathologies associated with cytoxic polyglutamine aggregates. In these experiments, Hsp70 isolated from bovine muscle was added to the culture of neuroblastoma SK-N-SY cells expressing either 25- or 103-glutamine chain proteins (25Q and 103Q, respectively). Transfection with 103Q caused the accumulation of aggresomes and progressive apoptosis in up to 80% of the cell population. As in the case of other cell cultures, extracellular Hsp70 was shown to accumulate inside of cells and to protect them from apoptosis (Novoselova *et al.*, 2005). In an attempt to uncover the target for the Hsp70-elicited protective effect we analyzed the size and the amount of aggresomes in cells expressing Q25 and Q103 and found that the above parameters were lower when Hsp70 was added simultaneously with or soon after transfection compared to cells whose treatment with Hsp70 started several hours later. We have concluded that the chaperone may act at earlier stages of aggregate formation probably preserving small oligomers from further "sticking" to each other. In any case, it is clear that exogenously administered Hsp70 can be a potent treatment capable of protecting cells or tissues from a variety of toxic agents.

## VI. Conclusions

Hsp70 as a chaperone is able to bind to a great number of polypeptides in cells affected by various stimuli. The interacting polypeptides include newborn proteins, mutant or denatured proteins, as well as mature cellular proteins that constantly change their conformation. A variety of pathogenic factors may cause protein denaturation. Among these are hypoxia, oxidative stress (all types of ischemia, diabetes, and neural pathologies), an increase in temperature (fever), the deregulation of protein-synthesizing and protein-modifying functions in aging, and certain mutations in genes (proteotoxic

diseases). As a result, proteins change their conformation, and hydrophobic domains, which are usually hidden inside the molecule, are being exposed on its surface. The exposure of hydrophobic domains almost inevitably leads to the aggregation of proteins that can result in the formation of insoluble protein aggregates, which is able to change the physiology of a cell completely. One of the key functions of Hsp70 class chaperones is the prevention of protein aggregation and the maintenance of the native conformation of cellular polypeptides. Cochaperones Hdj1, Bag-1, and CHIP help Hsp70 in this function. Together they perform many bind–release cycles aimed at correcting the conformation of target polypeptides. If the latter are irreparable, chaperones can bring them to proteasomes.

It is well established that the activation of chaperones is necessary to prevent aggregation of mutant proteins or their fragments. Cotransfection of the extended polyglutamine sequences along with Hsp70 or Hsp70 plus Hdj1 genes results in a reduction in apoptosis, which directly corresponds to the smaller number of cells forming aggregates (Wyttenbach *et al.*, 2002; Zhou *et al.*, 2001). Both chaperones were colocalized with mutant huntingtin in a dynamic manner, which suggests that chaperones are trying to pinch out the aggregate subunit or to separate the whole aggresome from the cytosol (Kim *et al.*, 2002). In our experiments, the ability of Hsp70 to penetrate through the plasma membrane was used instead of transfection with the gene. Since exogenously administered chaperone was found to interact with polyQ aggregates and prevent their formation, Hsp70 or preparations based on it may be a potential tool in the therapy of neurodegenerative pathologies (Novoselova *et al.*, 2005).

Hydrophobic domains can be found not only on the surface of denatured or mutant proteins, but also in normal proteins undergoing changes of conformation, e.g., due to phosphorylation or dephosphorylation. Interactions between Hsp70 and signaling molecules occur at various stages of apoptosis—upstream and downstream of mitochondria. It is remarkable that Hsp70 can act simultaneously at many different points of apoptotic signaling: from preventing the release of Bax (Ruchalski *et al.*, 2006) to the inhibition of caspase-activated DNase (Liu *et al.*, 2003). If the mitochondrial chain of signal transduction is broken because of Hsp70 binding to the CARD domain and the inhibition of the oligomerization of APAF-1 (Beere *et al.*, 2000, Saleh *et al.*, 2000), the process of apoptosis can proceed through the mechanism involving caspase-8-provoked activation of downstream effector caspases. However, Hsp70 also intervenes in this process by direct inhibition of these caspases (Komarova *et al.*, 2004). When the caspase cascade is fully switched off, Hsp70 can inhibit the caspase-independent death effector, AIF, which is being released from mitochondria (Cande *et al.*, 2002). That is why Hsp70 overexpression allows tumor cells to efficiently deal with any proapoptotic signals including the action of anticancer drugs.

Considering the data presented, it is possible to conclude that the overexpression of Hsp70 in cancer cells makes antitumor chemotherapy inefficient. However, during hyperthermia, the level of Hsp70 is increased in the cytoplasm and the amount of cells with surface expression of Hsp70 is also higher (Botzler *et al.*, 1996). It is difficult to increase locally the temperature of a tumor. However, new technology employing magnetic cationic liposomes, which can change the temperature inside a tumor, could help in anticancer therapy (Ito *et al.*, 2003). The effect was shown to be even stronger when purified Hsp70 was used. Approximately 20% of experimental tumors were totally regressed and others also showed growth suppression due to the activation of the immune response using a mechanism similar to that described in Section IV.E (Ito *et al.*, 2003).

It is clear that there are situations in which the enhanced content of Hsp70 is bad. Very often this happens in tumor cells. However, it can be turned into an advantage if the elevated Hsp70 level causes the appearance of tumor cells on the cell surface. In this case, tumor cells can be recognized and then killed by immune cells. Brain cells, most of which do not effectively express Hsp70, represent a completely different situation. In this case, the elevation in the amount of chaperone is likely to rescue neurons from the deleterious effects of protein aggregates. It is possible to elevate Hsp70 content in living cells by the administration of the pure protein. It is also important that new chemical inducers of Hsp70 synthesis and accumulation became available in the past few years. Among those recently discovered are geldanamycin, the inhibitor of Hsp90 ATPase (Shen *et al.*, 2005), and celastrol, the active part of Chinese herbal medicine (Westerheide *et al.*, 2004). Hopefully, the preparations based on Hsp70 or those that specifically induce the expression of this chaperone will find their way to the shelves of pharmacies in the near future.

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# Cell Biology of Mitochondrial Dynamics

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Mitochondria are the product of an ancient endosymbiotic event between an $\alpha$-proteobacterium and an archael host. An early barrier to overcome in this relationship was the control of the bacterium's proliferation within the host. Undoubtedly, the bacterium (or protomitochondrion) would have used its own cell division apparatus to divide at first and, today a remnant of this system remains in some "ancient" and diverse eukaryotes such as algae and amoebae, the most conserved and widespread of all bacterial division proteins, FtsZ. In many of the eukaryotes that still use FtsZ to constrict the mitochondria from the inside, the mitochondria still resemble bacteria in shape and size. Eukaryotes, however, have a mitochondrial morphology that is often highly fluid, and in their tubular networks of mitochondria, division is clearly complemented by mitochondrial fusion. FtsZ is no longer used by these complex eukaryotes, and may have been replaced by other proteins better suited to sustaining complex mitochondrial networks. Although proteins that divide mitochondria from the inside are just beginning to be characterized in higher eukaryotes, many division proteins are known to act on the outside of the organelle. The most widespread of these are the dynamin-like proteins, which appear to have been recruited very early in the evolution of mitochondria. The essential nature of mitochondria dictates that their loss is intolerable to human cells, and that mutations disrupting mitochondrial division are more likely to be fatal than result in disease. To date, only one disease (Charcot-Marie-Tooth disease 2A) has been mapped to a gene that is required for mitochondrial division, whereas two other diseases can be attributed to mutations in mitochondrial fusion genes. Apart from playing a role in regulating the morphology, which might be important for efficient ATP production, research has indicated that the mitochondrial division and fusion proteins can also be important

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during apoptosis; mitochondrial fragmentation is an early triggering (and under many stimuli, essential) step in the pathway to cell suicide.

**KEY WORDS:** Mitochondrial division, Mitochondrial fusion, Endosymbiosis, Apoptosis, FtsZ, Dnm1, Fzo1. 

## I. Introduction

Mitochondria are double membrane-bounded organelles that reside inside most eukaryotic cells. They are sites of oxidative energy metabolism (Kennedy and Lehninger, 1949) and are therefore essential for most eukaryote life. Mitochondria are derived from a bacterial endosymbiont that was taken up by an archaeal host nearly two billion years ago (Gray *et al.*, 1999; Martin *et al.*, 2001). Following endosymbiosis, and metabolic integration between the bacterium and the host, one of the most important events would have been controlling the reproduction of the symbiont by the host cell. As will be discussed in this review, it is likely that the original endosymbiont's division proteins were of bacterial origin, and at least one such protein still acts in this capacity alongside eukaryotic proteins. In multicellular eukaryotes, which perform extensive mitochondrial fusion, bacterial division molecules have been lost or replaced with different molecules derived from the host.

Mitochondria were first recognized as an elementary part of life at the end of the nineteenth century. Altmann (1890) found that the structures he termed "bioblasts" could be stained by fuchsin, and it was thus possible to demonstrate their existence in nearly all cell types. By 1914, it was recognized that mitochondria could take varying forms, including rods, threads, and networks (Lewis and Lewis, 1914). Electron microscopy (EM) has shown that mitochondria are bounded by a double membrane (Sjöstrand, 1953), the innermost of which is infolded to form *cristae mitochondriales*, or cristae (Palade, 1952). The area between the inner mitochondrial (IM) and outer mitochondrial (OM) membranes is termed the intermembrane space (IMS) and the area bounded by the cristae is the mitochondrial matrix.

That mitochondria may divide in a manner akin to prokaryotes has been hypothesized since the late nineteenth century (Altmann, 1890; Schimper, 1885). Based on their work using quantitative EM, Bahr and Zeitler (1962) proposed that mitochondria increased their weight by linear growth and then divided to produce two daughter organelles. This was supported by other EM observations of dumbbell-shaped mitochondria in rat liver cells (Bahr and Zeitler, 1962; Claude, 1965; Stempak, 1967), in the fungus *Neurospora crassa* (Hawley and Wagner, 1967), and in the slime mold *Physarum polycephalum* (Guttes *et al.*, 1968).

More recently, the use of fluorescence microscopy has led to a revival in light microscopic studies of mitochondria. Fluorophores such as dimethylaminostyryl pyridinium methiodide (DASPMI) (Bereiter-Hahn, 1976), Rhodamine 123 (Johnson *et al.*, 1980), its derivatives, the MitoTrackers (Guttes *et al.*, 1968), and green fluorescent protein (GFP), have provided a wealth of information on the dynamics of mitochondria in the living cell. Molecular techniques now allow us to identify and to understand the workings of the individual molecules involved in mitochondrial division. In many cells, mitochondria are anastomosing networks that are maintained by a balance between division and fusion events, both of which are controlled by nuclear-encoded proteins. In this review of mitochondrial dynamics, the role of the prokaryote-derived FtsZ protein will be explored, as well as the roles of the eukaryote-derived mitochondrial division and fusion systems, which both employ dynamin-like GTPases.

## II. The Dynamics of Mitochondrial Morphology

### A. The Architecture of Mitochondrial Cristae

Across eukaryotes, mitochondrial inner membrane protrusions, the cristae, form three morphological groups: discoidal, tubular, and flattened. Cristal morphology is generally in agreement with the classification of higher-order taxa, based on other morphological and genetic characteristics (Gray *et al.*, 1998), and has therefore been used as a phylogenetic character (Cavalier-Smith, 1981, 1993; Stewart and Mattox, 1980). Opisthokonts (animals and fungi) and green and red algae have flattened cristae; discoidal cristae are found in kinetoplastids and euglenoids. Organisms with tubular mitochondrial cristae include diatoms, chrysophyte algae, the Apicomplexa (the group which contains the malaria parasite), and some animals. Within individual groups, however, variations are seen in cristal arrangements (Cavalier-Smith, 1998); for example, within mammalian mitochondria, tubular cristae dominate in neuronal cells, fibroblasts, hepatocytes, and muscle tissue, but in muscle and brown adipose tissue, the mitochondria can also display flattened cristae (Lea *et al.*, 1994). As Cavalier-Smith (1998) has proposed, the mitochondrial cristae of specific mammalian tissues may have evolved from an ancestral form to meet the particular energy demands of that tissue. The preservation and appearance under EM of structures such as cristae are, of course, subject to the fixation technique used. An example of this can be seen in *Dictyostelium*, which has traditionally been thought of as an organism with tubular cristae, but when freezing methods were employed, cristae that have been interpreted as flattened have been observed (Fields *et al.*, 2002).

Thus, cristal morphology can be subject to the fixation techniques used, and can vary within individual cell types and at least in some cases, may not provide a reliable character for the classification of taxa.

The traditional textbook appearance of mitochondrial cristae has been of large infolded sections of the inner membrane, in agreement with the findings of Palade (1952). However, the advent of EM tomography of rapidly frozen cells has suggested that, rather than being large infoldings of the IM, cristae are connected to IM by tube-like conduits, termed cristae junctions (Perkins and Frey, 2000; Perkins *et al.*, 1997), the dimensions and positioning of which are consistent with the *pediculi crista* found by Daems and Wisse (1966). Junctions of the cristae have been associated with the tubular mitochondrial cristae of rat and chick neurons (Perkins *et al.*, 1997) and rat liver cells (Mannella *et al.*, 1994). More recently, cristae junctions have also been noted connecting flattened cristae of *Neurospora crassa* (Nicastroa *et al.*, 2000) and rat brown adipose tissue mitochondria (Perkins *et al.*, 1998). It is thought that cristae form by a budding of the IM to form tubular cristae, and that flattened cristae may be the result of tubular cristae fusing at their junctions (Perkins *et al.*, 1998). The number and structure of cristae are thought to be a reflection of a cell's energy requirement: in contracting skeletal muscle for example (which may have mitochondria with flattened cristae), numerous tightly packed cristae were found, greatly increasing the mitochondrion's internal surface area (Fawcett, 1966; Scheffler, 1999).

## B. Mitochondrial Morphology

The gross morphology of mitochondria, like that of their internal membranes, varies greatly between different cell types, and even within individual cells. In many cells, ovoid, rod-shaped organelles are observed; for example, they are sometimes seen in mammalian cell lines (Collins *et al.*, 2002) and *Arabidopsis* (Arimura and Tsutsumi, 2002; Logan and Leaver, 2000), and often in many single-celled organisms, such as the red alga *Cyanidioschyzon merolae* (Suzuki *et al.*, 1994) and the slime molds *Physarum polycephalum* (Kuroiwa, 1973) and *Dictyostelium discoideum* (Gilson *et al.*, 2003). However, the classical view of mitochondria as small individual organelles has, over the last 3 decades, given way to a more complicated picture. Serial sectioning of the yeast *Saccharomyces cerevisiae* in the early 1970s (Hoffmann and Avers, 1973), and later computer reconstructions (Stevens and White, 1979), produced a three-dimensional view of a single, giant reticulated yeast mitochondrion, whose configuration is adjusted to suit nutritional conditions (Egner *et al.*, 2002). Such mitochondrial reticula, or networks, have been seen in numerous mammalian cell types: for example, rat liver cells (Brandt *et al.*, 1974), cultured fibroblasts (Johnson *et al.*, 1980), and endothelial cells (Bereiter-Hahn *et al.*, 1983). Mitochondrial networks appear to be present in muscle cells

(Bakeeva *et al.*, 1978; Kirkwood *et al.*, 1986), where they are more likely to be found as the number or volume of mitochondria increases (Kayar *et al.*, 1988; Kirkwood *et al.*, 1987; Salmons *et al.*, 1978). This event has been linked to physiological stimuli such as exercise (Gollnick and King, 1969; Holloszy, 1967; Hoppeler *et al.*, 1973; Kiessling *et al.*, 1971). It is thought that an increased prevalence of reticulate networks may be caused by an increased incidence of mitochondrial fusion (Hood, 2001). Additionally, mitochondrial networks have been seen in members of various of micro-algal phyla at particular points of the cell cycle: for example, in *Euglena gracilis* (Calvayrac *et al.*, 1974; Osafune, 1973; Osafune *et al.*, 1975a; Pellegrini and Pellegrini, 1976), the green algae *Chlamydomonas reinhardtii* (Grobe and Arnold, 1977; Osafune *et al.*, 1975b), *Pyramimonas gelidicola* (McFadden and Wetherbee, 1982) and *Polytomella agilis* (Burton and Moore, 1974), and the haptophyte alga *Pleurochrysis carterae* (Beech and Wetherbee, 1984). Thus, the overall morphology of mitochondria in a cell may range from small ovoid organelles to large reticulated networks.

## C. Mitochondrial Division

Mitochondrial division is vital if future generations of cells are to inherit the organelles, and we now know that the process plays an important role during programmed cell death (Frank *et al.*, 2001). In diverse cell types, the ratio between mitochondrial volume and total cytoplasmic volume remains constant—this has been seen in the ciliate *Tetrahymena pyriformis* (Kolb-Bachofen and Vogell, 1975), human cell lines (Posakony *et al.*, 1977), *S. cerevisiae* (Grimes *et al.*, 1974), and *N. crassa* (Hawley and Wagner, 1967). Thus, as the cell enlarges, so does its mitochondrial complement, which is then evenly distributed to daughter cells at cytokinesis. Dumbbell-shaped mitochondria have been seen in many electron micrographs, and it is believed that they represent mitochondria in a state of fission (Kuroiwa, 1982). Observations of live cells have now confirmed that, at least in part, these observations are true. For example, in internodal cells of the green alga, *Nitella flexilis*, mitochondria in cells from a synchronous culture observed by scanning electron microscopy (SEM) showed the progression from elongated to dumbbell-shaped as they divided into two daughter organelles (Kuroiwa, 1982). Bereiter-Hahn and Vöth (1994) found that stretching of the mitochondrion preceded fission; however, some mitochondria appeared to constrict then deconstrict without dividing, a phenomenon also seen in yeast (Legesse-Miller *et al.*, 2003). In yeast, mitochondria are constantly dividing (approximately 1 division/2 minutes; Nunnari *et al.*, 1997), apparently at random points, both within the network, and at the periphery to pinch off daughter organelles (Nunnari *et al.*, 1997). By comparison, the mitochondria

of heat-shocked (synchronized) cells of *Tetrahymena pyriformis* were seen to divide synchronously in late S-phase (Kolb-Bachofen and Vogell, 1975). In many organisms, including those with reticulated networks and those with small ovoid mitochondria, it can be important that mitochondrial division is tightly coupled to that of the entire cell. Given the consequences of failure, it is perhaps not surprising that such is the case for the single mitochondrion of a *Cyanidioschyzon merolae* cell. The single "concave-lens–shaped" mitochondrion in *C. merolae* constricts at its center just after plastid division but prior to cytokinesis (Suzuki *et al.*, 1994).

In various other organisms containing mitochondrial networks, a link between the cell cycle and mitochondrial cycle is evident. Although the details differ for each group of organisms, a general trend has been noted by Bereiter-Hahn and Vöth (1994) for the mitochondrial networks in various algal protists. Interphase cells have reticulated mitochondria (with a few smaller organelles), which increase in size until mitosis. Mitosis brings about fission of the network to form mitochondrial clusters, and finally the daughter cells are bequeathed a reduced number of mitochondria (Bereiter-Hahn and Vöth, 1994). Thus, some organisms have the ability to significantly alter mitochondrial morphology throughout the cell cycle. This process involves changes in competence of the organelles to divide, fuse, and change their cellular distribution.

In contrast, early studies in *Saccharomyces cerevisiae* and HeLa cells, both of which contain mitochondrial networks, showed no fixed relationship between mitochondrial division and the cell cycle (Grimes *et al.*, 1974; Posakony *et al.*, 1977). In the chromophyte alga, *Mallomonas splendens*, the small ovoid mitochondria also appear to divide without any link to the cell cycle (Beech *et al.*, 2000; M. Williams, unpublished data). Presumably, in these organisms, the rate of mitochondrial division outpaces mitochondrial fusion (if present), leaving enough mitochondria for each daughter cell at cytokinesis. It has been shown that the mitochondrial network in *S. cerevisiae* simplifies prior to budding; during this process a mitochondrial tubule extends into the daughter cell and attaches, while other tubules appear to be divided and transported from mother to daughter cell (Jakobs *et al.*, 2003).

Mitochondrial division in apicomplexan parasites further reveals the variation across species. In *Toxoplasma gondii*, mitochondria of the tachyzoite (a rapidly dividing haploid stage that infects mammalian cells) are found as a single network (Melo *et al.*, 2000). *T. gondii* normally produces two daughter cells at division, however, upon the occasional production of multiple daughter cells, a mitochondrial branching system similar to that of *P. falciparum* is seen (Hu *et al.*, 2002). The tubular mitochondrion (McMillan *et al.*, 2005; Sato *et al.*, 2003; van Dooren *et al.*, 2005) of the multiple daughters of a *P. falciparum* (Morrissette and Sibley, 2002) is derived from

a single mitochondrion which extends throughout the forming cells and is divided prior to cytokinesis to provide each cell with a mitochondrion (van Dooren *et al.*, 2005).

Mitochondrial division, much like the organelles themselves, has evolved to suit the diversity of eukaryotic life, and thus the process is hard to generalize. The rod-like mitochondria of some organisms can be seen to divide by binary fission. In some cases, this division parallels the cell cycle, and in others the sheer numbers of mitochondria in a cell appears to ensure their continued inheritance. Likewise, cells with large reticulated mitochondrial networks have a general trend to their mitochondrial division seen in some species, but not in others. As will be discussed later, these dynamics are also required during various forms of programmed cell death, including apoptosis. Apoptotic pathways can be affected by perturbing mitochondrial division proteins, while lowering levels of the mitochondrial fusion proteins can commit cells to apoptosis—further strengthening the association between mitochondrial division and apoptosis.

## D. Mitochondrial Fusion

Large mitochondrial networks are generally highly dynamic structures which can undergo constant fusion and division events (Bereiter-Hahn and Vöth, 1994; Nunnari *et al.*, 1997; Rizzuto *et al.*, 1998). By using a variety of different mitochondrially targeted fluorescent proteins, it has been shown that mitochondria in yeast (Nunnari *et al.*, 1997), mammalian (Ishihara *et al.*, 2003; Legros *et al.*, 2002) and plant cells (Arimura *et al.*, 2004) fuse and mix contents. A precise balance between fusion and fission events is maintained, resulting in a stable network (Nunnari *et al.*, 1997). Mitochondrial fusion in cells is independent of a functional respiratory chain, but dependent upon inner membrane potential (Ishihara *et al.*, 2003; Legros *et al.*, 2002), and involves the tip of one mitochondrion coming into contact with the tip or side of another mitochondrion (Bereiter-Hahn and Vöth, 1994). Using correlative light and electron microscopy, Bereiter-Hahn and Vöth (1994) found the membranes of likely contact zones had a very high electron density and high concentrations of hexokinase and creatine kinase. The connection of the two organelles began at sites of contact between the inner and outer membranes, and fusion was followed by rearrangement of the cristae (Bereiter-Hahn and Vöth, 1994).

Using the photoconvertible protein Kaede (whose emission spectra are altered due to irradiation at a particular wavelength of light; Ando *et al.*, 2002; Arimura *et al.*, 2004), have shown that the mitochondria of onion epithelial cells (and presumably other plant mitochondria) undergo constant fusion. Nunnari and colleagues (1997) mated yeast cells in which the

mitochondria of one mating type were labeled with GFP, and in the other mating type the mitochondria were labeled with the mitochondrion-specific dye MitoTracker; upon mating the mitochondria fused and the contents of each mitochondrion were seen to mix. In a similar approach, Legros *et al.* (2002) used cells with either GFP or red fluorescent protein (RFP) targeted to the mitochondria, and showed that the contents of human mitochondria were mixed upon fusion, and that fusion was dependent upon inner-membrane potential. These data have since been backed up by Karbowski *et al.* (2004), who used the photoactivatable protein PA-GFP (which does not fluoresce until irradiated at a specific wavelength [Patterson and Lippincott-Schwartz, 2002]; for a review of photoactivatable and photoconvertible proteins see Lippincott-Schwartz *et al.*, 2003) to track a subpopulation of mitochondria in a number of mammalian cell lines, and found that because PA-GFP fluorescence became distributed among all the mitochondria in a cell, mitochondrial fusion must have taken place. Though mixing of protein content clearly occurs, experiments in yeast have shown that the mtDNA molecules remain segregated: When the mtDNA of one mating type was labeled with bromodeoxyuridine, mtDNA molecules remained segregated (Nunnari *et al.*, 1997). This is consistent with the findings of Birky, who found that heteroplasmic yeast strains can become homoplasmic for each mtDNA within 20 generations, which is far quicker than would be expected from stoichiometric predictions, suggesting mtDNA is not being mixed and daughter cells can inherit just one mtDNA type (Berger and Yaffe, 2000; Birky, 1994, 1995).

Not all experimental data, however, support the nonmixing of mtDNA. In cybrid HeLa cells containing mitochondria with wild-type mtDNA, and mtDNA with a large deletion, a rapid mixing of mtDNA molecules (probably facilitated by mitochondrial fusion) was observed, which restored respiratory ability to all mitochondria (Hayashi *et al.*, 1994). Also, when HeLaCOT cells, each with different mtDNA mutations (which prevented respiration), were fused, the resulting hybrid cells became respiration-competent after 10 days, and remained so for 3 months (Ono *et al.*, 2001). This contrasts sharply with the work of Birky (1994, 1995), but presumably the question of mtDNA mixing becomes critical when a strong selective pressure is introduced. Only the cells that had mitochondria containing the two genomes could respire and survive in the Hayashi (1994) and Ono (2001) experiments. Alternatively, there may be intrinsic differences between yeast and animal cells. Chen *et al.* (2003) have suggested that mitochondrial fusion protects individual mitochondria from the loss of mtDNA or metabolic substrates, allowing a level of functional homogeneity to be maintained within the cell (Chen *et al.*, 2003; Hayashi *et al.*, 1994). In those cells whose mitochondria fuse, fusion is probably providing cells with a mechanism to prevent the potentially deleterious effects of becoming

isolated from the population, allowing mixing of contents, which in some cases includes mtDNA. Taking the sharing of mtDNA one step further, Spees *et al.* (2006) have observed, *ex vivo*, the rescue of aerobically incompetent human cells by the transfer of whole mitochondria (or at least their mtDNA) from a donor cell. This process is active on the part of the donor, however the specifics are presently unclear (Spees *et al.*, 2006).

## III. The Origins of Mitochondria

We should consider the evolutionary origin of mitochondria before discussing the proteins involved in the dynamics of these organelles. Mitochondria and plastids are each the products of separate endosymbiotic events that took place over a billion years ago. Plastids arose from the engulfment of a cyanobacterium by a mitochondrion-bearing eukaryote approximately 1 billion years ago. The long-held belief has been that mitochondria arose when a primitive eukaryotic cell engulfed an aerobic α-proteobacterium upon which it became dependent for aerobic respiration (Gray *et al.*, 1998, 1999; Martin *et al.*, 2001). Recent phylogenies, however, indicate that the origin of eukaryotes began with the fusion of an archaebacterium and a proteobacterium (Martin and Embley, 2004; Rivera and Lake, 2004). Two similar and convincing arguments have proposed scenarios in which these organisms were a fermentative α-proteobacterium and a methanogenic archaebacterium (López-García and Moreira, 1999; Martin and Müller, 1998; Martin *et al.*, 2001; Moreira and López-García, 1998). According to these hypotheses, the original relationship was based on hydrogen metabolism, and was later replaced in many species with the more efficient oxygen-based aerobic respiration (López-García and Moreira, 1999; Martin and Müller, 1998; Moreira and López-García, 1998). Thus, the acquisition of mitochondria was a defining step in eukaryote evolution and, today, nearly all eukaryotes possess mitochondria. Eukaryotes that don't have mitochondria are thought to have lost them (Bui *et al.*, 1996; Roger *et al.*, 1996) or the organelles have instead become mitosomes or hydrogenosomes (Williams *et al.*, 2002)—some of which still maintain the molecular remnants of their mitochondriate past (Regoes *et al.*, 2005). Because plastids and mitochondria were once free-living bacteria it's not surprising that bacteria have provided clues to the molecular basis of plastid and mitochondrial division. In bacteria, binary fission is performed by a complex division apparatus which is almost universally centered on the protein FtsZ (Addinall and Holland, 2002; Errington *et al.*, 2003; Harry, 2001; Margolin, 2001b, 2003, 2005; Martin *et al.*, 2001).

## IV. Molecular Components of Division and Fusion

### A. Bacterial Division Proteins

Bacterial cell division, or cytokinesis, relies on over a dozen proteins (Addinall and Holland, 2002; Rothfield *et al.*, 1999). Several of these are involved with peptidoglycan synthesis but, because mitochondria have no trace of a bacterial cell wall, the focus will be on those division proteins that are not involved in peptidoglycan synthesis (for reviews of bacterial division, see Margolin, 2005; Vicente *et al.*, 2006). FtsZ was first discovered in *Escherichia coli*, in which experiments identified temperature-sensitive mutants that could not divide and thus formed filaments at the nonpermissive temperature (Lutkenhaus *et al.*, 1980). FtsZ is the first protein to act in the *E. coli* cell division pathway (Begg and Donachie, 1985) and, with the exception of a small number of organisms, appears to have been conserved across the prokaryotes (Erickson, 2000; Margolin, 2005; Vaughan *et al.*, 2004).

During much of the bacterial cell cycle, FtsZ exists as cytosolic monomers (or more likely short protofilaments; Anderson *et al.*, 2004) within the cell. Very early in *E. coli* cell division, FtsZ molecules form a ring (the Z-ring) located exactly around the plane of division and, as division progresses, the Z-ring contracts, presumably drawing the inner membrane to fission (Bi and Lutkenhaus, 1991). FtsZ is a GTPase (de Boer *et al.*, 1992), and the Z-ring is a dynamic structure with a high turnover of FtsZ molecules (Anderson *et al.*, 2004; Stricker *et al.*, 2002) that are capable of rapid exchange of GDP for GTP (Mingorance *et al.*, 2001). It has been suggested the hydrolysis of GTP to GDP may bring about a conformational change in the FtsZ polymers resulting in bending of the polymers and thus constriction of the ring, and ultimately the cell (Addinall and Holland, 2002; Lu *et al.*, 2000).

Placement of the Z-ring must be such that the cell is divided equally, and that the cytokinetic ring does not damage the nucleoid. This is thought to be achieved by the combination of the Min system of proteins and so-called nucleoid occlusion (NO; Errington *et al.*, 2003; Harry, 2001; Margolin, 2001b). Although poorly understood, NO is the process by which the bacterial chromosome (nucleoid) inhibits the formation of Z-rings (Harry, 2001; Sun and Margolin, 2001; Woldringh *et al.*, 1990, 1991). Recently, the first components of NO systems have been identified in *B. subtilis* and *E. coli*: Noc (Wu and Errington, 2004) and SlmA (Bernhardt and de Boer, 2005), respectively. Each of the proteins are members of DNA-binding protein families, albeit disparate ones (Bernhardt and de Boer, 2005). Noc and SlmA both localize to the nucleoid (Bernhardt and de Boer, 2005; Sievers *et al.*, 2002; Wu and Errington, 2004), and a direct interaction with FtsZ has been inferred for SulA (Bernhardt and de Boer, 2005). It may be, then, that these proteins are

capable of disrupting any Z-rings forming in the vicinity of (and thus threatening) the recently formed daughter nucleoids during division; the actions of nucleoid occlusion leave only the poles of the cell and the septum available for Z-ring formation (Bernhardt and de Boer, 2005; Wu and Errington, 2004). Two of the *Min* gene products, MinC and MinD, act in unison to inhibit FtsZ rings from forming at the cell poles (Harry, 2001; Kruse, 2002; Margolin, 2001a). MinE, on the other hand, blocks the action of MinCD by destabilizing the complex at the mid-cell region. It appears in *E. coli* that all three proteins oscillate as MinE "chases" MinCD from pole to pole leaving the highest concentrations of MinCD at cell poles, and the cell mid-point free for division (Autret and Errington, 2001; Errington *et al.*, 2003; Harry, 2001; Margolin, 2001a). Homologues of MinC, MinD, and MinE are identifiable in the α-proteobacteria, along with ZipA, ZapA, and the following members of the Fts family: FtsZ, FtsQ, FtsK, FtsA, FtsI, and FtsW (Andersson *et al.*, 1998; Galibert *et al.*, 2001).

FtsZ has no predicted membrane-spanning domains and must therefore be indirectly attached to the cell membrane that it is thought to constrict. In *E. coli*, the presence of either one of two proteins, ZipA or FtsA, is sufficient for Z-ring formation and thus both can provide some form of membrane tethering (septum development cannot continue without both proteins; Pichoff and Lutkenhaus, 2002). ZipA has an N-terminal transmembrane domain, and a C-terminus which interacts with FtsZ (Hale and de Boer, 1997). ZipA has been shown to promote bundling of FtsZ protofilaments and act in FtsZ stabilization (Hale *et al.*, 2000; Pichoff and Lutkenhaus, 2002; RayChaudhuri, 1999). However, ZipA has a limited phylogenetic distribution—being found only in the gram-negative bacteria (Hale and de Boer, 1997), and is thus unlikely to be a general anchor of FtsZ (Pichoff and Lutkenhaus, 2005). On the other hand, the actin-related FtsA (Bork *et al.*, 1992) is well conserved across prokaryotes (Rothfield *et al.*, 1999) and, in fact, can compensate for the loss of ZipA with a single gain-of-function mutation (Geissler *et al.*, 2003). FtsA has a conserved C-terminal motif predicted to form an amphipathic helix that is thought to insert into the membrane and provide FtsZ with the necessary anchor (Pichoff and Lutkenhaus, 2005). FtsA homologues are not apparent, however, in all bacteria; the cyanobacteria, for example, may use the protein Ftn2 to anchor FtsZ to the membrane (Mazouni *et al.*, 2004).

## B. Plastid Division

Plastids, like mitochondria, cannot be made *de novo*, and must therefore be divided prior to cell division. The division of plastids has recently been reviewed in this series (Hashimoto, 2003) and elsewhere (Aldridge *et al.*, 2005;

Hashimoto, 2005; Miyagishima, 2005; Osteryoung and Nunnari, 2003) but is briefly discussed here because of the obvious similarities to mitochondrial division. The genomes of plants and red algae do not encode any recognizable plastid versions of many bacterial division proteins but they almost universally encode plastid versions of FtsZ, and in some organisms versions of the Z-ring placement proteins MinD and MinE (Colletti *et al.*, 2000; Lemieux *et al.*, 2000; Moehs *et al.*, 2001), and the Z-ring interacting protein (found only in the cyanobacteria) ZipN (Vitha *et al.*, 2003). Additionally, the recently described *Arabidopsis* protein, GIANT CHLOROPLAST 1, appears to be involved in plastid division, despite sequence homology to the bacterial division inhibitor SulA (Maple *et al.*, 2004).

It was in the nuclear genome of *Arabidopsis thaliana* that the first eukaryotic *ftsZ* genes were found (Osteryoung and Vierling, 1995). *A. thaliana* has three *FtsZ* genes: *AtFtsZ1-1*, *AtFtsZ2-1* and *AtFtsZ2-2* (Osteryoung and Vierling, 1995; Osteryoung *et al.*, 1998) and anti-sense inhibition of either AtFtsZ1-1 or AtFtsZ2-1 leads to the impairment of normal plastid division (Osteryoung *et al.*, 1998). *In vitro*, *Nicotiana tabacum* FtsZ1 (but not FtsZ2 to any great extent) polymerizes to form filaments and rings not unlike those displayed by *in vitro* polymerized *E. coli* FtsZ (El-Kafafi *et al.*, 2005). The presence of FtsZ2 allows polymerization of FtsZ1 in GTP-free substrates, and a similar effect is seen with the addition of ZapA to GTP-free *B. subtilis* substrates (El-Kafafi *et al.*, 2005; Gueiros-Filho and Losick, 2002); thus, FtsZ2 may act to promote polymerization of FtsZ1(El-Kafafi *et al.*, 2005). AtFtsZ1-1 and AtFtsZ2-1 are located on the stromal side (the inside) of the inner of the two membranes surrounding chloroplasts (McAndrew *et al.*, 2001), and these and the plastid FtsZs of other species co-localize to ring structure(s) at the plastid division point (Miyagishima *et al.*, 2001c; Mori *et al.*, 2001; Vitha *et al.*, 2001). El-Kafafi *et al.* (2005) have also shown that a small amount of *N. tabacum* FtsZ2 associates with both the inner and outer chloroplast membranes, and thus proposed that this protein may act as a bridge connecting the two membranes during organelle division.

With the exception of *Plasmodium falciparum* (Gardner *et al.*, 2002)—this probably extends to all apicomplexans and possibly even their alveolate relatives, the dinoflagellates—plastid FtsZs are found in all plastid-bearing organisms for which genetic data are available: the glaucocystophytes (such as *Cyanophora*) (Sato *et al.*, 2005), the green algae/plants (Araki *et al.*, 2003; Koide *et al.*, 2004; Osteryoung *et al.*, 1998; Strepp *et al.*, 1998; Wang *et al.*, 2003), the red algae (Takahara *et al.*, 1999, 2000), and chromophytic algae with secondary plastids (Beech *et al.*, 2000; Gilson and Beech, 2001; Kiefel *et al.*, 2004; Miyagishima *et al.*, 2004). Plastid FtsZ proteins are always encoded in the nucleus, except in the cryptomonad *Guillardia theta*, in which the single FtsZ is encoded by the nucleomorph (Fraunholz *et al.*,

1998), the residual nucleus of the secondary endosymbiont (Douglas *et al.*, 1991; Gilson, 2001). FtsZ proteins in the green and red lineage plastids can each be divided into two phylogenetic groups: FtsZ1 and FtsZ2 for green algae/plants (Osteryoung *et al.*, 1998; Stokes and Osteryoung, 2003) and red FtsZA and red FtsZB for red algae (the red FtsZA group also contains FtsZs from secondary plastids of red algal origin; Miyagishima *et al.*, 2004). Each of the red and green FtsZs appear to be products of independent gene duplications. Analysis of the genome of *Ostreococcus tauri* (http://bioinformatics.psb.ugent.be), an early diverging prasinophyte (green) alga (Courties *et al.*, 1998), reveals two plastid FtsZs which can be clearly placed in the FtsZ1 and FtsZ2 groups, thus indicating FtsZ duplication in green algae occurred early in their evolution (Fig. 1). The red algal FtsZ pair, however, does not appear to have diverged until after the secondary endosymbiosis giving rise to the diatoms, or else only one of the pair was successfully transferred—the diatoms seem to have compensated for their single FtsZ allotment by twice duplicating their FtsZ for a total of three proteins (Fig. 1). Domain analysis points toward differing roles in plastid division for members of the differing FtsZ groups. The C-terminal region of FtsZ, which in *E. coli* is thought to be involved in protein-protein interactions (Ma and Margolin, 1999; Osteryoung and McAndrew, 2001), is present in one (or more) and missing in one (or more) version of plastid FtsZ in any given organism (Kiefel *et al.*, 2004; Miyagishima *et al.*, 2004, Osteryoung and McAndrew, 2001; Stokes and Osteryoung, 2003). The function of the C-terminal core domain in the green FtsZ2 and red FtsZA group proteins is unknown, given that there are no homologues of known bacterial binding partners (such as FtsA). Expression, however, of *N. tabacum* FtsZ1 and FtsZ2 in *E. coli* has shown that FtsZ2 severely disrupts bacterial division (creating large filaments) in a manner largely dependent on the presence of its C-terminal core domain (FtsZ1 expressing cells had a similar phenotype to *E. coli* FtsZ overexpressing cells), possibly due to the domain binding to the normal FtsZ partners FtsA and ZipA in bacteria (El-Kafafi *et al.*, 2005).

No MinC homologues have been identified in eukaryotes to date (Osteryoung and McAndrew, 2001). However, plastid-encoded versions of MinD and MinE are found in the protists *Chlorella vulgaris* (Wakasugi *et al.*, 1997) *Guillardia theta* (Douglas and Penny, 1999), and higher plants have nuclear-encoded versions of MinD (Colletti *et al.*, 2000; Moehs *et al.*, 2001; Osteryoung and McAndrew, 2001) and MinE (Itoh *et al.*, 2001; Reddy *et al.*, 2002). Changes in expression of the *Arabidopsis minD* homologue, *AtMinD1*, result in asymmetrical plastid division, akin to that seen in bacteria (Colletti *et al.*, 2000). Overexpression of *AtMinE1*, however, causes plastids to become large and misshapen (Itoh *et al.*, 2001; Reddy *et al.*, 2002), possibly the result of multiple division sites forming (Maple *et al.*, 2002). Taken together, these data suggest that, at least in the plastids of *Arabidopsis*, FtsZ placement

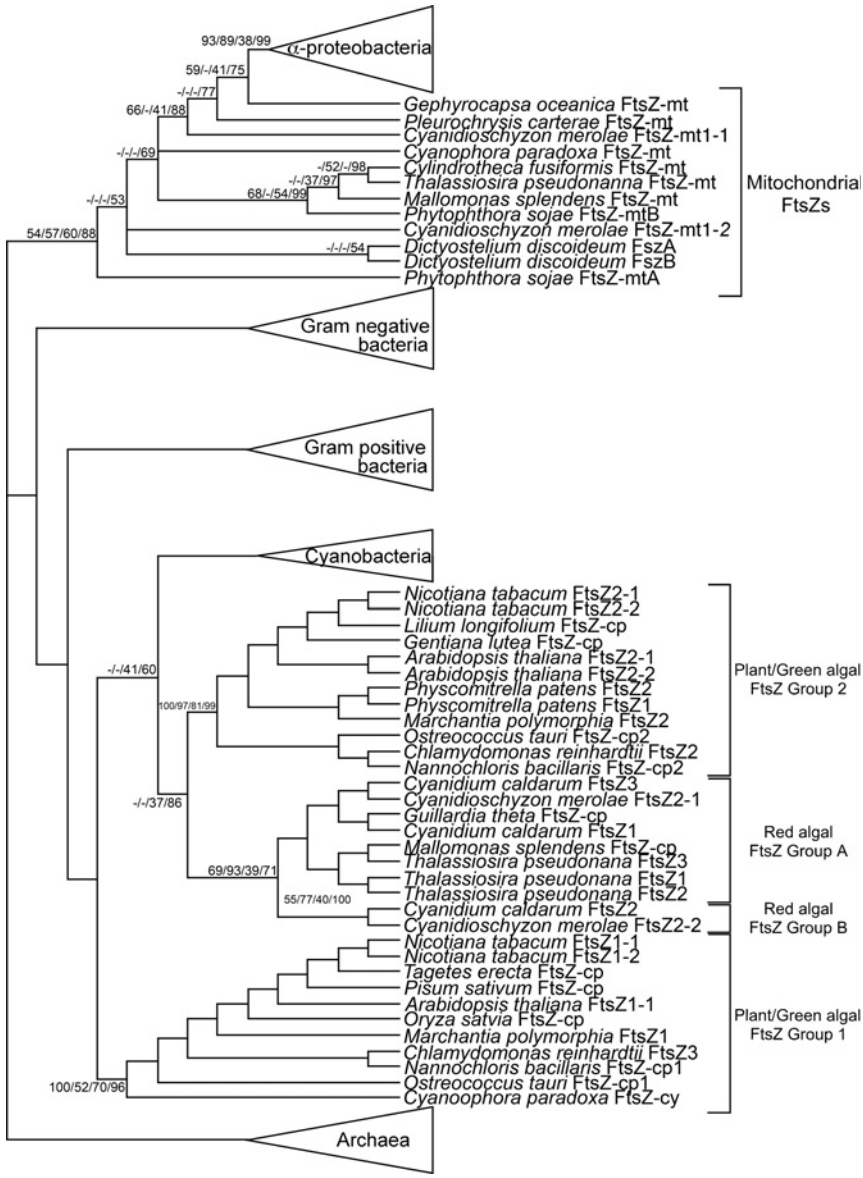


FIG. 1 Phylogenetic tree of FtsZ protein sequences. The values at each node represent bootstrap/confidence values for neighbor-joining/parsimony/quartet-puzzling/Markov Chain Monte Carlo analyses; a bootstrap value less than 50 is represented as "–." In all analyses performed, mitochondrial FtsZs clustered with the α-proteobacterial FtsZs, and the plastid FtsZs with the cyanobacterial FtsZs. The FtsZs of plastids form four clades: green-plastid FtsZ1, green-plastid FtsZ2, red-plastid FtsZ A, and red-plastid FtsZ B.

may still rely on a version of the Min system (lacking MinC) that acts in a similar manner to that in bacteria (Maple *et al.*, 2002).

Recently, the eukaryotic (host-derived) dynamin-like proteins, CmDnm2 and ARC5, were found to be associated with plastid division in *Cyanidioschyzon merolae* (Miyagishima *et al.*, 2003b) and *Arabidopsis* (Gao *et al.*, 2003), respectively. CmDnm2 forms a ring around the outside of the partly constricted plastid during division. Mutants of *arc5* have a greatly reduced number of plastids per cell (3–15, compared to 120 in wild-type cells) that are larger than wild-type and are constricted in the middle to form a dumbbell-shape (Gao *et al.*, 2003). Thus, plastid dynamins appear to be recruited after plastid constriction has commenced and probably act in final organelle severance.

So-called plastid-dividing (PD) rings have been observed in the dividing plastids of plants and green algae (Chida and Udea, 1991; Duckett and Ligrone, 1993a,b; Hashimoto, 1986; Hashimoto and Possingham, 1989; Oross and Possingham, 1989; Tewinkel and Volkmann, 1987; Udea and Nonaka, 1992), red algae (Kuroiwa *et al.*, 1993; Mita and Kuroiwa, 1988; Mita *et al.*, 1986), and chromophyte algae (Beech and Gilson, 2000; Hashimoto, 1997). Generally two rings are seen: one ring on the stromal side of the inner plastid membrane, and one on the cytoplasmic side of the outer plastid membrane (Hashimoto, 1986), with a third ring visible in the inter-membrane space of the *C. merolae* plastid (Kuroiwa *et al.*, 2002; Miyagishima *et al.*, 2001a,c). In the chromophyte alga, *Heterosigma akashiwo*, which has a secondary plastid derived from a red alga, the PD ring was found to be between the second and third plastid membranes—equivalent to the secondary symbiont outer plastid membrane (Hashimoto, 1997). The outermost PD rings of red algae are not composed of the dynamin-like protein CmDnm2 (Miyagishima *et al.*, 2003b), but rather of a 56-kDa protein that forms filaments 5 nm in diameter (Miyagishima *et al.*, 2001b). Although the gene encoding this 56-kDa protein has not yet been identified, it is thought to be of eukaryote origin due to its placement on the outside of the plastid (Miyagishima *et al.*, 2001c). Therefore, it appears that in the red algae and green algae/plants, plastid division is a cooperative process involving both prokaryotically and eukaryotically derived proteins (Miyagishima *et al.*, 2001c, 2003a).

## C. Mitochondrian Dividing (MD) Rings

The first mitochondrial division elements discovered were the MD rings in the unicellular red alga *Cyanidioschyzon merolae.* In these cells, Kuroiwa *et al.* (1993) noted electron-dense deposits at the equatorial regions of dividing mitochondria that formed two rings. The innermost of these MD rings resides in the mitochondrial matrix, and the outer ring on the cytoplasmic face of the mitochondrion (Kuroiwa *et al.*, 1995, 1998). The outer MD ring

forms prior to mitochondrial division (Miyagishima *et al.*, 1998) and rapidly begins to constrict the organelle, as plastid division ends (Miyagishima *et al.*, 1999). After mitochondrial fission is complete, the outer MD ring disassembles (Miyagishima *et al.*, 2001a) and the mitochondria are then segregated by a microtubule-based system (Nishida *et al.*, 2005). MD rings have been observed only in *C. merolae* and a few other unicellular organisms (Kuroiwa *et al.*, 1998; Hashimoto, 2004), and therefore may not be a common feature of mitochondrian division. Alternatively, MD rings may be too small or lacking in contrast to be visible in other eukaryotes (Miyagishima *et al.*, 2001a).

## D. MtFtsZ, An Endosymbiont-Derived Mitochondrial Division Protein

In the complete genomes of opisthokonts, plants, and *Plasmodium falciparum*, no mitochondrial versions of FtsZ have been found. However, across all eukaryotes, mitochondrial FtsZs (MtFtsZs) have been conserved in numerous lineages. Mitochondrial FtsZs were first described in the chromophyte alga *Mallomonas splendens* (MsFtsZ-mt; Beech and Gilson, 2000; Beech *et al.*, 2000) and *Cyanidioschyzon merolae* (CmFtsZ1-1; Takahara *et al.*, 2000), the former found as puncta or medial rings within mitochondria (Beech *et al.*, 2000), and the latter as a medial ring in the single *C. merolae* mitochondrion which constricts as mitochondrian division progresses (Nishida *et al.*, 2003, 2004). Genetic evidence of a role for FtsZ in mitochondrian division came from the slime mold *Dictyostelium discoideum*, which has two MtFtsZs, FszA and FszB (Gilson *et al.*, 2003). FszA, like MsFtsZ-mt and CmFtsZ1-1, forms puncta and ring-like structures at the midpoint of mitochondria and, consistent with a role in mitochondrian division, *Dictyostelium's* normally spherical mitochondria take on an elongate tubular morphology (indicative of an increase in length without division) (Fig. 2) and decrease in number when *fszA* is knocked out by insertional mutagenesis (Gilson *et al.*, 2003). The mitochondria of *Dictyostelium fszB* knockouts also show a reduction in number, and the same degree of elongation as *fszA* knockouts; however, FszB localization differs from that of other known FtsZs: FszB-GFP fusion proteins localize at the ends of mitochondria in an electron-dense structure, the submitochondrial body (SMB; Gilson *et al.*, 2003). The significance of this localization is not known, and it is unclear whether FszB transiently coaligns with FszA at the mitochondrial midline to bring about mitochondrian division (Fig. 2), or if the protein has a more indirect role in division. It should be pointed out that when *torA*, the other known gene whose product is located at the SMB, is knocked out, the normal mitochondrial morphology is lost (among other changes), becoming

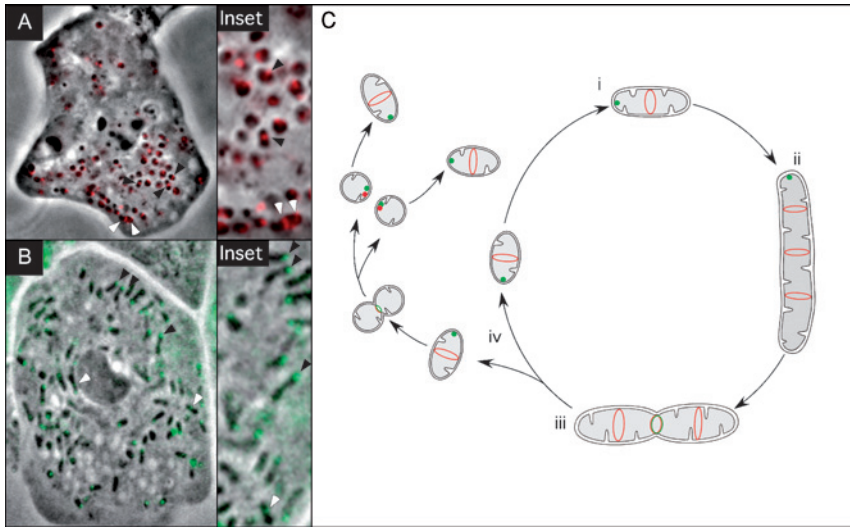


FIG. 2 (A) FszA-GFP (pseudo-colored red) localizes to the mitochondria in *Dictyostelium discoideum*, where it forms medial bands or rings (black arrows). Some mitochondria have multiple rings (white arrows). (B) FszB-GFP displays a polar mitochondrial localization (black arrows), however occasional medial bands or rings are seen (white arrows). (C) Proposed model of the roles of the two Fsz proteins in *D. discoideum*: FszA forms a medial ring in mitochondria, while FszB has a polar localization (i) multiple FszA rings may form as the mitochondrion elongates (ii), however it is not until FszB transiently localises with one of the FszA (iii) rings that division (iv) occurs.

larger and rounder (van Es *et al.*, 2001), and thus the effects seen for FszB knockout may be indicative of a greater mitochondrial defect, rather than a specific division defect. A second MtFtsZ, CmFtsZ1-2 has also been identified in the complete *C. merolae* genome (Matsuzaki *et al.*, 2004; Miyagishima *et al.*, 2004). The *C. merolae* MtFtsZ for which localization data are available, CmFtsZ1-1, acts at the mitochondrial mid-point during organelle division (Nishida *et al.*, 2004); as yet, it is not known where CmFtsZ1-2 is located.

The duplication of plastid FtsZs appears to have occurred independently at least three times, usually with one partner losing the variable C-terminal region (Kiefel *et al.*, 2004; Miyagishima *et al.*, 2004). Duplication is also a feature of mitochondrial FtsZs and, although no MtFtsZ has a true C-terminal core domain, FszA of *Dictyostelium* and the red-algal CmFtsZ1-2 both retain variable C-termini and a core domain-like sequence, whereas the C-termini of FszB and CmFtsZ1-1 are truncated; it is of note that it is FszB in *Dictyostelium* that does not show mid-point localization. Multiple MtFtsZs are not found in all organisms: only a single version is apparent in the genome (Armbrust *et al.*, 2004) of the diatom *Thallasiosira pseudonana* (Kiefel *et al.*, 2004;

TABLE I
Known Mitochondrial Fission and Fusion Proteins of *S. cerevisiae*

| Protein (yeast) | Homologues | Function | Human disease phenotype |
|---|---|---|---|
| **Fusion Proteins** | | | |
| Fzo1[1,2] | Fzo (*Drosophila*)[3]<br>Dm-FzoU/Dmfn (*Drosophila*)[4,5]<br>Mfn1 and Mfn2 (mammals)[6] | Mitochondrial fusion protein of the mitochondrial OM, with both the C- and N-termini exposed to the cytoplasm, thought to associate with other proteins/self-associate as part of the fusion process.[1,2,5–7] | Charcot-Marie-Tooth type 2A[8,9] |
| Ugo1[10] | Only found in fungi | Mitochondrial OM protein, with the C-terminus facing the cytosol and the N-terminus facing the intermembrane space.[10] *Interacts with Fzo1 and Mgm1.*[11] | None |
| Mgm1[11,12] | OPA1 (mammals)[13,14]<br>Msp1 (*S. pombe*)[15,16] | Dynamin-like protein of the mitochondrial IM and matrix, required for normal cristae morphology and mitochondrial fusion. May remodel IM during fusion, interacts with Fzo1 and Ugo1.[11, 17–20] | Dominant optic atrophy type 1[13,14] |
| **Division Proteins** | | | |
| Dnm1 | Drp1 (mammals)[21]<br>DRP-1 (*C. elegans*)[22]<br>DRP3A (*Arabidopsis*)[23]<br>DymA (*Dictyostelium*)[24]<br>CmDnm1 (*C. merolae*)[25] | Dynamin family member, remains mainly cytosolic and localizes to division point of constricting mitochondria to form puncta (puncta also contain Mdv1). May form a contractile ring, or a scaffold for other contractile proteins.[23,25–28] | None known |
| Mdv1[29–32] | Also known in yeast as: Fis2, Gag3, and Net2 | Localizes to Dnm1p puncta, at constricting mitochondria. May act as a molecular adapter between Dnm1 and Fis1[33] | None known |
| Fis1[29,30] | Also known in yeast as: Mdv2 | Distributed evenly over the outer mitochondrial membrane. Required for correct Dnm1 localization. May interact with Mdv1 to bring about conformational change[29,30,33] | None known |

Miyagishima *et al.*, 2004), and this protein lacks the variable C-terminal domain. Furthermore, incomplete MtFtsZs have been described for the glaucocystophyte *Cyanophora paradoxa*, the diatom *Cylindrotheca fusiformis*, the oomycete *Phytophthora infestans* and the haptophytes *Pleurochrysis carterae* and *Gephyrocapsa oceanica* (Kiefel *et al.*, 2004). Not surprisingly, phylogenetic analyses of these and all other MtFtsZ sequences indicate they are related to the FtsZs of the mitochondrial ancestor group, α-proteobacteria (Beech *et al.*, 2000; Gilson *et al.*, 2003: Kiefel *et al.*, 2004; Miyagishima *et al.*, 2003a; Takahara *et al.*, 2000) (Fig. 1). Additionally, genomic data indicate that mitochondrial *FtsZ* genes have been lost from eukaryote lineages at least three times: once after the diversification of plastid-bearing organisms, once during the evolution of opisthokonts, and once during Apicomplexan evolution (Arimura and Tsutsumi, 2002; Kiefel *et al.*, 2004; McFadden and Ralph, 2003; Miyagishima *et al.*, 2003a). Why these losses have occurred is unclear; however, it may be that the generally more bacterium-like mitochondrial morphology of FtsZ-containing organisms (with exceptions) is well suited to FtsZ-based division, whereas reticulated mitochondria that constantly fuse and divide may have found it necessary to dispense with an FtsZ-based mechanism of division (Kiefel *et al.*, 2004). In the past decade, studies on animal and yeast mitochondrial fusion and division have revealed a variety of proteins involved in these processes; these proteins are listed in Tables I and II, and are described later in more detail.

## E. Mitochondrial Fusion Proteins

### 1. Fzo1

The first protein shown to be involved in the maintenance of mitochondrial morphology was a mitochondrial fusion protein, *fuzzy onions* (Fzo) from *Drosophila melanogaster* (Hales and Fuller, 1997). *Drosophila* Fzo is targeted

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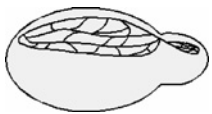
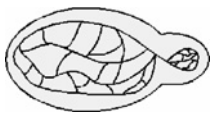
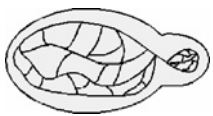
*Notes:* Homologues in other organisms and known diseases in man are also listed. Functions listed are based on work in *S. cerevisiae* and other systems. *IM,* Inner membrane; *OM,* outer membrane.

Table References: [1](Hermann *et al.*, 1998); [2](Rapaport *et al.*, 1998); [3](Hales and Fuller, 1997); [4](Hwa *et al.*, 2002); [5](Rojo *et al.*, 2002); [6](Santel and Fuller, 2001); [7](Fritz *et al.*, 2001); [8](Züchner *et al.*, 2004); [9](Kijima *et al.*, 2005); [10](Seskai and Jensen, 2001); [11](Wong *et al.*, 2003); [12](Jones and Fangman, 1992); [13](Alexander *et al.*, 2000); [14](Delettre *et al.*, 2000); [15](Pelloquin *et al.*, 1998); [16](Pelloquin *et al.*, 1999); [17](Shepard and Yaffe, 1999); [18](Wong *et al.*, 2000); [19](Olichon *et al.*, 2002); [20](Olichon *et al.*, 2003); [21](Smirnova *et al.*, 1998); [22](Labrousse *et al.*, 1999); [23](Arimura and Tsutsumi, 2002); [24](Wienke *et al.*, 1999); [25](Nishida *et al.*, 2003); [26](Otsuga *et al.*, 1998); [27](Bleazard *et al.*, 1999); [28](Smirnova *et al.*, 2001); [29](Fekkes *et al.*, 2000); [30](Mozdy *et al.*, 2000); [31](Tieu and Nunnari, 2000); [32](Cerveny *et al.*, 2001); [33](Tieu *et al.*, 2002).

TABLE II
Deletion Phenotypes of *S. cerevisiae* Mitochondrial Fission and Fusion Protein

| | **Δ Phenotypes** | |
|---|---|---|
| **Fusion** | | |
| *fzo1*Δ | Mitochondria fragment and tends to aggregate. Fragmentation leads to loss of mtDNA and inability to grow on nonfermentable carbon source[1,2] |  |
| *ugo1*Δ | Similar to mitochondria of fzo1Δ cells, with larger mitochondria[3] |  |
| *mgm1*Δ | Fragmentation, aggregation of mitochondria, loss of mtDNA,[4–7] and in mammals, loss of OPA1 leads to disruption on cristae, leakage of cytochrome *c*, and apoptosis[8] |  |
| **Division** | | |
| *dnm1*Δ | Formation of large interconnected networks of mitochondria. Mislocalization of Mdv1[9,10] |  |
| *mdv1*Δ | Similar phenotype to that of *dnm1*Δ cells[11–14] |  |
| *fis1*Δ | Similar phenotype to that of *dnm1* Δ cells, Dnm1 and Mdv1 mislocalization[11,12] |  |

| Double Δ Phenotypes | | | |
|---|---|---|---|
| *fzo1*Δ | *dnm1*Δ **or** *mdv1*Δ **or** *fis1*Δ | Phenotype similar to that seen in wild-type cells. Mitochondria do not fuse or divide[3,10–12] |  |
| *dnm1*Δ | *ugo1*Δ | Phenotype similar to that seen in wild-type cells[3] |  |
| *mgm1*Δ | *dnm1*Δ **or** *mdv1*Δ **or** *fis1*Δ | Phenotype similar to that seen in wild-type cells, however fusion is restored[5,11] |  |

*Notes:* Deletion of genes encoding mitochondrial fusion proteins brings about fragmentation and aggregation of mitochondria. Deletion of genes encoding mitochondrial division proteins creates large net-like mitochondria. Double deletion mutants (a fusion and a division protein) generally produce cells whose mitochondria appear close to wild-type (but do not function normally).

Table References: [1](Hermann *et al.*, 1998); [2](Rapaport *et al.*, 1998); [3](Seskai and Jensen, 2001); [4](Jones and Fangman, 1992); [5](Shepard and Yaffe, 1999); [6](Wong *et al.*, 2000); [7](Guan *et al.*, 1993); [8](Olichon *et al.*, 2002); [9](Smirnova *et al.*, 2001); [10](Sesaki and Jensen, 1999); [11](Fekkes *et al.*, 2000); [12](Mozdy *et al.*, 2000); [13](Tieu and Nunnari, 2000); [14](Cerveny *et al.*, 2001).

to mitochondria where it is required for developmentally regulated mitochondrial fusion during spermatogenesis—male *fzo* mutants are sterile (Hales and Fuller, 1997). *D. melanogaster* also encodes a second version of Fzo, *Dm-FzoU/dmfn*, which is expressed in both male and female flies, and probably mediates mitochondrial fusion events in most tissues (Hwa *et al.*, 2002; Rojo *et al.*, 2002). Homologues of Fzo are present in many members of the opisthokonts, with *S. cerevisiae* encoding a single Fzo homologue, Fzo1 (Hermann *et al.*, 1998; Rapaport *et al.*, 1998). Like that of *Drosophila,* mammalian genomes contain two Fzo homologues, the mitofusins Mfn1 and Mfn2 (Chen *et al.*, 2003; Eura *et al.*, 2003), which appear to have arisen from a separate gene duplication event. Mutations in the human *MFN2* gene have been found to cause Charcot-Marie-Tooth neuropathy type 2A (CMT2A; Kijima *et al.*, 2005; Züchner *et al.*, 2004)—one of a number of peripheral neuropathies that include sensory loss (Chen and Chan, 2005; Shy, 2004; Vance, 2000; Young and Suter, 2003).

Consistent with an early role in mitochondrial fusion, Fzo1 and the mitofusins are located on the outer mitochondrial membrane (Fig. 3B) (Hermann *et al.*, 1998; Rapaport *et al.*, 1998; Santel and Fuller, 2001). Fzo1 has a N-terminal GTPase domain, two membrane-spanning domains and a number of coiled-coil domains: one at the N-terminus, one following the GTPase domain, and one at the extreme C-terminus. The N-terminal coiled-coil domain is absent in the vertebrate mitofusins (Mozdy and Shaw, 2003). Thus, a small portion of Fzo1 lies within the IMS, and both the N- and C- termini, with their coiled-coil domains, are exposed to the cytoplasm; the N-terminal GTPase domain and the coiled-coil region contain most of the causative mutations of CMT2A (Kijima *et al.*, 2005). The IMS portion of Fzo1 associates with the IM (Fritz *et al.*, 2001) and, in both Fzo1 and Mfn2, is essential for normal functioning of the proteins; perturbations in the IMS portion of Fzo1 and Mfn2 cause mitochondrial fusion deficiencies (Fritz *et al.*, 2001; Neuspiel *et al.*, 2005; Shaw and Nunnari, 2002)—perhaps because of a failure to interact with as-yet-unknown components of the mitochondrial fusion machinery (Neuspiel *et al.*, 2005). The two coiled-coil domains on mitofusin molecules interact with each other (Honda *et al.*, 2005; Rojo *et al.*, 2002) in a GTPase-dependent manner (Honda *et al.*, 2005). These domains also interact with other mitofusin molecules to bring about mitochondrial clustering (Santel and Fuller, 2001); the regions form antiparallel structures with the coiled-coil regions of Mfn1 molecules on opposing mitochondria (Koshiba *et al.*, 2004).

Yeast *fzo*$\Delta$ cells have small fragmented mitochondria that lose their mtDNA (Hermann *et al.*, 1998; Rapaport *et al.*, 1998), lack cristae, and cluster together (Hermann *et al.*, 1998). Mice lacking Mfn1 or Mfn2 die early in gestation (in Mfn2-deficient embryos, death appears to be the result of abnormal placental development) and have fragmented mitochondria

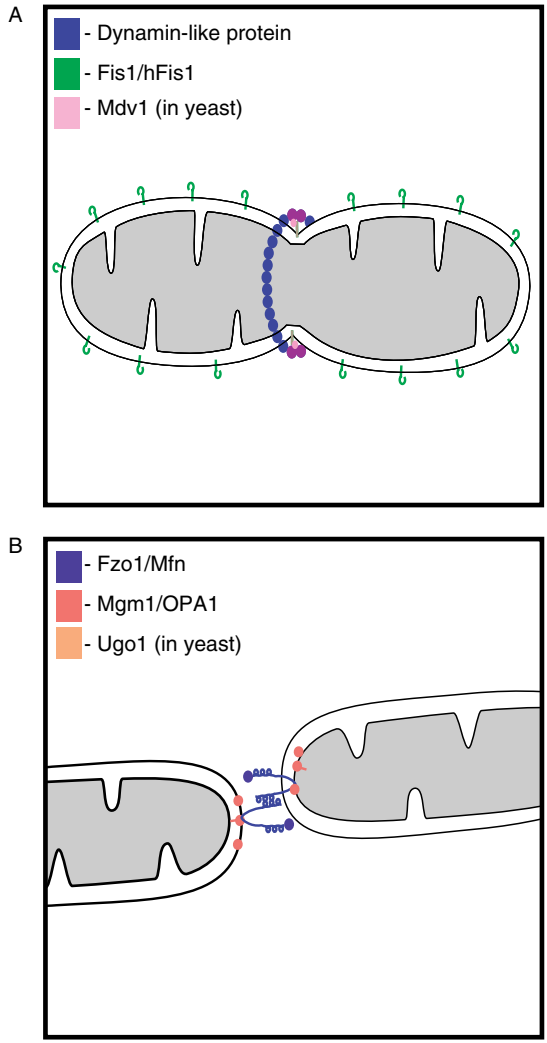



FIG. 3 A, Mitochondrial division: Fis1 (green) is distributed evenly over the mitochondrial outer membrane, where self-interactions may prevent binding with other division proteins. At the division site in yeast Mdv1 (pink) forms a complex with Fis1 and allows formation of a Dnm1 (blue) contractile ring. In mammalian systems no Mdv1 is found, and Fis1 interacts directly with Dnm1. B, Mitochondrial fusion: Fzo1 (or its homologues) molecules on opposing membranes interact via their coiled-coil domains. In coordination with Ugo1 (in yeast) and Mgm1, the mitochondria are fused.

(Chen *et al.*, 2003). Unlike yeast *fzo1* null mutants (*fzo1*Δ), the mitochondria in *mfn1*Δ or *mfn2*Δ cells maintain their mtDNA, and only some respiratory ability is lost: this may be attributable to the presence of two Mfn proteins in mammals that are partially functionally redundant and capable of buffering the loss of their partner (Chen *et al.*, 2003, 2005). Dominant-negative GTPase-deficient forms of Mfn2 (Eura *et al.*, 2003), or a mutant lacking the GTPase domain entirely (Neuspiel *et al.*, 2005), also cause mitochondria to cluster together—indicating that docking but not outer-membrane fusion has occurred (Chen and Chan, 2005; Koshiba *et al.*, 2004; Neuspiel *et al.*, 2005). Immuno-EM of mitochondria in Mfn2 GTPase-deficient cells showed clustering of Mfn2 into electron-dense patches between opposing mitochondrial membranes (Eura *et al.*, 2003)—a similar gap was noted in Mfn1 GTPase mutants (Koshiba *et al.*, 2004). Thus, Mfn GTPase activity is not required to dock mitochondria, but has a role in the pulling together of mitochondria during fusion. How exactly this is performed is a challenge for future research.

Overexpression of Mfn1, Mfn2, or Fzo1 produces cells with mitochondria that aggregate and cluster around the cells' nucleus (Eura *et al.*, 2003; Fritz *et al.*, 2003; Rojo *et al.*, 2002; Santel and Fuller, 2001), with some mitochondria extending to the cell periphery in Mfn2-overexpressing cells (Eura *et al.*, 2003). Though the mitochondrial outer membranes in Mfn2 overexpressing cells are in close contact, membrane integrity remains intact (Rojo *et al.*, 2002) and the membranes do not fuse—perhaps because another mitochondrial fusion component is limiting the rate of fusion. Two interesting Mfn2 mutants may help shed further light on the action of this molecule. Neuspiel *et al.* (2005) generated a dominant active version of Mfn2 (Mfn2 $Ras_{G12V}$) by mutating the GTP-binding motif to be identical to that of activated RasG12V (Barbacid, 1987; Franken *et al.*, 1993; Futatsugi and Tsuda, 2001), thus increasing the Mfn2 rate of nucleotide exchange and decreasing hydrolysis. Activated Mfn2 failed to form the puncta normally displayed by wild-type Mfn2 but was, instead, evenly distributed over the mitochondrial OM. Mitochondria in cells expressing $Mfn2Ras_{G12V}$ were interconnected networks indicative of an increased rate of fusion; indeed, upon fusion of cells, mitochondria in $Mfn2Ras_{G12V}$ cells rapidly migrated and initiated fusion. Taken together with data indicating that $Mfn2Ras_{G12V}$ can rescue a IMS mutant that was otherwise fusion deficient (similar to yeast Fzo1 mutants) (Fritz *et al.*, 2001; Shaw and Nunnari, 2002), a model has been presented wherein Mfn2 normally exists on the mitochondrial OM in its inactive GDP-bound form but, in response to stresses such as free radical production, is activated by IMS signaling to bring about mitochondrial fusion and this alleviates the effects of local damage to a single mitochondrion (Neuspiel *et al.*, 2005).

Yeast cells have a single version of Fzo1 (Hermann *et al.*, 1998; Rapaport *et al.*, 1998), as does *C. elegans* (Rojo *et al.*, 2002), yet vertebrates appear to require two interacting Mfns (forming both hetero- and homo-complexes)

(Chen *et al.*, 2003; Ishihara *et al.*, 2004; Legros *et al.*, 2002) for normal mitochondrial morphology (Chen *et al.*, 2003; Eura *et al.*, 2003; Rojo *et al.*, 2002). A collection of hybrid cells covering the possible combinations of Mfn1 and Mfn2 have shown that, provided a mitochondrion has at least one version of Mfn, any combination is sufficient to cause the fusion of opposing membranes (Chen *et al.*, 2005; Koshiba *et al.*, 2004) although, *in vivo*, Mfn1-Mfn1 interactions are far more common than Mfn2-Mfn2 interactions (Ishihara *et al.*, 2004). Differences also exist between the effects each mitofusin can have on the cell. The fusion protein OPA1 requires Mfn1, but not Mfn2 for mitochondrial fusion (Cipolat *et al.*, 2004): Legros and co-workers (2002) noted that Mfn1 appeared to stimulate fusion, while Mfn2 was responsible for mitochondrial clustering in transiently transfected cells; it is not clear, however, if differing expression levels account for the differences observed (Chen *et al.*, 2003). Work on mouse mitofusins has shown that while removal of Mfn1 causes mitochondria to fragment into small tubules, Mfn2-deficient cells form small spheres and have a lesser degree of fragmentation than Mfn1 knockouts, raising the possibility that Mfn2 may have a role in tubule maintenance (Chen *et al.*, 2003).

A number of proposals have been put forward to define the roles of the mitofusins. Given the differing mitofusin-specific effects on mitochondrial morphology, and the varied expression levels between tissue types, Eura *et al.* (2003) have proposed that mammals may use differential expression of the mitofusins to help produce the range of mitochondrial morphologies seen in different cell types. Alternatively, based on the rarity of Mfn2-Mfn2 complexes they found, Ishihara *et al.* (2004) suggested that Mfn2 may have a role in negatively regulating mitochondrial fusion, and thus when Mfn2 molecules are overexpressed they can outcompete Mfn1 in the formation of tethering complexes, but no fusion takes place.

## 2. Ugo1

The yeast Ugo proteins Ugo1 and Ugo2 (*ugo* is Japanese for fusion) were identified in a screen for mutants that lost mtDNA in a manner dependent on the mitochondrial division protein Dnm1 (Sesaki and Jensen, 2001). Similar to *fzo1*Δ cells, *ugo1*Δ cells have mitochondria that fragment, lose their mtDNA, and do not fuse after cells mate (Sesaki and Jensen, 2001). The mitochondria of *ugo1*Δ cells, however, are larger than those of *fzo1*Δ cells and have a tendency to aggregate (Sesaki and Jensen, 2001). Mitochondrial aggregation may be caused by the initial Fzo1-mediated docking steps taking place, but the loss of Ugo1 stops any further progression to fusion. Ugo1 is a 58-KDa integral mitochondrial OM protein that has its N-terminus facing the cytoplasm, and the C-terminus in the intermembrane space (Sesaki and Jensen, 2001). All three components of the yeast mitochondrial fusion system (Fzo1, Mgm1, and Ugo1)

co-immunoprecipitate (Sesaki *et al.*, 2003a; Wong *et al.*, 2003) and it appears it is Ugo1 that forms the basis of this interaction, in a manner independent of either Mgm1 or Fzo1's GTPase domain (Sesaki and Jensen, 2004). The intermembrane space C-terminal portion of Ugo1 interacts with Mgm1, while the cytoplasmic N-terminal region interacts with Fzo1 (Sesaki and Jensen, 2004). If the Fzo1-Ugo1 association is perturbed, yeast mitochondria fail to fuse and mtDNA is lost. Sesaki and Jensen (2004) have suggested that Ugo1 may regulate Mgm1 and Fzo1 at a key step in mitochondrial division by coordinating mitochondrial inner- and outer-membrane fusion.

Ugo2 is synonymous with Pcp1, a rhomboid-like protease, and is responsible for processing another mitochondrial fusion protein, Mgm1, into one of its isoforms, thereby maintaining normal mitochondrial morphology (Herlan *et al.*, 2003; McQuibban *et al.*, 2003; Sesaki *et al.*, 2003b).

## 3. Mgm1/OPA1

*Mgm1* was originally isolated as a gene involved in the maintenance of the yeast mitochondrial genome (Jones and Fangman, 1992) and a lack of Mgm1 causes aggregation of mitochondria and loss of mtDNA (Guan *et al.*, 1993). More recent studies have shown that mitochondria fragment in *mgm1*$\Delta$ cell, giving a phenotype very similar to cells lacking the mitochondrial fusion protein Fzo1 (Shepard and Yaffe, 1999; Wong *et al.*, 2000). Mgm1 is a dynamin-like GTPase (Sesaki *et al.*, 2003a; Wong *et al.*, 2003) which has homologues in mammals (OPA1; Alexander *et al.*, 2000; Delettre *et al.*, 2000) and fission yeast (Msp; Pelloquin *et al.*, 1998, 1999) that are also responsible for maintaining mitochondrial networks (Misaka *et al.*, 2002). Despite the low level of sequence conservation between the yeast and mammalian proteins, the two appear to be functional homologues given that human OPA1 is capable of restoring normal mitochondrial phenotype in *msp1*$\Delta$ fission yeast cells (Lenaers *et al.*, 2002). In humans, OPA1 is the gene mutated in Optic Atrophy Type 1, a major cause of blindness, and the first example of a disease caused by aberrations in mitochondrial networks (Alexander *et al.*, 2000; Delettre *et al.*, 2000).

A number of splicing variants of OPA1 (Delettre *et al.*, 2001) and at least two isoforms of Mgm1 (Herlan *et al.*, 2003; McQuibban *et al.*, 2003; Sesaki *et al.*, 2003b) exist that display differing submitochondrial localizations. Of the investigated OPA1 isoforms, the larger (93 kDa) associates with the mitochondrial IM, and the smaller (88 kDa) with the OM (Satoh *et al.*, 2003). The two yeast Mgm1 isoforms are the smaller 90-kDa form, s-Mgm1, and the larger 100-kDa form, l-Mgm1, and are localized to the intermembrane space (Herlan *et al.*, 2003; McQuibban *et al.*, 2003; Sesaki *et al.*, 2003b). The s-Mgm1 isoform is formed by the cleavage of l-Mgm1 by Pcp1 (also referred to as Ugo2). Pcp1 is a serine protease, homologous to the *Drosophila* rhomboid-1 protease (Urban *et al.*, 2001), that is known to act on two

substrates, cytochrome *c* peroxidase (Esser *et al.*, 2002; McQuibban *et al.*, 2003) and Mgm1 (Herlan *et al.*, 2003, 2004; McQuibban *et al.*, 2003; Sesaki *et al.*, 2003b). Following deletion of *pcp1* (*pcp1*Δ), yeast mitochondria become fragmented (resembling those of *mgm1*Δ cells) and mtDNA and respiratory ability are lost (Herlan *et al.*, 2003; McQuibban *et al.*, 2003; Sesaki *et al.*, 2003b). Examination of Mgm1 in *pcp1*Δ cells showed that the l-Mgm1 isoform was present, but not s-Mgm1 (Herlan *et al.*, 2003; McQuibban *et al.*, 2003; Sesaki *et al.*, 2003b); similar defects to those in *pcp1*Δ cells are attainable when Mgm1 is replaced by a version with a cleavage-resistant transmembrane domain (and thus no s-Mgm1 is formed) (McQuibban *et al.*, 2003). Partial complementation of *mgm1*Δ cells could be achieved with the introduction of constructs mimicking l-Mgm1 and s-Mgm1, but neither construct on its own could restore mitochondrial morphology (Herlan *et al.*, 2003).

Together, these experiments have shown that both isoforms of Mgm1 are essential for mitochondrial fusion in yeast, and thus the maintenance of normal mitochondrial morphology. Given the wide conservation of the rhomboid protease family (Koonin *et al.*, 2003; McQuibban *et al.*, 2003; Pascall and Brown, 1998; Urban *et al.*, 2002; Wasserman *et al.*, 2000), it is likely that Mgm1 homologues are processed in a similar way in other species. Two mitochondrially targeted members of the rhomboid protease family have been identified (McQuibban *et al.*, 2003): Rhomboid-7 (*Drosophila*) and PARL (human; Pellegrini *et al.*, 2001), the latter being capable of complementing *pcp1*Δ yeast (McQuibban *et al.*, 2003). Herlan *et al.* (2004) have presented a mechanism termed "alternate topogenesis," whereby the action of mitochondrial-processing peptidase and lateral insertion into the inner membrane by the TIM23 translocase results in l-Mgm1. In situations of higher matrix ATP, further ATP-driven import pulls Mgm1 further into the membrane making the cleavage site accessible to Pcp1; s-Mgm1 is then generated and released into the intermembrane space. Thus, when levels of matrix ATP are low (e.g., when there are mtDNA mutations brought about by oxidative stress), little or no s-Mgm1 would be formed and the resulting loss of fusion can act to isolate defective organelles from the population (Herlan *et al.*, 2004).

Temperature sensitive *mgm1* mutants ($mgm1^{ts}$) display a similar phenotype to $fzo1^{ts}$ or *fzo1*Δ cells and, in a similar manner to $fzo1^{ts}$/Δ cells, the $mgm1^{ts}$ phenotype can be rescued by disrupting the *dnm1* gene (Wong *et al.*, 2000). Unlike *fzo1*Δ *dnm1*Δ cells, however, $mgm1^{ts}$ *dnm1*Δ cells have mitochondria that are capable of fusion, which initially led researchers to believe that Mgm1 was not directly involved in mitochondrial fusion, and may have been part of the mitochondrial division system (Wong *et al.*, 2000). But further studies have shown that, in contrast to $mgm1^{ts}$ *dnm1*Δ cells, *mgm1*Δ *dnm1*Δ cells cannot fuse their mitochondria; presumably some residual activity was

present in *mgm1*$^{ts}$ cells at the nonpermissive temperature (Sesaki *et al.*, 2003b; Wong *et al.*, 2003). It thus appears that Mgm1 is a *bona fide* mitochondrial fusion protein, a deduction supported by its co-immunoprecipitation with the two other known components of the mitochondrial fusion machinery, Fzo1 and Ugo1 (Sesaki *et al.*, 2003a; Wong *et al.*, 2003).

Mgm1/Msp1/OPA1 appear to be required for normal cristal morphology and for mitochondrial fission (Olichon *et al.*, 2003; Sesaki *et al.*, 2003a; Wong *et al.*, 2003). EM of HeLa cells with OPA1 levels depleted by siRNA showed that the cristae were unstructured, vesicle-like structures, displaying larger than normal spaces between the membranes (Olichon *et al.*, 2003). The loss of normal cristal architecture was accompanied by a loss of membrane potential, the release of cytochrome *c* into the cytoplasm, and apoptosis (Olichon *et al.*, 2003). Mitochondria in *OPA1* siRNA cells were fragmented and had lost their ability to respire (Chen *et al.*, 2005; Cipolat *et al.*, 2004); however, when levels of OPA1 were returned to normal, respiration was restored, indicating that mtDNA wasn't lost and further suggesting that mitochondrial fusion is fundamentally required for respiration (Chen *et al.*, 2005). Similarly, in yeast, deletion of *mgm1* caused cristae to become unstructured; however, when these cells also had *dnm1* deleted, the cristae returned to their previous phenotype (Sesaki *et al.*, 2003a). This argues that the cristal malformation in *mgm1*$\Delta$ cells is not the cause of fusion deficiency. Correspondingly, when the *mmm1* (which encodes a mitochondrial OM protein required for mtDNA inheritance, Hobbs *et al.*, 2001) or *atp21* (encoding a subunit of ATP synthase, Paumard *et al.*, 2002) genes are mutated, malformed cristae are seen but the organelles are capable of fusion (Sesaki *et al.*, 2003a). Wong *et al.* (2003) have proposed two possible modes of action for Mgm1. The first is that Mgm1 is directly involved with remodeling events of the inner mitochondrial membrane that allow fusion to progress. In this scenario, Mgm1 could respond to fusion-promoting events on the OM via its interaction with OM proteins Fzo1 and Ugo1 (Sesaki *et al.*, 2003a; Wong *et al.*, 2003); it is thought possible that both these proteins interact with IMS or IM proteins (Neuspiel *et al.*, 2005; Sesaki *et al.*, 2003a). Given that OPA1 appears to have a role in shaping the mitochondrial IM (Olichon *et al.*, 2003), a role in membrane remodeling during fusion (and possibly division) is a possibility. Alternatively, Mgm1 may act to recruit Fzo1 and Ugo1 to the fission site, in a manner similar to other dynamins involved in endocytosis (Sever *et al.*, 1999, 2000; Wong *et al.*, 2003).

## F. Mitochondrial Division Proteins

None of the mitochondrial division proteins found to date are essential for cell survival; however, a recent screen has revealed that yeast has at least 119

essential genes that, when down-regulated, effect mitochondrial morphology (Altmann and Westermann, 2005). The genes fell into five general categories: those required for ergosterol biosynthesis, mitochondrial import, actin-based transport, vesicular trafficking, and ubiquitin/26S proteasome-dependent protein degradation (Altmann and Westermann, 2005). The role of ergosterol in the maintenance of mitochondrial morphology is particularly interesting. It has been suggested that ergosterol may play a part in activating and/or rearranging membrane proteins in the vacuolar fusion reaction (Fratti *et al.*, 2004; Kato and Wickner, 2001), and Altmann and Westermann (2005) have proposed that ergosterol may have a function in regulating mitochondrial division and fusion proteins in mitochondrial membrane.

## 1. Dnm1

The dynamin-like protein (DLP) Dnm1 is a yeast mitochondrial division protein that belongs to a family of membrane-severing proteins the archetype of which is dynamin-1 that constricts clathrin-coated pits during endocytosis (van der Bliek, 1999). The recruitment of DLPs for the constriction of mitochondrial membranes early in eukaryotic evolution is indicated by the diversity of organisms in which Dnm1 homologues have been identified (far more than dynamin-1 homologues, in fact): in addition to Dnm1 itself in yeast, mitochondrion-associated versions of DLPs are found in mammals (Drp1 or Dlp1; Smirnova *et al.*, 1998), *C. elegans* (DRP-1; Labrousse *et al.*, 1999), plants (ADL2b, now known as DRP3A; Arimura and Tsutsumi, 2002; Hong *et al.*, 2003), *Dictyostelium* (DymA; Wienke *et al.*, 1999) and red algae (CmDnm1; Nishida *et al.*, 2003, 2004) where they are associated with mitochondrial division, and homologues can also be identified in other organisms such as the diatom *Thalassiosira pseudonana*.

The bulk of Dnm1/Drp1 exists in cytoplasmic pools in yeast and mammalian cells (Legesse-Miller *et al.*, 2003; Otsuga *et al.*, 1998; Smirnova *et al.*, 2001) but, importantly, some of the protein localizes as puncta on the mitochondrial surface. In yeast, GFP-Dnm1 forms transient "hot spot" localization patches along mitochondria (Legesse-Miller *et al.*, 2003) and in *Arabidopsis*, ADL2b forms punctate structures at the tips of mitochondria and at points of constriction (Arimura and Tsutsumi, 2002). Immuno EM has localized Dnm1 puncta to points of mitochondria that appear to be dividing (Bleazard *et al.*, 1999) and, with GFP-tagged versions of Dnm1/Drp1, it has been shown that the puncta are associated with the division points of mitochondria (Smirnova *et al.*, 2001). Dnm1 initiates its own assembly by forming a structure composed of 8–12 Dnm1 sub-units, which can then form larger structures in a GTP-dependent manner (Ingerman *et al.*, 2005). In a manner similar to classical dynamin-1, Dnm1 can form spirals *in vitro* (Ingerman *et al.*, 2005); however, at a mean diameter of 109 nm, these spirals are a little over double the diameter measured of

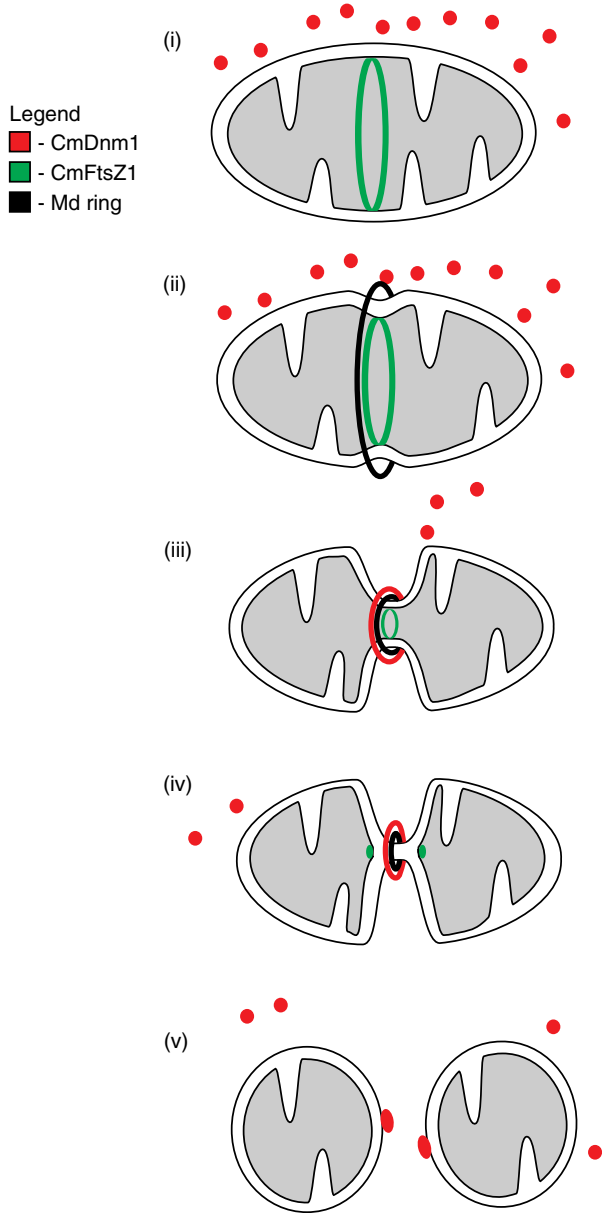



FIG. 4 Mitochondrial division in the red algae *C. merolae*. (i) The Z ring (composed of FtsZ; green) assembles first in the mitochondrial matrix, from soluble FtsZ monomers/-oligomers. CmDnm1 (red) is in cytosolic patches. (ii) The MD ring (black) forms on the outside of the outer membrane. Constriction of the mitochondrion begins. (iii) When the diameter of the constriction

dynamin-1 spirals (Chen *et al.*, 2004; Hinshaw and Schmid, 1995; Takei *et al.*, 1995; Zhang and Hinshaw, 2001). Mammalian Drp1 and yeast Dnm1 can also polymerize *in vitro* into rings that are approximately 50 nm in diameter (Legesse-Miller *et al.*, 2003; Smirnova *et al.*, 2001) and can sever artificial membranes (Sweitzer and Hinshaw, 1998; Yoon *et al.*, 2001); *in vivo*, such rings have been seen in cells where GTP hydrolysis was blocked (Ingerman *et al.*, 2005; Yoon *et al.*, 2001). Labrousse *et al.* (1999) noted that, with a diameter of 50 nm, Drp1 rings formed *in vivo* would be incapable of encircling a mitochondrial tubule, which can measure 700 nm in diameter, but this may be explained by the observation that mitochondrial constriction and localization of Dnm1 are independent events: it is only when Dnm1 is localized to a constriction point that mitochondrial division can take place (Legesse-Miller *et al.*, 2003). Such a constriction may be brought about by matrix separations, seen by Jakobs *et al.* (2003), that could pull apart the IM, causing a localized depression in the mitochondrial tubule that could then allow Dnm1 to encircle the organelle. Observations consistent with mitochondrial constriction prior to dynamin-like protein assembly have been made in the single-celled red alga *Cyanidioschyzon merolae* (Nishida *et al.*, 2003, 2004). This alga contains a single mitochondrion whose division is (necessarily) closely linked to that of the cell (Kuroiwa, 1998). *C. merolae* contains two versions of Mt-FtsZ, CmFtsZ1-1 and CmFtsZ1-2 (Miyagishima *et al.*, 1999; Takahara *et al.*, 2000), a single mitochondrial version of Dnm1, CmDnm1 (Nishida *et al.*, 2004), and, as mentioned earlier, MD-rings (Kuroiwa *et al.*, 1995)—all of which are involved in mitochondrial division. During mitochondrial division in *C. merolae*, Mt-FtsZ molecules form a ring on the matrix side of the organelle, followed by the formation of an MD ring on the cytoplasmic side. Once the mitochondrion has partially constricted, CmDnm1 is recruited from cytoplasmic patches to the division point, and final scission occurs (Nishida *et al.*, 2003) (Fig. 4). This appears to be paralleled in *Arabidopsis* plastid division. When the gene encoding the plastid dynamin-like protein (ARC5) was mutated, the plastids formed dumbbell-shaped organelles indicative of partial division lacking final scission (Gao *et al.*, 2003). Thus, as with dynamin-1 which is recruited late in endocytosis, dynamin-like proteins are apparently recruited to the mitochondria late in the division cycle, to assist in the organelle's final constriction (Griparic and van der Bliek, 2001; Labrousse *et al.*, 1999).

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point is small enough CmDnm1 is recruited to the mitochondrion. Dynamin may form rings or spirals (shown as ring). (iv) The inner-membrane constriction is finalized, wheras the outer-membrane continues constricting. (v) Final membrane severance is caused by dynamin's pinching. Some dynamin is left at the division point prior to its returning to cytoplasmic patches.

That Dnm1 was required for mitochondrial fission was discovered by Otsuga and co-workers (1998), who were screening mitochondrial morphology mutants. When the gene encoding Dnm1 was knocked out, the yeast mitochondria appeared to cease division, and collapsed to one side of the cell (Otsuga *et al.*, 1998). Subsequent studies found that the collapsed mitochondria in *dnm1*Δ cells were actually forming a net-like structure—the product of continual mitochondrial fusion, not balanced by mitochondrial division events (Bleazard *et al.*, 1999; Sesaki and Jensen, 1999). Overexpression of dynamin-like proteins in yeast and *C. elegans* lead to an increased number of mitochondrial fragments, presumably an effect of overactive division (Fukushima *et al.*, 2001; Smirnova *et al.*, 2001); also the mitochondrial fragmentation usually associated with loss of mitochondrial membrane potential can be blocked in mammalian cells by the expression of a dominant-negative Drp1 mutant (Legros *et al.*, 2002). Thus it seems that a balance between fission and fusion events, performed by Dnm1 and Fzo1/Ugo1 respectively, maintains normal mitochondrial morphology. This balance was evident in *fzo1*Δ *dnm1*Δ or *ugo1*Δ *dnm1*Δ double knockouts which displayed a phenotype similar to that of wild-type yeast mitochondria (Sesaki and Jensen, 1999, 2001). Dominant-negative mutations of the GTPase domain of both Drp1 and Mfn1 or Mfn2 also resulted in cells that had a phenotype resembling wild-type (Chen *et al.*, 2003; Santel and Fuller, 2001), indicating that other proteins exist capable of maintaining normal mitochondrial morphology in the absence of the known mitochondrial fission and fusion elements. With the exception of the loss of the mixing of mitochondrial contents following mating, yeast mitochondria do not seem to be adversely affected by the loss of these proteins.

In yeast, peroxisomes also use a dynamin-like protein, Vsp1, for their division (Hoepfner *et al.*, 2001). Mammals and *Arabidopsis* do not appear to have homologues of Vsp1 and, intriguingly in these organisms, homologues of Dlp1 and DRP3A are not only essential for mitochondrial division, but are required for peroxisomal division (Koch *et al.*, 2003, 2004; Li and Gould, 2003; Mano *et al.*, 2004) where the molecule presumably acts by pinching membranes.

## 2. Mdv1 and Caf4

By looking for null-mutants that suppress division-induced fragmentation in *fzo1* knockouts, four groups have identified two proteins involved in yeast MD. These proteins are capable of restoring a normal mitochondrial phenotype, and therefore block the loss of mtDNA. The first of these proteins is Mdv1/Fis2/Gag3/Net2 (Cerveny *et al.*, 2001; Fekkes *et al.*, 2000; Mozdy *et al.*, 2000; Tieu and Nunnari, 2000; hereafter referred to as Mdv1). Mdv1 was discovered as a suppressor of mitochondrial fragmentation in *fzo1*Δ (Mozdy

*et al.*, 2000; Tieu and Nunnari, 2000) and *mgm1*Δ cells (Fekkes *et al.*, 2000; see following) and from yeast two-hybrid interactions with Dnm1 (Cerveny *et al.*, 2001; Uetz *et al.*, 2000). Unlike Dnm1 and Fis1 (the second protein found in these screens; see later), there do not appear to be Mdv1 homologues outside the fungi. In knockouts of *mdv1*, the mitochondria form networks, similar to those seen in cells lacking Dnm1—indicating that, like *dnm1*Δ cells, *mdv1*Δ cells lack the ability to divide their mitochondria; therefore mitochondrial fusion continues unopposed (Cerveny *et al.*, 2001; Fekkes *et al.*, 2000; Mozdy *et al.*, 2000; Tieu and Nunnari, 2000). The mitochondrial networks formed are not collapsed to one side of the cell, as seen in *dnm1* knockouts, which suggests impairment of mitochondrial division is not as severe in *mdv1* knockouts as it is in *dnm1* knockouts (Griffin *et al.*, 2005)—similar to that seen in some Dlp1 mutants, where division is not abolished (Griffin *et al.*, 2005; Smirnova *et al.*, 2001).

Like *dnm1*Δ cells, *mdv1* mutants are also resistant to the mitochondrial fragmentation observed when cells are treated with sodium azide (Fekkes *et al.*, 2000). Consistent with a role in mitochondrial division, Mdv1 is associated peripherally with the mitochondrial OM at constriction points where it forms puncta that co-localize with Dnm1 puncta (Cerveny *et al.*, 2001; Fekkes *et al.*, 2000; Tieu and Nunnari, 2000). The localization of Mdv1 to puncta is reliant on Dnm1, as Mdv1 is evenly distributed over the mitochondria in *dnm1*Δ cells. On the other hand, the loss of Mdv1 does not alone affect Dnm1 localization (Fekkes *et al.*, 2000; Tieu and Nunnari, 2000). A paralogue of Mdv1 protein, Caf4 (Liu *et al.*, 2001), has been further investigated (Griffin *et al.*, 2005). Although knockouts of *caf4* alone do not produce noticeable mitochondrial defects, the combined phenotype of *caf4*Δ *mdv1*Δ cells point to a role for Caf4 in mitochondrial division. *mdv1*Δ cells have been described as having less severe mitochondrial morphological alterations than *fis1*Δ (see later) and *dnm1*Δ cells; however, when *CAF4* is also knocked out, the resulting *caf4*Δ *mdv1*Δ cells have collapsed mitochondrial networks akin to *fis1*Δ or *dnm1*Δ cells, and Dnm1 localization is lost. Thus, it seems likely that either Caf4 or Mdv1 can recruit Dnm1 to the mitochondrial membrane (Griffin *et al.*, 2005). Caf4 has high homology to Mdv1, and is predicted to have a similar tertiary structure (Griffin *et al.*, 2005). Mdv1 has a novel N-terminal region (NTE), a central coiled-coil domain, and seven C-terminal WD40 repeats predicted to form a β-propeller; the β-propeller and coiled-coil domains have been implicated in protein-protein interactions (Garcia-Higuera *et al.*, 1996; Lupas, 1996).

Experimental evidence indicates that the Mdv1 N-terminal domain interacts directly with Fis1, while the WD40 repeats are required for an interaction with Dnm1 (Cerveny and Jensen, 2003; Karren *et al.*, 2005; Tieu *et al.*, 2002); similar interactions are predicted for Caf4 (Griffin *et al.*, 2005). It has been proposed that, *in vivo*, Mdv1 co-assembles with Dnm1 puncta, at points defined

by Fis1, and acts as a molecular adaptor (Tieu and Nunnari, 2000) or, that a direct interaction between Fis1 and Mdv1 facilitates Dnm1 recruitment to division points (Karren *et al.*, 2005). Further work with Caf4 confirms an Mdv1 role in the recruitment of Dnm1 (albeit one that can be compensated for by Caf4), and a second Caf4-independent role downstream required for fission (Griffin *et al.*, 2005). This is consistent with temperature-sensitive *mdv1* mutants whose mitochondria rapidly divide once returned to the permissive temperature (Tieu and Nunnari, 2000), indicating that Caf4 has been able to recruit Dnm1 to the division site, and that it is the second role of Mdv1 that has been impaired.

Despite their importance in yeast mitochondrial division, no recognizable homologues of Mdv1 or Caf4 are evident in metazoan genomes. If Mdv1 does act as a molecular adaptor with Dnm1 and Fis1 in yeast, as proposed by Tieu and Nunnari (2000), then this intermediary is not required outside the fungi.

## 3. Fis1

The second protein found to suppress *fzo* and *mgm1* knockouts was Fis1/Mdv2 (Mozdy *et al.*, 2000; Tieu and Nunnari, 2000; hereafter referred to as Fis1), which interacts with Dnm1 as a membrane anchor for DRPs. The human homologue of Fis1 is hFis1 (James *et al.*, 2003; Karbowski *et al.*, 2002; Stojanovski *et al.*, 2004; Yoon *et al.*, 2003). In *Arabidopsis,* two Fis1 homologues have been identified: BIGYIN/AtFis1a (At3g57090) and AtFis1b (At5g12390), the knockout of BIGYIN resulted in fewer and larger mitochondria, indicative of a block in mitochondrial division (Scott *et al.*, 2006). Database searches reveal Fis1 homologues in other metazoans, *C. elegans*, and *Drosophila* (James *et al.*, 2003; Mozdy *et al.*, 2000; Stojanovski *et al.*, 2004), and we note recognizable homologues in the genomes of *Dictyostelium* (Eichinger *et al.*, 2005) and *C. merolae* (Matsuzaki *et al.*, 2004). In *S. cerevisiae* lacking *fis1*, the mitochondria form large nets akin to those of *dnm1*$\Delta$ cells (Mozdy *et al.*, 2000; Tieu and Nunnari, 2000); however, *fis1*$\Delta$ yeast do have some residual mitochondrial division as shown by time-lapse confocal microscopy (Jakobs *et al.*, 2003). Upon micro-injection of hFis1 antibodies (Yoon *et al.*, 2003), and when hFis1 levels are lowered with RNAi (Stojanovski *et al.*, 2004), mitochondria were elongated and collapsed around the nucleus (Stojanovski *et al.*, 2004; Yoon *et al.*, 2003). In yeast (but not human cells—Lee *et al.*, 2004), *fis1* knockouts produce fewer but larger Dnm1 and Mdv1 puncta, and some Mdv1 remains cytosolic (Mozdy *et al.*, 2000; Tieu and Nunnari, 2000). Thus, in yeast lacking Fis1, neither Dnm1 nor Mdv1 can assemble properly at constricting mitochondria and fission cannot progress. Unlike Dnm1 and Mdv1, Fis1 does not localize exclusively to the mitochondrial division points, but rather is distributed evenly over the outer mitochondrial membrane (Mozdy *et al.*, 2000; Tieu and Nunnari, 2000), as is

hFis1 in human cell lines (James *et al.*, 2003; Karbowski *et al.*, 2002; Stojanovski *et al.*, 2004). Fis1 and hFis1 are anchored to the mitochondrial OM by their C-terminus and have their N-terminus exposed to the cytosol (James *et al.*, 2003; Mozdy *et al.*, 2000; Stojanovski *et al.*, 2004; Yoon *et al.*, 2003). Mutants lacking the C-terminus remain cytosolic and cause a defect in mitochondrial division and, given Drp1-hFis1 interactions, the unanchored Fis1 is probably sequestering cytosolic Drp1 and thus rendering division impossible (Yoon *et al.*, 2003).

The exposed portion of Fis1 contains 6 α-helices: helices 2-3 and 4-5 are predicted to form two tetratrico-peptide repeat (TPR)-like bundles (Dohm *et al.*, 2004; Suzuki *et al.*, 2003) which together form a concave structure that allows binding of other proteins (D'Andrea and Regan, 2003; Dohm *et al.*, 2004) and is required for the binding of hFis1 to Dlp1 in human cells (Yu *et al.*, 2005). The extreme N-terminus of Fis1 varies greatly between species: compared to the human Fis1 sequence, the *Drosophila* sequence lacks the first 2 α-helices, the *Arabidopsis* sequence contains an extra 18 residues, and the yeast sequence an extra 8 (Suzuki *et al.*, 2005). Notably, the extra yeast residues form a rigid domain, which self-interacts with the concave surface of the TPR-like bundles and, given the propensity for TPR-like domains to be involved in protein-protein interactions, it has been suggested that Fis1 may interact with itself to regulate other molecular interactions (Suzuki *et al.*, 2005) at an early step in mitochondrial division (Karren *et al.*, 2005). Indeed, the domain is required for the localization of Mdv1 (Suzuki *et al.*, 2005).

Expression of Fis1-GFP in yeast (in conjunction with native expression, thus increasing the total levels of Fis1 in the cell) does not appear to affect mitochondrial morphology (Mozdy *et al.*, 2000). In human cell lines, however, the effects of elevated hFis1 can be profound. Overexpression of hFis1 has been found to cause mitochondria to fragment and degenerate into small punctiform organelles surrounding the nucleus (James *et al.*, 2003; Stojanovski *et al.*, 2004; Yoon *et al.*, 2003)—differing from the more distributed pattern of fragmented mitochondria seen in Drp1 overexpressers (Oakes and Korsmeyer, 2004; Szabadkai *et al.*, 2004). hFis1 overexpression can be counteracted by dominant-negative forms of Drp1 (James *et al.*, 2003; Stojanovski *et al.*, 2004). The punctiform mitochondrial phenotype of hFis1 overexpressing cells (James *et al.*, 2003; Stojanovski *et al.*, 2004; Yoon *et al.*, 2003) is similar to that seen in apoptotic cells (see following) (Desagher and Martinou, 2000; Frank *et al.*, 2001), which is consistent with the finding that these cells release cytochrome *c* from their mitochondria and eventually undergo apoptosis (James *et al.*, 2003). Apoptosis in hFis1 overexpressing cells was blocked by coexpression of the anti-apoptotic protein Bcl-$x_L$, greatly reducing cytochrome *c* release, but not affecting punctiform mitochondrial morphology. Expression of $Drp1^{K38A}$ also greatly reduced cytochrome *c* loss, but did not prevent apoptosis (James *et al.*, 2003). Taken together, these data indicate that in human cells, hFis1 levels are

important for maintaining mitochondrial morphology, perturbation of which may lead to apoptosis.

Several models for the action of Fis1 have been put forward. Oakes and Korsmeyer (2004) suggested that Fis1 may act as an anchor for proteins besides Dnm1 and, as such, may also act in Dnm1-independent roles. Based on the dramatic increase in mitochondrial fission on hFis1 overexpression, it has been proposed that hFis1 is a limiting factor in mitochondrial fission in mammalian systems (Stojanovski *et al.*, 2004; Yoon *et al.*, 2003); further, based upon the lack of phenotypic change in overexpressed N-terminal truncated hFis1, at least one role of hFis1 may be to recruit Drp1 to the outer mitochondrial membrane via interactions at the hFis1 N-terminus (Yoon *et al.*, 2003). It has been suggested that Fis1 has two roles in yeast mitochondrial division (Tieu *et al.*, 2002). Given that Fis1 is required for Dnm1 localization to mitochondria, Fis1's first role is thought to be in the assembly of Dnm1 into higher-order structures. Dimeric Mdv1 is then thought to co-assemble with Dnm1 and Fis1, allowing fission to take place. Tieu and co-workers (2002) have proposed that the NTE region of Mdv1 may act as a molecular switch. In this model, the NTE region of Mdv1 interacts with Fis1, causing a conformational change in the Dnm1 structures with which Mdv1 interacts via its WD-repeat domain; these changes in Dnm1 may bring about division of the mitochondrial membranes (Tieu *et al.*, 2002). Yu *et al.* (2005) have suggested a compatible scenario, where the $\alpha$1 helix of hFis1 has a role in the regulation of Dlp1 via a two-step process involving a hFis1-Drp1 binding stage and an hFis1-independent stage. In such a model, self-interaction of hFis1 $\alpha$1 (and, most likely, the rigid domain of Fis1 in yeast) would block the Dlp1 binding site for much of the Fis1 distributed across the membrane, except at the point of division; Fis1 would thus define the mitochondrial division site. Appropriate levels of Dlp1 could then be accumulated to nucleate self-assembly (Ingerman *et al.*, 2005), before the second stage which would see Fis1 release Dlp1 and allow it to directly bind to the outer mitochondrial membrane for scission (Yu *et al.*, 2005).

As mentioned earlier, peroxisomes in some organisms require Dnm1 for fission. It may not be surprising then, that at least in mammalian cells, Fis1 is also necessary for peroxisomal division. Koch *et al.* (2005) showed that mammalian peroxisomes undergo elongation and increased division when Fis1 is overexpressed. Interestingly, when Fis1 overexpression is coupled with the dominant-negative $Drp^{K38A}$, despite a cease in peroxisome division, the elongation effect was still seen, indicating that Fis1 overexpression is affecting other components responsible for peroxisomal division and/or morphology (Koch *et al.*, 2005).

## 4. GDAP1

Charcot-Marie-Tooth disease 2A has been associated with mutations in the mitochondrial fusion protein Mfn2 (Kijima *et al.*, 2005; Züchner *et al.*,

2004) and, interestingly, a gene implicated in another form of CMT, CMT 4A, has recently been identified as being required for mitochondrial division. Ganglioside differentiation-associated protein1 (GDAP1) was identified as a gene whose expression was increased after induction of differentiation in mouse neuroblastomas (Liu *et al.*, 1999); it is primarily expressed in neuronal cells (Pedrola *et al.*, 2005) and, over the last few years, GDAP1 has also been implicated in CMT 4A (Baxter *et al.*, 2002; Cuesta *et al.*, 2002; Di Maria *et al.*, 2004; Nelis *et al.*, 2002). GDAP1 is anchored to the outer mitochondrial membrane by C-terminal transmembrane domains (Marco *et al.*, 2004; Niemann *et al.*, 2005; Pedrola *et al.*, 2005). Perturbations in GDAP1 expression were seen to affect the morphology of the mitochondrial network: over expression led to Drp1-dependent fragmentation of the mitochondrial network and GDAP1 knock-down resulted in mitochondrial elongation (Niemann *et al.*, 2005). No mitochondrial fragmentation was observed, however, in cells overexpressing previously identified CMT 4A variants of the *GDAP1* gene (Ammar *et al.*, 2003; Azzedine *et al.*, 2003; Baxter *et al.*, 2002; Boerkoel *et al.*, 2003; Cuesta *et al.*, 2002; Di Maria *et al.*, 2004; Nelis *et al.*, 2002; Senderek *et al.*, 2003), strongly suggesting that it is these mutations that are underlying pathology in CMT 4A patients (Niemann *et al.*, 2005). Thus, GDAP1 is a component of the mitochondrial division apparatus in mammalian neuronal cells and, though the effects of the loss of GDAP1 are not as drastic as those seen following the loss of other division proteins such as Drp1 (which is presumably still active), the loss does lead to a deterioration of axonal nerves (Niemann *et al.*, 2005).

## 5. Mdm33

Extensive screening of a yeast deletion library has revealed 10 new mitochondrial distribution and morphology (Mdm) proteins in *S. cerevisiae* (Dimmer *et al.*, 2002). Mdm30 is thought to be required for the regulation of Fzo1 (Fritz *et al.*, 2003). Mdm31 and Mdm32 are IM proteins that are similar to each other and likely to be the result of a recent gene duplication and it has been proposed that they may play a role in the attachment of mtDNA to the mitochondrial segregation machinery (Dimmer *et al.*, 2005). Perturbations of Mdm31, Mdm32, or both proteins, give rise to cells with giant spherical mitochondria that are deficient in segregation to daughter buds, and whose cristae are typically absent (Dimmer *et al.*, 2005). These defects clearly extend beyond the division defects seen when disrupting proteins such as Dnm1 or Fis1; such mitochondria also lack normal mobility, and it is possible that Mdm31 and Mdm32 are linked to the mmm1-containing mitochondrial segregation machinery in the OM (Dimmer *et al.*, 2005).

Mdm33, on the other hand, appears to be a *bona fide* mitochondrial division protein, knockouts of which cause the mitochondria to take a giant ring-like morphology (Dimmer *et al.*, 2002; Messerschmitt *et al.*, 2003).

Three-dimensionally, the ring-like morphology is seen as a hollow sphere, which Messerschmitt and co-authors (2003) propose is the result of mitochondria failing to divide and continuing to fuse in three dimensions (the giant ring-like mitochondria of *mdm33*Δ cells are fusion competent). This phenotype is not observed when other elements of the mitochondrial division apparatus are removed, and implies that the tightly controlled integrity of the mitochondrial tubule is somehow lost in *mdm33*Δ cells. In *mdm33*Δ *fis1*Δ cells, the mitochondria continue to form large ring-like structures, indicating that Mdm33 acts in constriction earlier than Fis1 does. Conversely, when *mdm33*Δ *fzo1*Δ cells are generated, the mitochondria have a *fzo1*Δ phenotype. Overexpression of Mdm33 causes fragmentation and/or aggregation of mitochondria, presumably as a result of excess mitochondrial division. EM of mitochondria in Mdm33 overexpressing cells shows numerous IM partitions within the mitochondrion. IM partitions have been noted in the past, generally in chemically treated cells, but also in rapidly dividing cells (Griparic and van der Bliek, 2001). It is important to note that the human and yeast mitochondrial division proteins mentioned thus far are all thought to act in the division of the mitochondrial OM. No IM division proteins have been described in yeast or animals (MtFtsZs are absent in these organisms), and Mdm33 may represent the first such protein. The model presented by Messerschmitt *et al.* (2003) describes IM constriction being mediated by Mdm33, which forms bridging interactions with other Mdm33 molecules on the opposing side of the mitochondrion causing IM constriction and subsequent inward deformation of the OM. This allows Dnm1 to encircle the mitochondrion (in a Fis1-dependent manner) and sever the organelle.

## 6. Clu1

The *Dictyostelium* protein, CluA, is required for the normal distribution of mitochondria (Zhu *et al.*, 1997). When the *cluA* gene is deleted (*cluA*$^-$), the mitochondria, which in wild-type amoebae are distributed evenly throughout the cytoplasm, cluster together near the center of the cell (Zhu *et al.*, 1997). The mitochondrial defect seen in *Dictyostelium cluA*$^-$ cells appears to have an effect on the cell as a whole, as the overall growth rate slows and cytokinesis sometimes fails (Zhu *et al.*, 1997). The clustering of mitochondria in *cluA*$^-$ amoebae does not appear to result from a defect in the cytoskeleton, as actin or microtubule destabilizing agents fail to alter the mitochondrial distribution, and peroxisomes (which use the same transport system) showed a wild-type distribution (Fields *et al.*, 2002). Examination of mitochondrial clusters in *cluA*$^-$ amoeba using EM showed that the individual organelles appeared to have failed to complete division: the outer mitochondrial membrane being continuous between two neighbors (Fields *et al.*,

2002). The mitochondria within clusters were often connected to neighboring mitochondria by numerous narrow constrictions, suggesting a failure of division late in the process (Fields *et al.*, 2002).

Homologues of *cluA* have been found in the genomes of mammals, insects, *C. elegans,* and *S. cerevisiae* (Fields *et al.*, 1998; Zhu *et al.*, 1997). Partial complementation of *Dictyostelium cluA*$^-$ amoebae with the yeast *clu1* was found to be possible: growth rates were restored, but aggregates were still formed, albeit smaller, more distributed ones (Fields *et al.*, 1998). Knockouts of *S. cerevisiae clu1* had an effect on yeast mitochondrial distribution similar to that seen in *Dictyostelium*, as the mitochondria aggregated, collapsing to one side of the cell (Fields *et al.*, 1998). Unlike *cluA*$^-$ *Dictyostelium* cells, however, *clu1*$\Delta$ yeast did not suffer growth defects, and the inheritance of mitochondria to buds appeared to progress normally (Fields *et al.*, 1998). It is unknown whether the mitochondrial aggregates in yeast have membrane connections similar to those in *Dictyostelium cluA*$^-$ amoebae (Fields *et al.*, 2002).

## V. Mitochondrial Division and Apoptosis

Apoptosis, an essential process for normal development of metazoan tissues (Vaux and Korsmeyer, 1999), has been conserved in yeast (Madeo *et al.*, 2002) and various forms of cell death are now recognized in plants (van Doorn and Woltering, 2005). In animals, the release of cytochrome *c* from the intermembrane space of mitochondria is recognized as an early step in apoptosis (Budihardjo *et al.*, 1999; Liu *et al.*, 1996) and one that is necessary for apoptosis under many stimuli (Li *et al.*, 2000). The method by which cytochrome *c* is released into the cell is not yet known, but studies using cytochrome *c*-GFP have shown that the process is fast (Goldstein *et al.*, 2000; Green and Kroemer, 2004) and does not appear to damage the organelles (Frey *et al.*, 2002). An increasingly important link exists between mitochondrial division and apoptosis. Upon induction of apoptosis by various stimuli, mitochondria stretch, then undergo division approximately 10 minutes after the release of cytochrome *c* (Arnoult *et al.*, 2005; Esseiva *et al.*, 2004; Gao *et al.*, 2001), resulting in small punctate mitochondria that cluster around the nucleus (Desagher and Martinou, 2000; Frank *et al.*, 2001). During this process, an important pro-apoptotic factor, Bax (Wei *et al.*, 2001), becomes localized to dividing mitochondria (Karbowski *et al.*, 2002) and is associated with the cessation of mitochondrial fusion and the release of Smac/DIABLO-CFP (Karbowski *et al.*, 2004), which is known to be released concurrently with cytochrome *c* (Rehm *et al.*, 2003). The Bcl-2 family members, Bax and Bak, coalesce during apoptosis at mitochondrial foci (Nechushtan *et al.*, 2001), and that in turn colocalize with both Drp1 and Mfn2 (Karbowski *et al.*, 2002), Drp1 itself being required in the

apoptotic process. After treatment with numerous apoptotic stimuli, Drp1 localizes to mitochondria where it is required for mitochondrial scission (Frank *et al.*, 2001; Karbowski *et al.*, 2002). Expression of dominant-negative Drp mutants (that disrupt mitochondrial division) or Drp deletions give rise to cells that have decreased mitochondrial fragmentation and delayed apoptosis (Frank *et al.*, 2001; Karbowski *et al.*, 2002). Frank *et al.* (2001) found that $Drp1^{K38A}$ cells (dominant-negative mutants) maintained their mitochondrial membrane potential and that loss of cytochrome *c* to the cytoplasm was prevented. In *C. elegans*, it has recently been found that expression of DRP-1 dominant-negative mutants caused the inappropriate survival of cells that would normally die during worm development (Jagasia *et al.*, 2005). Over-expression of Drp1, on the other hand, has been shown to sensitize cells to $Ca^{2+}$-independent apoptosis (Karbowski *et al.*, 2002; Szabadkai *et al.*, 2004). Conversely, Drp1 overexpression can protect cells against $Ca^{2+}$-mediated apoptosis, as excessive fragmentation renders the mitochondria incapable of propagating calcium waves (Szabadkai *et al.*, 2004). In mammalian cells, Drp1 overexpression alone does not induce apoptosis (Szabadkai *et al.*, 2004), however overexpression of yeast Dnm1 can bring about cell death (Fannjiang *et al.*, 2004) and *C. elegans* DRP-1 over-expression brings about inappropriate programmed cell death (Jagasia *et al.*, 2005).

Perhaps not surprisingly, overexpression of hFis1, another mitochondrial division protein, also has pro-apoptotic affects (James *et al.*, 2003; Lee *et al.*, 2004; Stojanovski *et al.*, 2004). hFis1 depletion can stop Bax translocation, and cytochrome *c* release, and protect cells from death to a greater degree than reduction of Drp1 levels (Lee *et al.*, 2004). In yeast, Fannjiang *et al.* (2004) found the opposite: *fis1*$\Delta$ cells were more susceptible to programmed cell death, and the presence of Fis1 protected cells from programmed cell death through its interactions with Dnm1. Given the role of hFis1/Fis1 in the binding of Dlp1 in mitochondrial division, it is not unexpected that this interaction is required during apoptosis; Kong *et al.* (2005) have shown that the anti-apoptotic factor Bcl-2 can compete with Dlp1 for binding of hFis1. Bcl-2 was shown to co-immunoprecipitate with Fis1 and, in Bcl-2 overexpressing cells, Dlp1 binding of hFis1 is lost—suggesting that the binding of Bcl-2 and Dlp1 to hFis1 are mutually exclusive events (Kong *et al.*, 2005). Thus, given the requirement of Dlp1 for apoptosis, it may be that Bcl-2 can block apoptosis by blocking the ability of Dlp1 to interact with its most important partner, Fis1. Yeast lack components of the mammalian programmed cell death pathway, such as the Bcl-2 family of proteins, and the relationship between their Fis1 and Dnm1 homologues differ (Lee *et al.*, 2004). Intriguingly, expression of the anti-apoptotic Bcl-2 in *fis1*$\Delta$ yeast helps protect the cells from death (Fannjiang *et al.*, 2004); presumably Bcl-2 is still capable of binding Fis1, and therefore blocking Dnm1.

Mammalian mitochondrial division is usually balanced by mitochondrial fusion, and thus the role of mitochondrial fusion regulators in apoptosis is of interest. The Fzo1 homologue, Mfn2, colocalizes with Bax in apoptotic cells (Karbowski *et al.*, 2002), accompanied by a cessation of mitochondrial fusion (Karbowski *et al.*, 2004). The dominant-active $Mfn2_{G12V}$ protects cells from mitochondrial fragmentation, and Bax localization is lost (Neuspiel *et al.*, 2005). In light of these events, two alternate modes of Mfn2 action in apoptotic mitochondrial events have been proposed (Neuspiel *et al.*, 2005). First, inactive Mfn2 molecules form channels, for permeability transition, to which Bax localizes (but cannot do in $Mfn2_{G12V}$ cells), or second that fission and fusion events are mutually exclusive (and thus a constitutively active Mfn2 blocks the fission required for apoptosis).

The mitochondrial fusion protein, OPA1, appears to be necessary to prevent unwanted apoptosis; OPA1 depletion causes cells to become sensitive to apoptosis, to the point where some OPA1-depleted cells will undergo apoptosis spontaneously; this can be counteracted by overexpression of Bcl-2 (Lee *et al.*, 2004; Olichon *et al.*, 2003). In keeping with this, Arnoult *et al.* (2005) have shown that OPA1 is released together with cytochrome *c*, and have postulated that the loss of OPA1 causes a remodeling of mitochondrial cristae, and that this allows the release of cytochrome *c* (and thus OPA1 knockdown cells are more susceptible to cytochrome *c* release and apoptosis). The subsequent loss of OPA1 can also explain the fragmentation of mitochondria after cytochrome *c* loss, and the protection from apoptosis provided by $Drp^{K38\AA}$ as these cells suffer more broad malformations to their mitochondria, which may extend to the cristae (Arnoult *et al.*, 2005). Thus it has been inferred, not unreasonably, that the lack of normal OPA1 in dominant optic atrophy may cause cell death leading to blindness (Karbowski *et al.*, 2004).

## VI. Concluding Remarks

Mitochondrial division is a process essential to all eukaryotes; it is required to continue the inheritance of mitochondria to future generations. Mitochondrial division is also essential for maintaining normal organellar morphology in organisms that have mitochondria that constantly fuse. Following the acquisition of the original α-proteobacterium over a billion years ago, it is likely that the endosymbiont initially controlled its own division process. The division of mitochondria would have been mediated by the endosymbiont's FtsZ-based division apparatus. It is not hard to imagine that a mechano-enzyme such as dynamin, that already had a role in membrane pinching (and was probably part of the machinery that endocytosed the α-proteobacterium), could be harnessed

to divide the OM (van der Bleik, 2000). It is likely that the utilization of dynamin in outer mitochondrial membrane constriction was an early event in mitochondrial evolution, as dynamin-like proteins can be seen in a vast array of eukaryotes, including animals, yeast, slime molds, red algae, and plants (Fig. 5). When one contrasts the ancient and ubiquitous nature of dynamin-like proteins to the paucity of dynamin-1 homologues (e.g., Praefcke and McMahon, 2004), the possibility arises that mitochondrial dynamin-like proteins were the ancestral

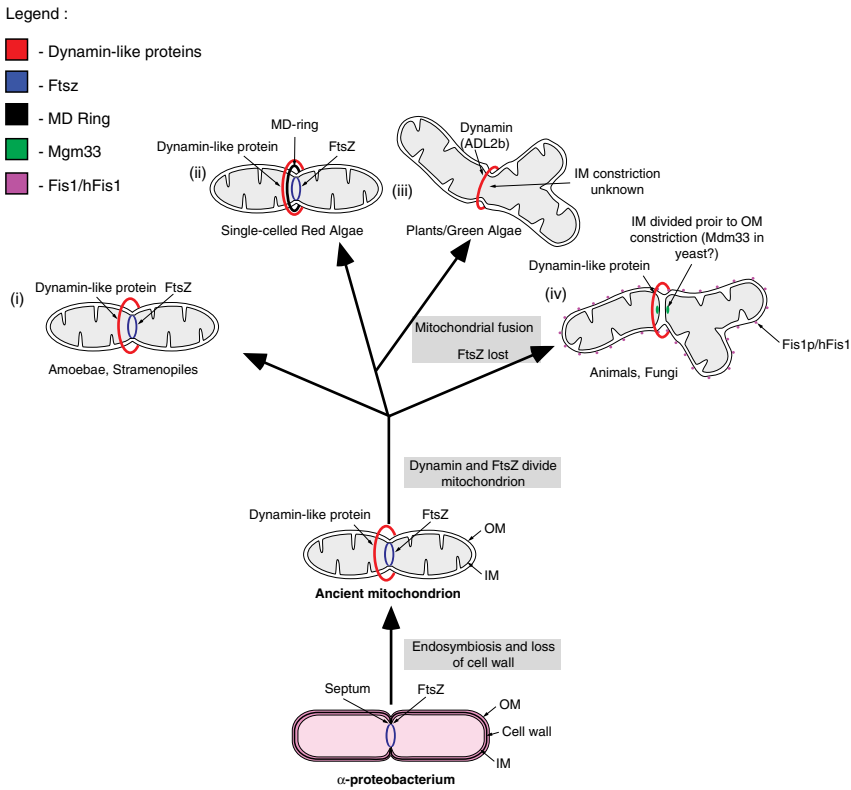


FIG. 5 Evolution of mitochondrial division mechanisms: the original α-proteobacterial endosymbiont would have used FtsZ as a major component of its division system. Early in mitochondrial evolution, dynamin-like proteins were recruited to divide the outer mitochondrial membrane. (i) In some (and perhaps many) protists, FtsZ has been maintained and is used in conjunction with dynamin-like proteins. (ii) The single-celled red algae also still use mitochondrial FtsZ, and dynamin-like proteins, in conjunction with MD (mitochondrion dividing) rings. (iii) Plants have retained dynamin, and lost FtsZ; there are no known elements of the plant mitochondrial IM division apparatus. (iv) Animals and yeast use dynamin-like proteins, in conjunction with Fis1 to divide the outer mitochondrial membrane. Yeast may use Mgm33 to divide the IM.

dynamins (Kuroiwa *et al.*, 2006) that have since given rise to the other dynamin family members.

While the ancestral α-proteobacterial–derived division protein FtsZ has been maintained in a diverse assemblage of protists (Gilson and Beech, 2001), it has been lost at least three separate times (animal, green algal/plant, and apicomplexan lineages). When looking at organisms that have retained MtFtsZs, two common points are striking. First, the mitochondria of these organisms are, in many (but not all) cases, decidedly bacterial-shaped (Kiefel *et al.*, 2004). Second, the mitochondrial fusion seen in organisms such as yeast or animals is not seen, or at least occurs at a much lower level. Across broad groups, mitochondrial fusion appears to use different molecules. *A. thaliana*, for example, has no identified versions of Fzo1, Ugo1, or Mgm1 (Praefcke and McMahon, 2004)—this may indicate an independent origin and evolution of mitochondrial fusion in plants (and other organisms). It is tempting to speculate on the origins of mitochondrial fusion and the loss of MtFtsZs. As has been noted in the past (Sesaki *et al.*, 2003a), mitochondrial division and fusion are essentially the same reactions running in opposite directions, the order in which membranes are fused is simply reversed (Fig. 6). It may be, then, that components of the IM fusion apparatus have evolved to perform two different membrane fusion reactions (division and fusion) depending on which other molecules they interact with. In such circumstances one can imagine how the mitochondrial division protein, FtsZ, could become redundant. One possible candidate for such a dual-function protein is Mgm1. Mgm1 is clearly a major component of the mitochondrial fusion machinery (Sesaki *et al.*, 2003b; Wong *et al.*, 2003); however, because we can think of mitochondrial fission and fusion as being

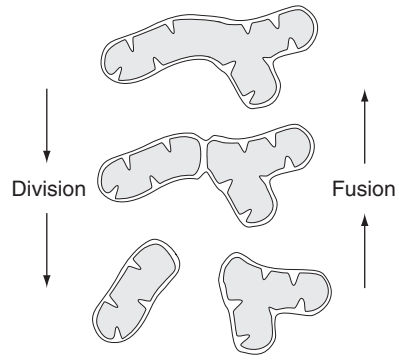


FIG. 6 Mitochondrial division and fusion are essentially the same processes, running in opposite directions. Mitochondrial division requires the fusion of the IM, followed by the outer. Mitochondrial fusion involves fusion of OMs and then fusion of the IMs.

similar processes working in opposite directions, a protein involved in both processes would need to be in the intermembrane space. Mgm1 is a dynamin-like GTPase (Sesaki *et al.*, 2003a; Wong *et al.*, 2003) which resides in the intermembrane space (Wong *et al.*, 2000, 2003) precisely the location it would need to be in to perform an action like that of dynamin, which needs to constrict from the outside.

Speculation aside, in all the organisms that lack MtFtsZ, only one protein, Mdm33, has been identified, which may be involved in mitochondrial IM division. Mdm33 is involved in the partitioning of the mitochondrial IM, and probably IM division (Messerschmitt *et al.*, 2003). No homologues of Mdm33, however, are apparent in animal genomes, and this protein may have evolved to fill a unique role in yeast mitochondrial division.

The balance between division and fusion maintains the morphology of mitochondrial networks, and the evolution of this dynamic balance has allowed cells to quickly shift their morphology should the need arise. In the case of programmed cell death, mitochondrial fragmentation is a frequently noted step which requires mitochondrial division proteins. Increased expression of hFis1 can help promote apoptotic events (James *et al.*, 2003; Lee *et al.*, 2004; Stojanovski *et al.*, 2004) and overexpression of dynamin-like proteins in *S. cerevisiae* (Fannjiang *et al.*, 2004) and *C.elegans* (Jagasia *et al.*, 2005) can have similar effects, suggesting not only that mitochondria fragment prior to apoptosis, but mitochondrial fragmentation can trigger the process. Apoptosis has been suggested to have a role in cell death associated with dominant optic atrophy; the mutation in *OPA1* renders fusion incapable of balancing mitochondrial division, the resulting fragmentation in turn promotes apoptosis (Karbowski *et al.*, 2004).

It should be pointed out that recently (Delivani *et al.*, 2006) the events of mitochondrial division and apoptosis in human cells have been proven separable via the expression of *C. elegans* CED-9 (a Bcl-2–related protein) in human cells. In these experiments, CED-9 was shown to be capable of inducing mitochondrial fusion (and was associated with Mfn-2), and thus preventing division, but not apoptosis. It is also noteworthy that the CED-9 antagonist, EGL-1, appears to be able to fragment *C. elegans* mitochondria (Jagasia *et al.*, 2005), and thus the EGL-1 and CED-9 proteins appear to have dual but separable roles in the maintenance of mitochondrial morphology, and apoptosis (Delivani *et al.*, 2006; Estaquier and Arnaud, 2006).

The finding that *GDAP1* is required for mitochondrial division in neuronal cells (Niemann *et al.*, 2005) identifies the first known mitochondrial division defect to cause a human disease: Charcot-Marie-Tooth disease 4A. GDAP1 is expressed primarily in neurons (Niemann *et al.*, 2005; Pedrola *et al.*, 2005) and its loss does not appear to halt mitochondrial division, but causes mitochondrial elongation; this possibly reflects Drp1 and hFis1 continuing to divide mitochondria to some extent. No known human conditions are associated

with *Drp1* or *Fis1* mutations, which stresses the importance of mitochondrial division, as mutations in its core components are presumably lethal. Additionally, two mitochondrial fusion defects are associated with disease: Charcot-Marie-Tooth disease: 2A and dominant optic atrophy are caused by mutants of *mfn2* and *OPA1,* respectively. Again these conditions are characterized by the degeneration of neurons, the lack (or lowered level) of mitochondrial fusion (and the associated increase in local mtDNA mutations) being less tolerable in the heavy energy requirements of neurons. Future research will no doubt continue to tease out the specifics of these mitochondrial morphology-based disorders, as well as identify other diseases whose underpinnings lie in the proteins responsible for mitochondrial fusion and division.

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# A Two Decade Contribution of Molecular Cell Biology to the Centennial of Alzheimer's Disease: Are We Progressing Toward Therapy?

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Alzheimer's disease (AD), described for the first time 100 years ago, is a neurodegenerative disease characterized by two neuropathological hallmarks: neurofibrillary tangles containing hyperphosphorylated tau and senile plaques. These lesions are likely initiated by an imbalance between production and clearance of amyloid β, leading to increased oligomerization of these peptides, formation of amyloid plaques in the brain of the patient, and final dementia. Amyloid β is generated from amyloid precursor protein (APP) by subsequent β- and γ-secretase cleavage, the latter being a multiprotein complex consisting of presenilin-1 or -2, nicastrin, APH-1, and PEN-2. Alternatively, APP can be cleaved by α- and γ-secretase, precluding the production of Aβ. In this review, we discuss the major breakthroughs during the past two decades of molecular cell biology and the current genetic and cell biological state of the art on APP proteolysis, including structure–function relationships and subcellular localization. Finally, potential directions for cell biological research toward the development of AD therapies are briefly discussed.

**KEY WORDS:** Alzheimer's disease, APP, ADAM10, BACE1, Presenilin, γ-Secretase, Regulated intramembrane proteolysis, Amyloid β. 



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## I. Alzheimer's Disease: A 100-Year History

Of all neurodegenerative diseases, Alzheimer's disease (AD), ranked third in the list of causes of death in the industrialized world after cardiovascular diseases and cancer, is by far the most common form of senile dementia. Exactly 100 years ago, during a psychiatry meeting in Tübingen on November 3 1906, Alois Alzheimer reported neuropathological lesions, described as "*miliary foci in the cerebral cortex*" and "*dense bundles of fibrils within the cytoplasm of neurons*," in the autopsied brain of the 51-year old patient Auguste D., who had been diagnosed with "presenile dementia." These two main neuropathological hallmarks of AD were later called senile or amyloid plaques and neurofibrillary tangles (NFT), respectively, but the precise nature of these aggregates was unknown. For several decades, little progress was made toward understanding of AD. After the development of electron microscopy, ultrastructural studies in the 1960s revealed the amyloid core of senile plaques, as well as the paired helical nature of NFTs in the cytoplasm of neuronal cell bodies and neurites. In the late 1970s, the deficit in central cholinergic transmission caused by degeneration of cholinergic neurons was recognized as an important pathological and neurochemical feature of AD (Davies and Maloney, 1976; Whitehouse *et al.*, 1982). This led to the development of therapies aimed at improving cholinergic transmission. Together with an NMDA-channel blocking drug, these drugs are still the only approved treatments for AD. However, they can only delay the progress of the disease.

A major advance in our understanding of the genetic and molecular aspects of AD was made in 1984 with the biochemical identification of amyloid β peptide (Aβ) as the main component of senile plaques (Glenner and Wong, 1984; Masters *et al.*, 1985). A few years later, amyloid β peptides were shown to be generated by proteolytic cleavage of a larger amyloid precursor protein (APP) (Fig. 1A) and the APP gene was mapped to chromosome 21q21 (Goldgaber *et al.*, 1987; Kang *et al.*, 1987; Robakis *et al.*, 1987; Tanzi *et al.*, 1987). At about the same time, the NFTs were shown to consist of insoluble polymers of hyperphosphorylated tau (Grundke-Iqbal *et al.*, 1986), a protein that in normal conditions coassembles with tubulin on microtubules to stabilize them [therefore also referred to as microtubule-associated protein tau (MAPT)] (Iqbal *et al.*, 2005).

Following its identification, the Aβ peptide was proposed to be the initiator of a complex multistep pathological cascade leading to plaques, but also to NFTs, neuronal dysfunction, and finally dementia. This "amyloid-tangle cascade hypothesis" is by far the dominant theory in the field (Hardy, 2006) (http://www.alzforum.org/res/adh/cur/knowntheamyloidcascade.asp).
According to this theory, AD pathogenesis is initiated by an imbalance between the production and clearance of Aβ. This may lead to a relative

higher ratio of Aβ42 [containing 42 amino acids (aa)] over the usual Aβ40. These two extra amino acids make Aβ42 more prone to misfolding, aggregation, and precipitation (Jarrett and Lansbury, 1993), explaining why Aβ42 is a plaque core component in classical late onset AD. Indeed, excessive Aβ42 oligomerizes in limbic and association cortices, and these soluble Aβ oligomers are thought to be the major neurotoxic species, compromising, e.g., synaptic function (Cleary *et al.*, 2005). Afterward, as Aβ oligomers are gradually deposited as diffuse plaques (which probably function as reservoirs of insoluble Aβ maintaining an equilibrium with soluble Aβ), local microglia and astrocytes in its vicinity get activated, leading to inflammatory responses, oxidative injury, and altered neuronal ionic homeostasis. Moreover, the activity of several kinases and phosphatases is also altered, causing abnormal tau phosphorylation and NFT formation. All these effects lead to neuronal and synaptic dysfunction, potentially preceded by dysfunctions in axonal transport (Section II.C.2) (Roy *et al.*, 2005) and in the endosomal/lysosomal system (Section IV.B.3) (Glabe, 2001; Nixon, 2005). This selective neuronal loss results in neurotransmittter deficits and finally in dementia in AD patients. However, the exact mechanisms linking Aβ to neuronal dysfunction are not fully understood (Selkoe, 2004; Small *et al.*, 2001), as well as the (probably multifactorial) physiological events initiating or leading to this pathogenic process. Misregulations in cholesterol homeostasis (Section II.C.3) (Grimm *et al.*, 2005; Koudinov and Koudinova, 2005) deranged neuronal calcium signaling (Section IV.B.3) (Tu *et al.*, 2006) or combined oxidative stress and aberrant mitotic stimuli [the "two-hit" hypothesis (Zhu *et al.*, 2004)] may represent only a few of the possibilities. However, damage of the vascular system is also an important factor contributing to AD pathogenesis (Dermaut *et al.*, 2005). (For an overview of current AD pathogenesis hypotheses see http://www.alzforum.org/res/adh/cur/default.asp.)

The identification of Aβ in amyloid plaques pointed to the respective *APP* gene as the site to search for pathogenic mutations in families with an autosomal dominant early onset form of the disease (familial AD or FAD). Indeed, the first FAD-linked mutations were found in the early 1990s and about 20 mutations have since been identified (Fig. 1B). All mutations appear to be clustered at or near the proteolytic cleavage sites in APP and cause an increase in either total Aβ or in the ratio of Aβ42 to Aβ40, both *in vitro* as well as *in vivo* in transgenic mice and affected individuals (Annaert and De Strooper, 2002; Selkoe, 2004). In addition, some *APP* mutations near the middle of the Aβ region enhance Aβ oligomerization and fibril formation, while the Dutch, Flemish, Italian, and Arctic mutations render Aβ more resistant to proteolytic degradation by neprilysin, the peptidase with the most important role in catabolism of Aβ in the brain (Tsubuki *et al.*, 2003). Hence, these mutations are pathogenic by facilitating fibrillogenesis and by extending the half-life of Aβ in the brain, disturbing the balance between production and clearance of Aβ. Interestingly, gene dosage variability resulting from the duplication of

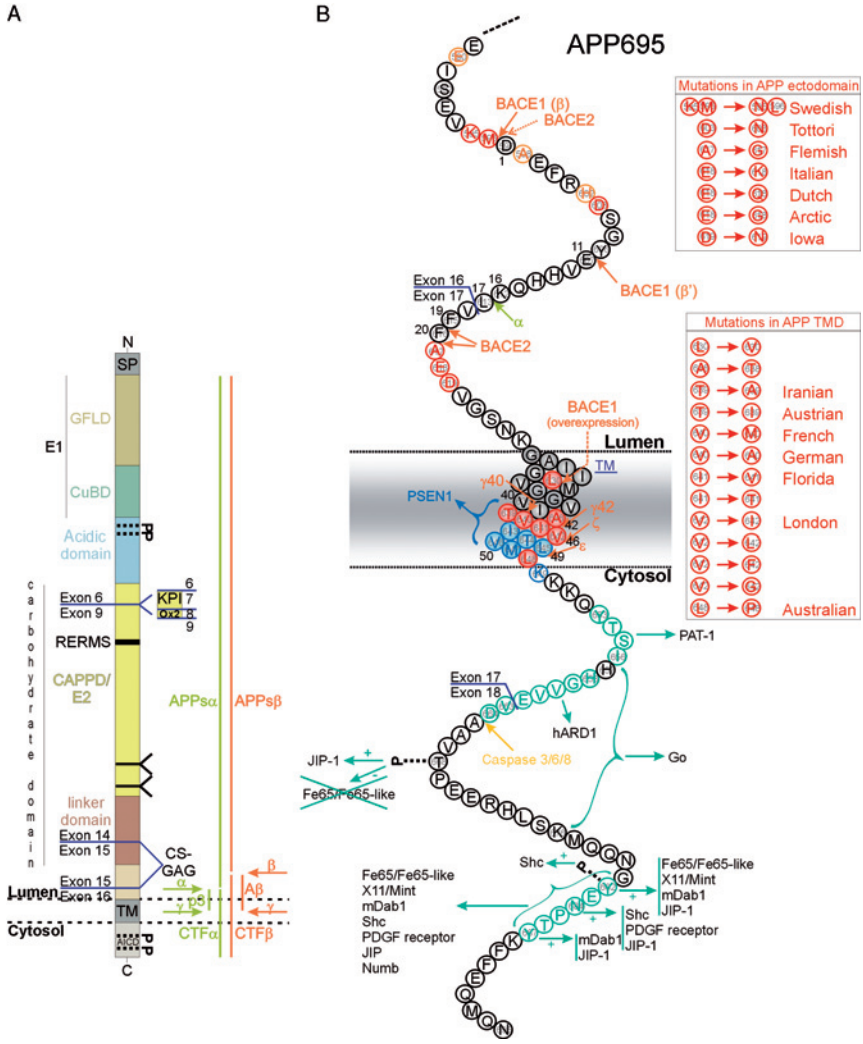


FIG. 1 APP is a type I transmembrane protein with several functional domains. (A) Overall domain structure and processing products of APP, showing some exon boundaries. The KPI domain, Ox2 sequence, and CS-GAG attachment site in APP751/770, resulting from alternative splicing of exons 7, 8, or 15, are indicated. Glycosylation sites are depicted by ⋎ and phosphorylation sites by "". The E1 region contains a GFLD and a CuBD, and is separated via an acidic domain from the carbohydrate domain. This latter domain is subdivided into an E2 or CAPPD, which contains the RERMS sequence, and a linker domain. SP, signal peptide; TM, transmembrane domain; AICD, APP intracellular domain. Cleavage of APP by α-secretase (light green) or β-secretase (orange) leads to the secretion of APPsα or APPsβ and generates APP-CTFα or APP-CTFβ, respectively. These fragments are subsequently cleaved by γ-secretase, producing p3 and Aβ, respectively. (B) Detailed amino acid representation of the human APP695 IC domain,

the *APP* locus or from mutations in the *APP* promoter leading to increased APP expression, was recently shown to be associated with AD and/or cause early onset AD and cerebral amyloid angiopathy, (Rovelet-Lecrux *et al.*, 2006; Theuns *et al.*, 2006) providing an additional strong argument for the amyloid tangle cascade hypothesis.

In the past decade, all genes encoding the enigmatic secretases generating Aβ or preventing its production were discovered, starting with the *presenilin* (PSEN) genes in 1995. Currently, over 150 FAD mutations have been found in *PSEN1*, but only about 10 in *PSEN2* (Section IV.A). In parallel, mutations related to frontotemporal dementia (FTD) were found in the *tau* gene (http://www.molgen.ua.ac.be/ADMutations/; http://www.alzforum.org/res/com/mut/default.asp). With respect to late onset AD, the only gene that has reached the status of a genetic risk factor for AD is the *apolipoprotein E (ApoE) ε4* allele (www.alzgene.org) (Hardy, 2005). Furthermore, two polymorphisms in the promoter region of the *APP* gene (Lahiri *et al.*, 2005) and genetic variations in *APBB2* (Fe65L, an APP binding protein) were recently shown to influence the risk of late onset AD (Li *et al.*, 2005).

Although AD is hereditary in less than 5% of the cases and aging is still the main cause of sporadic AD (Tanzi and Bertram, 2005), these genetic defects have been very instructive in modeling the molecular bases of AD. Indeed, brain pathology in hereditary and sporadic forms of AD appears to be identical, pointing toward a common molecular mechanism. Throughout the years, a polarized discussion between the "amyloidocentric" and "tauocentric" views prevailed, both attempting to convince the community of the true culprit, Aβ or tau, in AD. This discussion was recently fuelled with the extension of the phenotypic spectrum of PSEN mutations from phenotypes showing Aβ pathology, over the coexistence of AD and Pick body pathology (Halliday *et al.*, 2005), to FTD without amyloid plaque pathology (Dermaut *et al.*, 2004; Hutton, 2004). Recent genotype–phenotype correlation studies demonstrated that AD and FTD are indeed genetically

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TMD, and part of the ectodomain. Amino acid numbering of human APP695 is indicated inside circles and amino acid numbering starting from the first residue (D597) of the Aβ sequence is indicated outside the circles. Exon boundaries are depicted. FAD-linked mutations are indicated in red and enumerated in the table, while nonpathogenic mutations are marked in orange. The α-secretase cleavage site is shown by a light green arrow, while BACE1, BACE2, γ- (after V40 or A42), and ε- and ζ-cleavage sites are indicated by orange arrows. The additional BACE1 cleavage site (after L34) within the TM domain resulting from overexpression of BACE1 is indicated by a dotted orange arrow. T668 and Y682 in the AICD can be phosphorylated. The PSEN1-binding domain in the TM region is depicted in blue. Four functional motifs in the AICD and their interacting proteins are indicated in dark green; + signs indicate (phosphorylated) amino acid residues that are necessary for or stimulate this interaction, while the – sign shows that phosphorylation of T668 reduces the interaction with Fe65 (see text). The caspase 3/6/8 cleavage site in AICD is shown as well (yellow arrow).

interconnected on the spectrum of presenile degenerative brain disorders, with tau possibly being the major player (Dermaut *et al.*, 2005).

In this review, we will discuss more intensively the current cell biological knowledge on the intimate partnerships between APP and its proteolytic enzymes α-, β-, and γ-secretase, the latter being a multiprotein complex consisting of four essential components: PSEN1 or PSEN2, nicastrin (NCT), APH-1, and PEN-2.

## II. APP, Victim or Culprit?

### A. APP Family

APP is the most renowned member of this family of type I transmembrane proteins. They all share a large extracellular domain, one transmembrane domain (TMD), and a relatively short cytosolic domain. The APP gene contains 19 exons. Alternative splicing of exon 7, 8, or 15 results in eight potential splice variants (Fig. 1A). The 695-amino acid-long APP (APP695) is predominantly expressed in neurons, while the APP751/770 splice variants, containing a Kunitz-type protease inhibitor (KPI) domain, are nonneuronal (Kitaguchi *et al.*, 1988; Ponte *et al.*, 1988; Tanzi *et al.*, 1988). A high-molecular-mass APP variant, appican, that is expressed in astrocytes, results from the splicing of exon 15, creating a chondroitin-sulfate-glycosaminoglycan attachment site (CS-GAG) (Shioi *et al.*, 1995).

In mammals, APP has two homologues, the APP-like proteins APLP-1 and APLP-2, which can also be alternatively spliced (Wasco *et al.*, 1993). APP orthologues have been identified in many species such as *Xenopus* (APP) (Okado and Okamoto, 1992), *Drosophila* (APPL) (Martin-Morris and White, 1990), *Caenorhabditis elegans* (APL-1) (Daigle and Li, 1993), and *Danio rerio* (Musa *et al.*, 2001). In *Xenopus*, an APLP-2 homologue can also be found (Collin *et al.*, 2004).

### B. APP, Destined for Proteolysis?

During trafficking from their site of biosynthesis in the endoplasmic reticulum (ER) through the Golgi to the plasma membrane, APP, APLP-1, and APLP-2 undergo several posttranslational modifications including N- and O-glycosylation, sulfation, phosphorylation, and sialylation; the latter promotes the metabolic turnover of APP and thus enhances Aβ secretion (Nakagawa *et al.*, 2006). However, the best studied posttranslational modification of APP is its proteolytic processing (Fig. 1A).

Holo-APP is generally proteolyzed in a two-step mode. First, the APP ectodomain is shed by α- or β-secretase, generating a soluble 100–120 kDa

APP ectodomain (APPsα or APPsβ) and a membrane-associated 10–12 kDa C-terminal fragment (APP-CTFα or APP-CTFβ). Second, the APP-CTFs undergo γ-secretase-mediated intramembrane proteolysis, leading, respectively, to the secretion of the p3 and Aβ peptides and the release of the cytosolic domain (Haass *et al.*, 1993). So α-secretase cleavage, occurring between Lys-16 and Leu-17 of the Aβ sequence, precludes Aβ generation and is therefore referred to as the nonamyloidogenic pathway. This pathway is predominant in most cell types, whereas APP processing by β- and γ-secretase leading to amyloidogenesis is favored in neurons.

Originally, two major sites for cleavage of the APP-CTFs by γ-secretase were described, generating 40- and 42-amino acid-long Aβ peptides (Fig. 1B). However, recent studies have identified additional intramembrane cleavages occurring sequentially from the C-terminus of the APP TMD. First, ε-cleavage between residues 49 and 50 of the Aβ sequence leads to the generation of Aβ49 and the release of the APP intracellular domain (AICD) at the cytoplasmic side (Sastre *et al.*, 2001; Weidemann *et al.*, 2002). Aβ49 is further processed by ζ-cleavage, producing an Aβ46 species, the immediate precursor of Aβ40/42 (Qi-Takahara *et al.*, 2005; Zhao *et al.*, 2005). Indeed, Aβ46 was shown to be more stably associated with PSEN1, the catalytic component of γ-secretase, than APP-CTFβ. To date, it is not known whether these three subsequent cleavages are catalyzed by different enzymes or by one enzyme. In the latter case, the enzyme needs to bear minimum two different catalytic sites or activities. Indeed, ε/ζ- cleavages and γ-cleavage are differentially regulated *in vitro* by increased activities of α- and β-secretase (Kume *et al.*, 2004) and by TMP21 levels (Section IV.F.2) (Chen *et al.*, 2006), and they are differentially inhibited by transition state analogues and non-transition state inhibitors, respectively (Zhao *et al.*, 2005). The molecular basis of the consecutive intramembrane proteolysis from the ε- to the γ-cleavage site may be related to the requirement water molecules to hydrolyze peptide bonds. So ε-cleavage might create a path for the water molecules to have access to the ζ-cleavage site and then to the γ-secretase cleavage site (Zhao *et al.*, 2005).

Similarly, APLP-1 and APLP-2 are processed by several sheddases followed by γ-secretase-dependent intramembrane proteolysis. However, no Aβ peptide is generated, because the amino acid sequence in that region is too divergent from APP (Li and Sudhof, 2004; Naruse *et al.*, 1998; Slunt *et al.*, 1994), and the same is true for *C. elegans* APL-1 (Daigle and Li, 1993) and *Drosophila* APPL (Rosen *et al.*, 1989). In contrast, all amino acids implicated in amyloidogenesis are conserved in *Xenopus* (Section II.C.3) (van den Hurk *et al.*, 2001).

Besides ectodomain shedding and intramembrane proteolysis, APP can also undergo cleavage in its intracellular domain by caspase 3, 6, and 8, generating the C31 peptide and the Jcasp domain, C- and N-terminal to aa 664, respectively. Both peptides can in turn induce apoptosis (Lu *et al.*, 2000; Madeira *et al.*, 2005), just like AICD-C59 and AICD-C57 produced by γ-secretase cleavage of APP (H. S. Kim *et al.*, 2003).

The functions of APP and its homologues *in vivo* remain poorly understood, essentially because single knockouts did not show any gross abnormalities, suggesting functional redundancies. For instance in *Drosophila*, *APPL* null mutant flies are viable and fertile and display only subtle behavioral deficits which can be rescued by APPL as well as by human APP (Luo *et al.*, 1992). Similarly, homozygous *APP*-deficient mice show a mild phenotype, although motor dysfunction, brain gliosis, and impairment in spatial learning and exploratory behavior can be observed (Muller *et al.*, 1994; Zheng *et al.*, 1995). Cultured neurons derived from *APP*-deficient mice display defects in axonogenesis and arborization (Perez *et al.*, 1997) as well as increased duration of mitosis, indicating a role for APP in controlling cell cycle progression during cortical development (Lopez-Sanchez *et al.*, 2005).

Available data from transgenic mice double deficient in *APP*, *APLP-1,* and/or *APLP-2* indicate a functional redundancy between APLP-2 and both other family members, suggesting a key physiological role for APLP-2, as well as diverse nonoverlapping functions for APP versus APLP-1 (Heber *et al.*, 2000; von Koch *et al.*, 1997). The triple knockout mice die shortly after birth and display cranial abnormalities and cortical dysplasias resembling human type II lissencephaly. Moreover, a partial loss of cortical Cajal Retzius cells is observed, indicating that APP and APLPs play a crucial role in the survival of these cells and in neuronal adhesion. However, since *APP*/*APLP* triple knockout mice survive through embryonic development, APP function is apparently not essential in all neurons during embryogenesis (Herms *et al.*, 2004). So APP family members play a major role in early postnatal development, but also in adulthood, for example, in regulation of neuronal progenitor proliferation in the central nervous system (Caille *et al.*, 2004) and in brain repair after injury (Leyssen *et al.*, 2005).

Most of these functions are likely mediated through the multiple protein interactions with the ecto- and/or intracellular domain of APP. Hence, processing by secretases can serve a dual role. It can either abrogate these protein interactions and therefore also the associated functions (considering APP as a ''victim''), or it can generate soluble fragments that may independently initiate new interactions as a ligand (e.g., soluble ectodomains) or as a cytosolic scaffold destined for nuclear translocation, degradation, etc. (considering APP as a ''culprit''). In the next section, we highlight the most important protein–protein interactions and their functional repercussions.

## C. Interactome of APP

### 1. APP Ectodomain

Within the APP ectodomain, two main domains with different putative functions can be discerned, i.e., the E1 region and the carbohydrate region, bridged via an unstructured highly flexible acidic stretch (Fig. 1A) (Reinhard

*et al.*, 2005). In the APP751/770 splice variants, the KPI and Ox2 sequences (Tanaka *et al.*, 1988) are situated between this acidic region and the carbohydrate domain.

In the E1 region, two domains can be distinguished, the cystein-rich N-terminal growth factor-like domain (GFLD) (aa 23–128) and the copper-binding domain (CuBD) (aa 124–189) (Fig. 1A). The crystal structure of the **GFLD** (Rossjohn *et al.*, 1999) revealed a disulfide-bonded β-hairpin loop also found in other growth factors, which is thought to be involved in MAP kinase activation (Greenberg *et al.*, 1995) and/or in the formation of a heparan sulfate proteoglycan (HSPG) binding domain (Small *et al.*, 1999). In addition, a neighboring hydrophobic surface patch may be required for ligand or proteoglycan binding and APP–APP dimerization. Because of its resemblance to other growth factors, this GFLD domain is suggested to be responsible for the growth factor-like properties of APP. Indeed, APP can activate neurite outgrowth and neural stem cell proliferation, and these stimulating effects can be modulated by binding of several HSPGs or fibulin-1 to the GFLD (Ohsawa *et al.*, 2001; Small *et al.*, 1999; Williamson *et al.*, 1996).

The **CuBD** contains His-147, His-151, Tyr-168, and Met-170 arranged in a tetrahedral orientation, allowing this domain to act as a neuronal metallotransporter reducing Cu(II) to Cu(I) (Barnham *et al.*, 2003). The idea that APP is a major regulator of neuronal copper homeostasis, preventing metal-mediated oxidative stress in the brain, is fuelled further by the elevation and reduction of cerebral copper levels in APP knockout and APP overexpressing transgenic mice, respectively (Maynard *et al.*, 2005).

The carbohydrate domain bears the two N-glycosylation sites and is subdivided into the **E2** or **central APP domain (CAPPD)** and an essentially structureless linker or juxtamembrane domain (aa 507–589) (Fig. 1A) (Dulubova *et al.*, 2004; Wang and Ha, 2004). Interestingly, the secreted neuronal glycoprotein F-spondin, thought to be involved in axonal pathfinding, binds to this E2 domain. F-spondin may be an endogenous regulator of APP proteolysis, since binding to APP leads to inhibition of β-secretase cleavage (Ho and Sudhof, 2004).

Besides E1, the E2 domain also contributes to the growth-promoting activity of APP through its RERMS sequence (aa 328–332) (Ninomiya *et al.*, 1993). In addition, it bears a conserved HSPG-binding site (Small *et al.*, 1999; Wang and Ha, 2004). Its structure resembles that of spectrin or α-actinin, and the E2 region can likewise form antiparallel dimers in solution (Wang and Ha, 2004). While dimerization generates a better groove for the HSPG-binding site, it disables the RERMS sequence. Thus dimerization and subsequent dissociation of APP might be a way to regulate different APP functions. Although attractive, some conflicting data exist on whether the secreted APP ectodomain exists as monomer or dimer in solution (Scheuermann *et al.*, 2001; Wang and Ha, 2004). Interestingly, Aβ production is significantly increased when APP molecules are linked via

juxtamembrane disulfide bridges, suggesting that dimerization may regulate APP cleavage (Scheuermann *et al.*, 2001). In addition, also heterooligomerization between APP, APLP-1, and APLP-2 seems to be possible. Recently, homo- or heterodimerization *in trans* was shown to promote cell–cell adhesion in mouse brain and in synaptically enriched membrane compartments. However, it should be noted that in this case the E1 domain was identified as the major interaction interface (Soba *et al.*, 2005). Interestingly, the same group previously suggested that APP interferes with cell adhesion, since overexpression of human FL-APP in *Drosophila* induced a blistered-wing phenotype, pointing to a dysfunction in cell–cell adhesion between the two epithelial cell layers forming the wing (Fossgreen *et al.*, 1998). Therefore, this role of APP—in monomeric or dimeric form—in the regulation of cell–cell adhesion requires further investigation.

Finally, growth-promoting activity of the APP ectodomain was confirmed *in vivo*. Indeed, abundants sAPP 695-binding sites are found in the subventricular zone of the lateral ventricle, a neurogenic area of the adult brain, and APP binds to and regulates the proliferation of adult progenitor cells important for adult neurogenesis (Caille *et al.*, 2004). Moreover, intracerebroventricular injection of sAPP can improve a form of spatial memory, implying a role in early memory processes as well (Bour *et al.*, 2004).

## 2. Scaffold of the APP Intracellular Domain

Several short stretches of amino acids are identified in the intracellular domain of APP that mediate the assembly of functionally different protein networks. It is unlikely that all of them occur simultaneously, so a major effort has to be made in the future to correlate specific interactions with their location in the cell or neuron. At least four functional motifs can be identified in this APP region (Fig. 1B).

In the proximal region of APP, a tyrosine-based basolateral sorting motif (YTSI, aa 653–656) is able to recruit PAT-1, a microtubule-binding protein interacting with APP tail 1. Because of its homology with the kinesin light chain, PAT-1 is said to mediate the transport of APP to the cell surface (Gao and Pimplikar, 2001; Zheng *et al.*, 1998). Adjacent to this motif, the sequence aa 657–676 is required for the binding of the major brain-specific GTP-binding protein Go (Nishimoto *et al.*, 1993). More recently, a part of this motif (aa 658–664) was also shown to interact with hARD1 (human homologue of yeast amino terminal acetyltransferase ARD1). Interestingly, coexpression with another acetyltransferase hNAT1 suppresses Aβ40 secretion, suggesting a role for these enzymes in Aβ generation (Asaumi *et al.*, 2005).

However, the most important and most complicated motif in APP is the highly conserved YENPTY motif (aa 682–687), which comprises the YENP endocytosis signal (Perez *et al.*, 1999). Essentially, it is a competitive scaffolding motif for adaptor proteins with phosphotyrosine-binding (PTB) domains, such as the Fe65(like), X11/Mint, and JIP [c-Jun N-terminal kinase (JNK)-interacting protein or Janus kinase-interacting protein] families. Other examples are mDab1 (mammalian homologue of *Drosophila* Disabled 1), Shc (SH2-containing sequence), the PDGF receptor, and Numb. Different amino acid residues in this YENPTY motif show some binding specificity for different adaptor proteins, explaining the multitude of interactions. Indeed, Fe65 and X11/Mint binding depends on Y682, ShcA/C and the PDGF receptor need N684 for binding to this motif, mDab1 requires both Y682 and Y687, while all three residues are essential for JIP-1 binding (Borg *et al.*, 1996; Matsuda *et al.*, 2001; Merdes *et al.*, 2004; Trommsdorff *et al.*, 1998; Zambrano *et al.*, 2004). The phosphorylation status of the tyrosine or threonine residues provides a second level of specificity. For instance, JIP-1 preferentially interacts with T668-phosphorylated APP (Muresan and Muresan, 2005), while this phosphorylation reduces the interaction with Fe65 and has little or no impact on X11 or mDab1 binding (Ando *et al.*, 2001). Phosphorylation of Y682, executed, e.g., by Abl tyrosine kinase, promotes the interaction with ShcA/ShcC (Zambrano *et al.*, 2004).

These adaptor proteins are mostly multimodular proteins that can recruit other binding partners. Consequently, several functions have been described for the APP cytoplasmic domain complexed with these adaptor proteins in different locations in the cell.

The **Fe65**–APP interaction is clearly implicated in regulating cell motility. Fe65 binds to Mena, the mammalian homologue of *Drosophila* enabled, which negatively regulates cell motility through its interaction with profilin (Bear *et al.*, 2000; Ermekova *et al.*, 1997; Lanier *et al.*, 1999) and colocalizes with APP and β1-integrin in dynamic focal complexes at the leading edge of moving cells (Sabo *et al.*, 2001). As the binding sites for profilin and Fe65 overlap in Mena, the balance between the different protein interactions is decisive for cell movement. Moreover, APP and Fe65 colocalize also *in vivo* in actin-rich lamellipodia in growth cones and associate with Rab5-containing synaptic organelles in nerve terminals, suggesting a role of the APP/Fe65 complex in growth cone motility and synaptic plasticity (Sabo *et al.*, 2003).

In addition, Fe65 appears to functionally link APP with low-density lipoprotein (LDL) receptor-related protein (LRP). Indeed, Fe65-induced increase in secretion of APPs and Aβ requires the last 50 amino acids of the LRP cytoplasmic domain (Yoon *et al.*, 2005). The putative role of Fe65 in the translocation of APP to the cell surface (Sabo *et al.*, 1999) or the role of LRP in APP endocytosis (Kounnas *et al.*, 1995) might also contribute to the effects of Fe65 and LRP on APP processing.

A second compelling role for Fe65–APP interaction is fuelled by the initial observation of a nuclear Fe65 pool and its ability to recruit transcription factors of the CP2/LSF/LBP1 family (Zambrano *et al.*, 1998). In analogy to the Notch intracellular domain (NICD) forming a transcriptional active complex with a member of the CSL [CBF1, Su(H), Lag-1] family of DNA-binding proteins (Schroeter *et al.*, 1998), much effort has been made to demonstrate a similar mechanism for the AICD after intramembrane proteolysis. Initially, several groups reported that AICD is stabilized by complex formation with Fe65 and is translocated to the nucleus (Cupers *et al.*, 2001b; Kimberly *et al.*, 2001), where it recruits the histone acetyltransferase Tip60 to stimulate transcription (Cao and Sudhof, 2001). This tripartite complex was shown to activate, e.g., the tetraspanin KAI1 gene, possibly by binding of the Fe65 transactivation domain to the nucleosome assembly factor SET (Baek *et al.*, 2002; Telese *et al.*, 2005). Furthermore, induced AICD expression identified *APP*, *BACE*, *Tip60*, *GSK3*β, and *thymidilate synthase* as transcriptional target genes (Bruni *et al.*, 2002; von Rotz *et al.*, 2004). Recently, AICD (and also the ICD of APLP-1 and -2) was shown to regulate the expression of the Aβ degrading enzyme neprilysin, providing a negative feedback loop to control Aβ generation (Pardossi-Piquard *et al.*, 2005).

However, Cao and co-workers recently reported that although γ-secretase cleavage of APP is required for transactivation, nuclear translocation of AICD itself is dispensable (Cao and Sudhof, 2004). Transcriptional transactivation by APP and Notch may thus involve distinct mechanisms: whereas the NICD directly functions in the nucleus, the AICD acts indirectly by recruiting and activating Fe65. Moreover, another study even argues that γ-secretase-dependent production of AICD is not required for activation of transcription (Hass and Yankner, 2005). Both studies claim that APP (and AICD) exerts its role outside the nucleus while still membrane bound, by recruiting and stabilizing Tip60 through cyclin-dependent kinase phosphorylation. In a recent study, even no direct role for AICD in transcription regulation of putatitive AICD target genes was found. Instead, Fe65 was shown to have a general, not AICD-dependent, weak stimulating effect on a wide variety of promoters (Hebert *et al.*, 2006; Yang *et al.*, 2006).

Finally, the importance of the APP–Fe65 interaction was recently confirmed by the observation that the phenotype of $Fe65^{-/-}Fe65L1^{-/-}$ mice is very similar to that of $APP^{-/-}APLP\text{-}1^{-/-}APLP\text{-}2^{-/-}$ mice, suggesting that this phenotype might be caused by a decreased transmission of an APP-dependent signal through Fe65 proteins (Guenette *et al.*, 2006).

In addition, Fe65 interacts not only with APP, but also with tau in a phosphorylation-dependent manner. This interaction requires an intact microtubule network, providing an exciting new link with tau pathology in AD (Barbato *et al.*, 2005).

The neuronal adaptor protein **X11α/Mint-1** competes with Fe65 for APP binding and has opposite effects on its proteolytic processing, so it decreases

APPs and Aβ secretion (Lau *et al.*, 2000). Moreover, this processing is modulated via interactions of X11α/Mint-1 with other proteins that implicate APP in functions such as cell adhesion, synapse formation, and remodeling (Mueller *et al.*, 2000). For instance, the tripartite complex Mint-1/CASK/Veli acts as a nucleation site for proteins involved in synaptic vesicle exocytosis and synaptic junctions (Butz *et al.*, 1998). Similarly, the interaction of Mint-1 with CASK and a subunit of a presynaptic $Ca^{2+}$ channel suggests a role in synaptic transmission (Maximov *et al.*, 1999). Moreover, in the fly, interaction of APPL with dX11/Mint/Lin-10 regulates synapse expansion downstream of Fasciclin II (Ashley *et al.*, 2005).

The adaptor protein **JIP-1**, a scaffold protein for JNK, can also bind to the AICD YENPTY motif. Similar to Fe65, Jip-1b is able to bind Tip60 and to form complexes involved in nuclear translocation and gene activation, but the mechanism involved is different from that used by Fe65 (Scheinfeld *et al.*, 2003; von Rotz *et al.*, 2004).

The more exciting role for JIP-1 is probably outside the nucleus where it functions as a linker between APP and the light chain of kinesin-1 (Matsuda *et al.*, 2003). A role for APP in fast axonal transport was demonstrated earlier when it was noticed that *APPL* deficiency in *Drosophila* or high overexpression of human APP695 or APPL causes axonal transport "jams" within neurons, reminiscent of kinesin and dynein mutants, although these studies favored a direct interaction of APP with kinesin-1 (Gunawardena and Goldstein, 2001; Kamal *et al.*, 2000). This apparent controversy may be solved by a recent study demonstrating that phosphorylation of APP at Thr-668 is a prerequisite for APP and JIP-1 to be cotransported by kinesin-1 to the neurites, so the transport of nonphosphorylated APP is independent of JIP-1 (Muresan and Muresan, 2005). Although the issue of whether the axonal transport compartment also contains the Aβ-producing secretases BACE1 and PSEN1 is under debate (Kamal *et al.*, 2001; Lazarov *et al.*, 2005), all studies in general support the hypothesis that disrupted axonal transport can contribute to AD (Section I). Indeed, in AD mouse models and patients, axonal swellings and accumulation of motor proteins are often observed. Moreover, impairing axonal transport by reducing the kinesin dosage enhances the frequency of axonal defects and increases Aβ peptide levels and amyloid deposition (Stokin *et al.*, 2005). Finally, increased APP expression in mice leads to abnormal retrograde transport of nerve growth factor and degeneration of basal forebrain cholenergic neurons (Salehi *et al.*, 2006).

Both mammalian **mDab1** and *Drosophila* **Dab** adaptor proteins bind to their respective YENPTY motifs in APP/APPL. Furthermore, APP interacts with Abl tyrosine kinase, either indirectly via Dab (Howell *et al.*, 1997), or directly, provided that APP is phosphorylated (by Abl) at Tyr-682 (Zambrano *et al.*, 2001). These interactions imply that APP (and APPL) is involved in the regulation of axonal outgrowth and arborization. Abl probably fulfills its role through interaction with profilin, an essential downstream signaling molecule

of APPL, and subsequent modification of the actin cytoskeleton. This role for APP in axonal outgrowth is especially important after traumatic brain injury. Furthermore, fly brain injury not only upregulates neuronal APPL, but the trauma also activates the JNK signaling stress cascade. This leads to an activation of profilin and other cytoskeletal regulators, ensuring a permissive environment for actin remodeling. So APP and the JNK stress cascade signaling are tightly linked in the context of axonal outgrowth (Leyssen *et al.*, 2005).

The phosphorylation of Tyr-682 of APP is also important for binding of **ShcA and ShcC**. Interaction with these adaptor proteins leads to an activation of APP processing, probably through a pathway that involves Grb2 and ERK1,2 and that is dependent on γ-secretase cleavage of APP (Russo *et al.*, 2002; Tarr *et al.*, 2002). Similarly, interaction of AICD with the **PDGF receptor** activates APP processing. In this case, the nonreceptor tyrosine kinase Src and the small G-protein Rac1 are involved (Zambrano *et al.*, 2004). The adaptor proteins **Numb and Numb-like** can repress Notch activity by binding to the YENPTY motif in APP (Roncarati *et al.*, 2002). In this way, the same catalytic γ-secretase activity can exert opposing effects on Notch signaling by generating NICD (De Strooper *et al.*, 1999) and AICD. Finally, expression levels of the prolyl isomerase Pin1 regulate APP processing through binding to the phosphorylated Thr-668-Pro motif in APP (Pastorino *et al.*, 2006). Pin1 also binds to and isomerizes a similar motif in tau, indicating Pin1 deregulation as a potential link between tangle and plaque pathologies in AD.

## 3. Aβ: Not Only Harmful?

Following the surprising observation that Aβ is generated throughout life in all cells as part of its normal metabolism (Busciglio *et al.*, 1993; Haass *et al.*, 1992; Seubert *et al.*, 1992; Shoji *et al.*, 1992), the idea originated that this peptide may also have physiological functions.

Next to its neurotrophic activities and its stimulating effects on neuronal viability (Whitson *et al.*, 1990; Yankner *et al.*, 1990), Aβ was also identified as a regulator of ion channel function (Plant *et al.*, 2005) and calcium homeostasis (Abramov *et al.*, 2004). Furthermore, Aβ was shown to participate in a negative feedback loop involved in synaptic homeostasis (Kamenetz *et al.*, 2003).

Recently, it was demonstrated that Aβ42 can downregulate sphingomyelin levels, while Aβ40 can reduce *de novo* cholesterol synthesis, suggesting a biological role for APP processing in cholesterol and sphingomyelin metabolism (Grimm *et al.*, 2005). Aβ is also capable of oxidizing cholesterol to the proapoptotic 7β-hydroxycholesterol species, a selective α- but not β-secretase cleavage inhibitor. So higher levels of this neurotoxic cholesterol species produced by increased amounts of Aβ might contribute to the oxidative stress and cell loss observed in AD (Section I) (Nelson and Alkon, 2005). However, some caution is needed in assigning physiological relevance to Aβ, since this peptide sequence is very poorly conserved between humans and rodents,

although it is conserved among other mammals (Johnstone *et al.*, 1991) and in *Xenopus* (Section II.B) (van den Hurk *et al.*, 2001), which indicates that Aβ might not have critical physiological functions.

Finally, only a few studies have reported protein interactions of APP occurring via its TMD. One of these interacting proteins is BRI2, which is thought to be a modulator of APP processing (Fotinopoulou *et al.*, 2005). Another study showed that an 11-amino acid-long sequence in APP located C-terminally from the γ-cleavage site is essential for the interaction of APP with PSEN1 (Fig. 1B) (Annaert *et al.*, 2001). This domain is also the region in APP where the AD-causing mutations that affect γ-secretase cleavage are found. Therefore, we speculate that the observed shift in cleavage site preference between the γ40 and the γ42 site is caused by an altered binding of APP to PSEN1, as supported by a phenylalanine mutagenesis study in this region (Lichtenthaler *et al.*, 1999). Consequently, this binding domain in APP may serve as a therapeutic target for specific inhibition of APP proteolysis.

## III. Biology of APP Sheddases

### A. Diversity of α-Secretase

The shedding of APPsα is similar to the processing of other integral membrane proteins like transforming growth factor-α (TGF-α), tumor necrosis factor-α (TNF-α), the cell adhesion molecule L-selectin, and the growth factor coreceptor syndecan. All these proteins, including APP, are cleaved or "shed" from the plasma membrane by an activity that consists of a constitutive and a regulated component.

The regulated pathway can be activated via protein kinase C (PKC), which in turn is stimulated by phorbol esters or by intracellular diacylglycerol (DAG) generation. Other receptor-mediated second messenger cascades can also regulate the nonamyloidogenic processing of APP, e.g., those including the presynaptic DAG receptor Munc13-1, the serotonin 5-HT4 receptor, the G-protein-coupled P2Y2 receptor, and the G-protein-coupled receptor PAC1 (Camden *et al.*, 2005; Hooper and Turner, 2002; Kojro *et al.*, 2006; Robert *et al.*, 2005; Rossner *et al.*, 2004). Finally, competitive sorting events influencing APP internalization or transport may also affect α-cleavage, as demonstrated for the binding of APLP-1 to LRP and the resulting increase in α-secretase cleavage (Neumann *et al.*, 2006).

#### 1. ADAM Family

Several (if not all) candidates for α-secretase are members of the ADAM (a desintegrin and metalloprotease) family of metalloproteases, including

ADAM9 (also called MDC9 or meltrin-γ), ADAM17 [also called TACE (TNF-α converting enzyme)] and ADAM10 (Kojro and Fahrenholz, 2005).

It is now becoming clear that **ADAM10** accounts most likely for the majority of α-secretase activity. This conclusion is not only based on the expected subcellular localization of this enzyme in the secretory pathway and at the plasma membrane, but also on molecular evidence (Lammich *et al.*, 1999; Marcinkiewicz and Seidah, 2000). Both basal and PKC-stimulated activity is assigned to ADAM10, and its activity can be abolished by mutating its zinc binding site. Moreover, ADAM10 is capable of cleaving synthetic substrates mimicking the α-secretase site with the correct specificity (Lammich *et al.*, 1999). These findings were corroborated *in vivo* in transgenic mice overexpressing ADAM10 (Postina *et al.*, 2004). Furthermore, in chronic hypoxia, the reduction in constitutive and regulated α-secretase cleavage of APP correlates with a decrease in ADAM10 expression (Webster *et al.*, 2004), an effect that is also observed in AD patients (Colciaghi *et al.*, 2002).

The role of **ADAM17** in α-secretase activity seems to be limited to the regulated component of the nonamyloidogenic pathway, as evidenced by *ADAM17*$^{-/-}$ fibroblasts and by the use of ADAM17 inhibitors (Blacker *et al.*, 2002; Buxbaum *et al.*, 1998), although this latter study still requires confirmation (Parkin *et al.*, 2002). Finally, **ADAM9** as well as hADAM9s, a short form lacking its C-terminus, are also associated with α-secretase-like activity. Like ADAM17, ADAM9 seems to account for the regulated (phorbol ester-stimulated) component of α-secretase (Hotoda *et al.*, 2002). However, recent studies revealed a more complicated regulation. First, downregulation of ADAM9 expression drastically reduces sAPPα secretion, indicating that ADAM9 is an important regulator of the physiological processing of APP. Second, in an *ADAM10*$^{-/-}$ background, ADAM9 can increase sAPPα production only when cooverexpressed with ADAM10. Therefore, ADAM9 probably exerts its effect through ADAM10, likely by contributing to the shedding of ADAM10 (Cisse *et al.*, 2005). This soluble ADAM10 ectodomain might in turn function as α-secretase together with ADAM17, fuelling the idea of tissue-specific "teams" of different proteases that exert α-secretase activity (Asai *et al.*, 2003; Cisse *et al.*, 2005). Moreover, the recent suggestion that the ADAM10 CTF becomes a substrate for γ-secretase after shedding, may add another level of regulation of α-secretase, the nature of which is under investigation.

## 2. Ubiquitous Sheddases

Beyond APP, ADAM family members mediate the ectodomain shedding of many more membrane-anchored proteins, rendering them substrates for γ-secretase-mediated intramembrane proteolysis. APLP-2 is shed by both ADAM10 and 17 (Endres *et al.*, 2005), but APLP-1 and -2 are not cleaved

by ADAM9 (Li and Sudhof, 2004). Very similar to the processing of APP, the team of ADAM10/9 and 17 contributes, respectively, to the constitutive and regulated shedding of the cellular prion protein (Cisse *et al.*, 2005; Vincent *et al.*, 2001). Furthermore, the involvement of ADAM17 in the S2 cleavage of Notch and the shedding of the Notch ligand Jagged (Brou *et al.*, 2000; LaVoie and Selkoe, 2003), as well as the role of ADAM10 in Delta-1 shedding (Six *et al.*, 2003), clearly suggest an important function for ADAMs in Notch signaling. Indeed, *ADAM 10*-deficient mice die at day E9.5 with multiple defects of the developing central nervous system, somites, and cardiovascular system, together with a reduced expression of the Notch target gene *hes-5* in the neural tube and an increased expression of Delta-1 (Hartmann *et al.*, 2002). Finally, ADAM10 is involved in the ectodomain shedding of the voltage-gated sodium channel β2-subunit and the MHC class I protein HLA-A2 (Carey *et al.*, 2006; Kim *et al.*, 2005a), while ADAM17 catalyzes the cleavage of the growth hormone receptor (Cowan *et al.*, 2005) and sorLA (Bohm *et al.*, 2006).

## B. β-Secretase Cleavage, the Point of No Return

### 1. BACE1 and BACE2 Held Responsible

Six years after the discovery of a soluble APPβ (Golde *et al.*, 1992; Seubert *et al.*, 1993), the "β-*secretase*" gene was discovered on chromosome 11q23–24 almost simultaneously and independently by five laboratories (Hussain *et al.*, 1999; Lin *et al.*, 2000; Sinha *et al.*, 1999; Vassar *et al.*, 1999; Yan *et al.*, 1999). Although several acronyms were proposed, consensus was reached for the term BACE1 (β-site APP cleaving enzyme or β-secretase β-amyloid-converting enzyme). Shortly afterward, BACE2 emerged from genome databases showing 64% homology with BACE1 (Saunders *et al.*, 1999; Yan *et al.*, 1999). The *BACE2* gene maps to chromosome 21q22 within the critical Down region. Recently, two *Drosophila* homologues DASP1 and DASP2 were found, displaying 59% and 50% similarity to human BACE1 (Kotani *et al.*, 2005). Surprisingly, no or very little evidence for genetic linkage, mutations, or allelic association with familial or sporadic AD is found, except for a few *BACE1* polymorphisms and a *BACE2* haplotype association with late-onset AD (www.alzgene.org) (Tanzi and Bertram, 2005).

Both at the mRNA and protein level, the pattern of BACE1 expression is mainly restricted to the brain and particularly to neurons, and more closely resembles that of APP and ADAM10 than the BACE2 expression pattern (Marcinkiewicz and Seidah, 2000; Zohar *et al.*, 2003). This BACE1 expression pattern might explain the anatomical predilection of AD for the brain. Interestingly, BACE1 was recently found to be expressed in reactive astrocytes in AD brain but not in normal brain, indicating astrocyte activation as a potential therapeutic target (Rossner *et al.*, 2005).

BACE1 can cleave APP at two sites of Aβ, the Asp1 (β) and the Glu11 site (β′), depending on its intracellular localization (Fig. 1B) (Fluhrer *et al.*, 2002; Huse *et al.*, 2002). Overexpression of BACE1 shifts the preference to the β′ site (Creemers *et al.*, 2001; Liu *et al.*, 2002). Moreover, the detection of significant levels of Aβ11-40/42 in amyloid plaques indicates that β′-cleavage may play a role in cerebral amyloid deposition (Huse *et al.*, 2002). Overexpressed BACE1 generates an additional C-terminal cleavage, resulting in Aβ1-34 and a decrease of Aβ40 and Aβ42 (Fluhrer *et al.*, 2003). However, this cleavage is dependent on prior γ-secretase-mediated cleavage of APP-CTFs (Shi *et al.*, 2003). In contrast to BACE1, BACE2 acts preferentially at Phe-19 and Phe-20 within the Aβ sequence close to the α-secretase site (Farzan *et al.*, 2000). Therefore, BACE2 overexpression reduces Aβ formation, suggesting that it may act as an alternative α-secretase (Yan *et al.*, 2001b). Interestingly, the Swedish APP mutation increases C99 production by both BACE1 and BACE2, while the Flemish mutation substantially enhances cleavage at Asp-1 by BACE2 (Farzan *et al.*, 2000; Sinha *et al.*, 1999; Yan *et al.*, 1999).

BACE1 and BACE2 are type I transmembrane proteins with an overall similar structural organization. They belong to a new family of aspartyl proteases closely related to the pepsin family. Their luminal domain contains two catalytic Asp residues in a conserved D(T/S)G(T/S) sequence motif and mutation of one of them is sufficient to abrogate β-secretase cleavage of APP (Farzan *et al.*, 2000; Hussain *et al.*, 1999). The cocrystal structure of BACE1 with a substrate-based transition state analogue inhibitor revealed a conserved general folding of aspartyl proteases with the inhibitor in the substrate-binding cleft (Hong *et al.*, 2000). Analysis of the unbound BACE1 protease domain revealed a flap region that appears to be locked in an ''open'' position, which permits entrance of substrates into this cleft (Hong and Tang, 2004). Furthermore, interaction with APP occurs via Asp-1 of the Aβ sequence, which may form a salt bridge with Arg-296 in BACE1 (or Arg-310 in BACE2) near the DT/SG motif (Sauder *et al.*, 2000). Moreover, the main determinant for BACE1 to cleave at the β or β′ site, in addition to the sequence, is not the distance of these sites to the membrane, but the structural compatibility between BACE1 and APP. The β′ site is better positioned to access the BACE1 catalytic cavity, explaining why overexpression shifts the cleavage to the β′ site (Qahwash *et al.*, 2004). Finally, a ligand-binding pocket distinct from the enzymatic active site was discovered within the catalytic domain of BACE1, a finding that may lead to the development of novel and selective BACE1 inhibitors (Kornacker *et al.*, 2005).

Both BACE proteins undergo multiple posttranslational modifications, but the degree to which these modifications are required for BACE activity differs. For instance, mature glycosylation is a prerequisite for β-secretase

activity (Charlwood *et al.*, 2001), whereas the disulfide bonds in the catalytic domain are critical but not essential (Fischer *et al.*, 2002). Both BACE1 and BACE2 are synthesized as proenzymes, but they differ in the mechanism of prodomain cleavage. This event is autocatalytic for BACE2 (Hussain *et al.*, 2001; Yan *et al.*, 2001b) and mediated by furin (and possibly other proconvertases), recognizing the RLPR motif flanking the prodomain, for BACE1 (Bennett *et al.*, 2000; Creemers *et al.*, 2001). However, BACE1 and BACE2 are most likely not true zymogens, since preventing prodomain cleavage in BACE1 does not influence its activity (Benjannet *et al.*, 2001; Creemers *et al.*, 2001). The different regulation of BACE1 and BACE2 cleavage may therefore govern specificity to folding and sorting of the respective proteins rather than regulating their activity (Shi *et al.*, 2001).

Furthermore, in post-Golgi compartments, BACE1 can undergo endoproteolysis of a surface-exposed α-helix, which is a cell- and tissue-specific event and does not take place in neurons. This cleavage generates a stable N-terminal fragment (NTF) and C-terminal fragment (CTF) that remain covalently associated via a disulfide bond and probably retain some β-secretase activity (Huse *et al.*, 2003). BACE1 also undergoes true ectodomain shedding, releasing a soluble protease, but the sheddase remains to be identified (Hussain *et al.*, 2003). This shedding can be increased by mutating the palmitoylation sites in BACE1, suggesting that palmitoylation helps to better anchor BACE1 in the membrane or, alternatively, mediates the association with specific microdomains (Benjannet *et al.*, 2001).

In addition, BACE1 can exist as a dimer, which may be the true active enzyme, since this dimeric species has a higher substrate affinity than the monomeric form (Schmechel *et al.*, 2004; Westmeyer *et al.*, 2004). This dimerization (and Aβ production) seems to be promoted by lipidation (Parsons and Austen, 2005). Indeed, several lipids including neutral glycosphingolipids (cerebrosides), anionic glycerophospholipids, and sterols (cholesterol) stimulate BACE1 activity (Kalvodova *et al.*, 2005). Furthermore, ceramide, a lipid second messenger, stabilizes BACE1 and hence promotes Aβ generation (Puglielli *et al.*, 2003a).

Finally, BACE1 activity can also be modulated via protein interactions. For instance, heparan sulfate binding to BACE1 blocks access of APP to the active site, thereby selectively inhibiting β-cleavage (Scholefield *et al.*, 2003). In addition, the leucine zipper protein PAR-4, initially associated with neuronal degeneration and aberrant Aβ production in models of AD, regulates BACE1 activity through binding to its cytosolic tail (Xie and Guo, 2005). Surprisingly, two components of the γ-secretase complex, PSEN1 (Hebert *et al.*, 2003a) and NCT (Hattori *et al.*, 2002), may also be involved in the maturation and activity of BACE1. Targeting some of these natural regulators of BACE1 activity might be a potential therapeutic strategy for AD.

## 2. BACE Cleavage: Not a Privilege for APP

Besides APP and APLPs (Section II.B), several other BACE1 substrates have been identified, such as the sialyltransferase ST6Gal (Kitazume *et al.*, 2005), the P-selectin glycoprotein ligand 1 (PSGL-1) (Lichtenthaler *et al.*, 2003), neuregulin (Willem *et al.*, 2006) the β-subunits of voltage-gated sodium channels (Wong *et al.*, 2005), LRP, (von Arnim *et al.*, 2005). In the last two cases, BACE1 cleavage renders the remaining C-terminal fragments substrates for γ-secretase (Section IV.F.1). However, whether all these substrates play a role in the pathology of AD still needs to be determined.

*BACE1* deficiency results in an (almost) complete block of Aβ generation in neurons, without a compensatory increase in BACE2 expression, confirming that it is the major β-secretase (Harrison *et al.*, 2003; Laird *et al.*, 2005; Roberds *et al.*, 2001; Luo *et al.*, 2003b). Interestingly, glia cells derived from $BACE1^{-/-}$ embryos still produce Aβ (Dominguez *et al.*, 2005). This observation suggests that BACE2 might be responsible for this activity and may therefore contribute to Aβ generation in AD and Down syndrome patients (the latter having three *BACE2* alleles).

However, some contradiction exists on the $BACE1^{-/-}$ phenotypes. While initially no apparent anatomical or behavioral abnormalities up to 14 months of age were noticed, more recent studies describe complex phenotypes, with early postnatal lethality (that is enhanced in $BACE1^{-/-}BACE2^{-/-}$ mice), smaller size, and hyperactive behavior (Dominguez *et al.*, 2005). Also subtle alterations in the steady-state inactivation of voltage-gated sodium channels were noticed, in agreement with β-subunits of voltage-gated sodium channels being BACE1 substrates. Moreover, *BACE1*-deficient mice are more timid and less exploratory than transgenic mice overexpressing BACE1, suggesting that BACE1 may play a role in neurotransmission (Harrison *et al.*, 2003). Furthermore, the alterations in hippocampal synaptic plasticity as well as in tests of cognition and emotion implicate BACE1 in cognitive, emotional, and synaptic functions (Laird *et al.*, 2005). Recently, *BACE1* mice were diagnosed with peripheral nerve hypomyelination caused by the lack of neuregulin 1 processing (Willem *et al.*, 2006).

In contrast with the *BACE* knockout mice, transgenic mice overexpressing BACE1 show higher β-cleavage of APP (Bodendorf *et al.*, 2002). However, amyloid deposition in these mice correlates better with moderate than with high BACE1 overexpression (Section III.B.3) (Lee *et al.*, 2005). Moreover, the mice display degeneration of neurons in the neocortex and hippocampus and degradation of myelin (Rockenstein *et al.*, 2005). In double transgenic mice overexpressing both human BACE1 and APP, Aβ levels are increased and more Aβ depositions are observed (Bodendorf *et al.*, 2002; Chiocco *et al.*, 2004; Willem *et al.*, 2004).

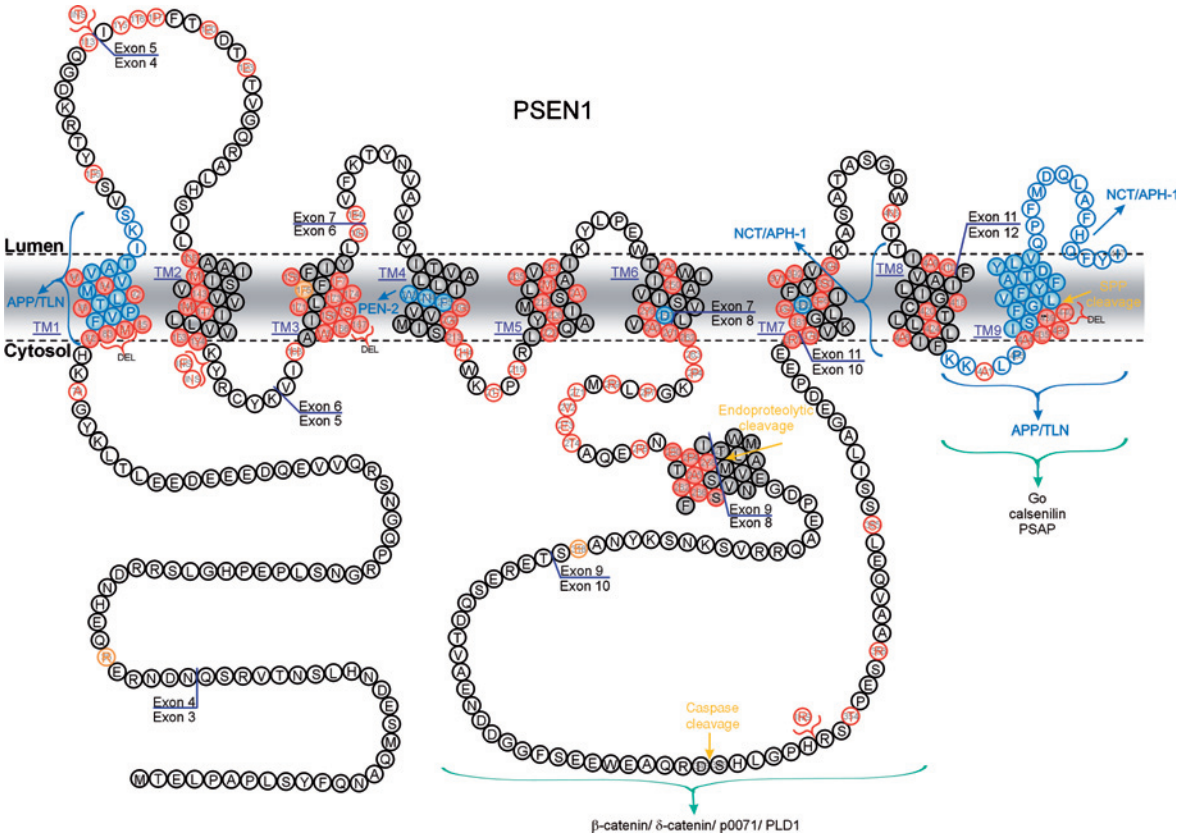
## 3. Sorting out BACE

There is a subtle difference in subcellular localization patterns between BACE1 and BACE2. BACE2 is broadly localized from early Golgi toward

the trans-Golgi network (TGN), including obvious vesicular staining reminiscent of early endosomes, while endogenous BACE1 and thus also most of the β-secretase activity is mainly restricted to the later Golgi/TGN (Yan *et al.*, 2001a,b). This localization probably depends on the BACE1 TMD, whereas the cytoplasmic tail of BACE1 is required for efficient maturation and trafficking through the Golgi (Capell *et al.*, 2000b). Indeed, BACE1 bears a highly conserved DDXXLL binding motif for interaction with the VHS (Vps-27, Hrs, and STAM) domain of GGA1 and GGA2 (Golgi-localized γ-ear-containing ADP-ribosylation factor-binding) adaptor proteins, and this interaction is increased upon phosphorylation of Ser-498 within this motif. This suggests that BACE1 exits the TGN via GGA- and AP1/clathrin-mediated budding transport carriers and is directly delivered to the endosomes, reminiscent of the sorting of mannose-6-phosphate receptors (Bonifacino, 2004). In accordance with the acid pH optimum of the BACE1 enzyme, small amounts are indeed localized in early endosomes. Furthermore, endosomal BACE1 can be retrogradely recycled to the TGN or sorted to lysosomes for degradation. Both routes depend, as expected, on (the phosphorylation status of the serine residue within) the GGA-binding dileucine motif (He *et al.*, 2005; Koh *et al.*, 2005; Shiba *et al.*, 2004; von Arnim *et al.*, 2004; Wahle *et al.*, 2005). The same sorting motif is also required for the internalization of small amounts of cell surface localized BACE1 (Huse *et al.*, 2000; Walter *et al.*, 2001), which is initiated through ectodomain interactions with APP (Huang *et al.*, 2004).

Furthermore, the majority of β-secretase activity seems to reside in different subcellular compartments than α-secretase activity, and several lines of evidence indicate that a differential sorting of the substrate APP to these α- and β-secretase-containing compartments is a mechanism to control Aβ production.

First, delivery of APP to endocytic compartments seems to favor β-secretase processing, while delivery of APP to the plasma membrane promotes α-secretase processing (Daugherty and Green, 2001; Rajendran *et al.*, 2006). Second, in polarized cells, BACE1 is sorted to the apical compartment, whereas APP undergoes basolateral sorting. Similarly, in hippocampal neurons, BACE1 is targeted to axons, while α-secretase cleavage of APP occurs in soma and dendrites (Capell *et al.*, 2002). However, high BACE1 overexpression causes a shift of β-secretase cleavage from the axonal/synaptic compartment to the neuronal cell body, thereby decreasing APP processing (and hence Aβ production) in post-Golgi compartments. This might explain why high BACE1 overexpression can decrease amyloid deposition in transgenic mice (Section III.B.2) (Lee *et al.*, 2005). Third, the neuronal sorting receptor sorLA (sorting protein-related receptor) was recently shown to regulate the sorting and access of APP to BACE1. SorLA confines APP to the Golgi by impairing its transition to the cell surface or by shuttling APP from early endosomes back to the Golgi. In this way, APP is protected from amyloidogenic processing in these Aβ-generating compartments


PSEN1
Lumen
Cytosol
APP/TLN
TM1
TM2
TM3
TM4
TM5
TM6
TM7
TM8
TM9
PEN-2
NCT/APH-1
NCT/APH-1
Exon 5
Exon 4
Exon 7
Exon 6
Exon 6
Exon 5
Exon 7
Exon 8
Exon 11
Exon 10
Exon 11
Exon 12
Exon 9
Exon 8
Exon 9
Exon 10
Exon 4
Exon 3
DEL
DEL
DEL
Endoproteolytic cleavage
Caspase cleavage
APP/TLN
Go
calsenilin
PSAP
β-catenin/ δ-catenin/ p0071/ PLD1

(Andersen *et al.*, 2005). Moreover, sorLA can also interact with BACE1, and this interaction prevents complex formation between BACE1 and APP in the Golgi, leading to a further inhibition of β-secretase cleavage of APP and Aβ production (Spoelgen *et al.*, 2006). Fourth, α-secretase is suggested to reside outside lipid rafts, whereas β-secretase cleavage of APP is thought to occur in lipid rafts, so the processing of APP via the amyloidogenic or nonamyloidogenic pathway seems to be regulated by its sorting to these compartments (Ehehalt *et al.*, 2003; Vetrivel *et al.*, 2005), although much debate about this hypothesis is still going on (Section IV.F.4).

## IV. Complex-ity of γ-Secretase

### A. General

Originally referring to the enzyme cleaving APP within its TMD, γ-secretase finally turned out to be a highly hydrophobic multiprotein complex consisting minimally of four transmembrane proteins: PSEN (Section IV.B), NCT (Section IV.C), APH-1, and PEN-2 (Section IV.D).

The ***PSEN1*** and ***PSEN2*** genes, located on chromosomes 14q24 and 1q42, were first discovered by geneticists searching for causative genes in early-onset AD (Levy-Lahad *et al.*, 1995; Rogaev *et al.*, 1995). They encode polytopic membrane proteins of 467 and 448 amino acids, respectively (Fig. 2). The fact that many more pathogenic mutations are found in the *PSEN1* gene than in the *PSEN2* gene (Section I) suggests more critical roles for PSEN1-bearing complexes in the onset of the disease. Except for the Δexon9 mutation, most FAD mutations are missense mutations resulting in single amino acid changes within or flanking the conserved hydrophobic TMDs. The TMD preference of these mutations, together with the general finding that they increase the relative production of the amyloidogenic Aβ42, suggests that they affect the overall

FIG. 2 Detailed amino acid representation of the predicted nine TMD topology and functional domains of human PSEN1, showing exon boundaries and amino acid numbering of human PSEN inside circles. FAD-linked mutations are indicated in red, while nonpathogenic mutations are marked in orange. Interaction domains with APP/TLN or NCT/APH-1/PEN-2 are marked in blue, as well as the conserved residues D257 and D385 forming the putative catalytic site. Interactions of the C-terminal domain and the hydrophilic loop domain with several other proteins, such as the brain G-protein Go, the calcium-binding protein calsenilin, the PSEN1-associated protein (PSAP), β- and δ-catenin, p0071, and PLD1, are shown in dark green. The endoproteolytic cleavage site separating PSEN1-NTF and -CTF in the seventh hydrophobic region, the SPP cleavage site in the ninth TMD, and the caspase cleavage site in the hydrophilic loop domain are indicated as well (yellow arrows).

structure and integrity of PSEN in the complex, leading to subtle alterations in substrate-binding properties or in the presentation of the substrate to the catalytic site. However, it was only until the dramatic effect of *PSEN1* deficiency on total Aβ production was recognized (De Strooper *et al.*, 1998) that the idea of PSEN being the enzyme itself surfaced. Indeed, ablation of both *PSEN1* and *PSEN2* genes even eliminates γ-secretase activity (Herreman *et al.*, 2000; Zhang *et al.*, 2000).

However, several findings, including (1) the requirement of unknown limiting factors for endoproteolysis and the formation of stable PSEN1-NTFs and -CTFs (Section IV.F.3) (Thinakaran *et al.*, 1997), (2) the cofractionation of γ-secretase activity and PSEN1 in a high-molecular-weight complex (Section IV.F.2) (Li *et al.*, 2000a; Yu *et al.*, 1998), and (3) the apparent "spatial paradox" between the preferential localization of PSEN1 in early secretory compartments versus γ-secretase activity essentially situated in post-Golgi compartments (Section IV.F.3) (Annaert and De Strooper, 1999) clearly suggested that additional proteins are involved in regulating γ-secretase activity and localization.

Surprisingly, the first such candidate was not discovered through genetic analysis, but biochemically, as a major protein coimmunoprecipitating with anti-PSEN1 antibodies. It was named **nicastrin** after the Italian village Nicastro where over 40 years ago FAD cases were described in an extended family, marking the start of the quest for causes of these rare inherited forms of AD (Yu *et al.*, 2000). In addition, a genetic screen aimed at identifying Notch pathway components required for the normal development of the anterior pharynx in the *C. elegans* embryo identified the genes *anterior pharynx defective-1* (***APH-1***) and the NCT orthologue *APH-2* (Goutte *et al.*, 2002). Independently, Francis and co-workers (2002) performed a genetic screen in *C. elegans* for genes interacting with *C. elegans* PSEN homologues. In this way, the presenilin enhancer genes *PEN-1*, which is identical to *APH-1*, and a novel gene ***PEN-2*** were identified. In the latter study, strong interactions of *APH-1* and *PEN-2* with *SEL-1* (PSEN orthologue) and *APH-2* were found, while RNAi-mediated downregulation of *APH-1* or *PEN-2* reduced γ-secretase cleavage of APP and Notch, showing that both proteins are essential for γ-secretase activity.

Since the discovery of these four γ-secretase complex members, several groups succeeded in reconstituting γ-secretase activity in yeast or in Sf9 insect cells by cooverexpression of these four components (Edbauer *et al.*, 2003; Hayashi *et al.*, 2004; Zhang *et al.*, 2005a). Moreover, in mammalian cells, cooverexpression results in increased γ-secretase activity (Kimberly *et al.*, 2003), indicating that PSEN, NCT, APH-1, and PEN-2 most likely comprise the minimal limiting factors that coassemble in an active γ-secretase complex (Section IV.F).

## B. Presenilin, a First Corner of the Veil Lifted

### 1. Current Face of Presenilin

The *PSEN* genes are conserved throughout the animal kingdom from mammals to worms, in plants and even in the slime mould *Dictyostelium* (Annaert and De Strooper, 2002) (www.ensembl.org). In *C. elegans*, three PSEN family members exist, SEL-12, HOP-1 (homologue of presenilin) and SPE-4, showing, respectively, 42%, 27%, and 17% similarity to human PSEN1, and the latter is a diverged member of the PSEN family functioning in proper localization of macromolecules during spermatogenesis. In contrast, *Drosophila* bears only one PSEN homologue. Interestingly, many studies show that human *PSEN* genes can functionally rescue deficiencies of their orthologues in *C. elegans*, *Drosophila,* and zebrafish (and vice versa), underscoring the high functional conservation in this family.

Within this PSEN family, a remarkably high degree of similarity or even identity is seen in the regions encoding the hydrophobic domains, suggesting that the major functions of PSEN are directed by these hydrophobic regions (HR) (Fig. 2). In contrast, the hydrophilic region of the N-terminal domain of PSEN and the large hydrophilic loop domain between HR seven and eight are quite divergent in the different PSENs, even within the same species, or even absent (e.g., in *Dictyostelium*). Therefore, it is likely that these domains convey some functional specificity. In support, both domains have been found to be dispensable for γ-secretase activity (Soriano *et al.*, 2001).

In total, 10 HR can be delineated in PSEN (Fig. 2). The determination of which HRs cross the lipid bilayer as TMDs is essential for a correct understanding of structure–function relationships. However, the exact topology of PSEN remains an issue of controversy (Kim and Schekman, 2004). Over the years, various topology models claiming six to nine TMDs have been proposed based on high overexpression of FL-PSEN or truncated or single HR fragments fused to reporter molecules. Thus, these experiments exceeded the normal physiological context of PSEN expression. The most widely accepted model predicted eight TMDs, with the N- and C-termini and the large hydrophilic loop domain facing the cytosol. This model was recently jeopardized by the finding that the C-terminus has a luminal orientation (Oh and Turner, 2005b). Subsequent studies identified the tenth HR as a TMD, leading to an overall nine TMD topology with the N-terminus facing the cytosol and the C-terminus facing the lumen (Henricson *et al.*, 2005; Laudon *et al.*, 2005; Oh and Turner, 2005a; Spasic *et al.*, 2006). This time, the conclusion is based on several independent less invasive strategies. Indeed, glycosylation consensus motifs, cell surface biotinylation, and cystein derivatization were used on FL-PSEN1 expressed in a *PSEN* knockout background. Furthermore, the data

on the glycosylation status of the PSEN1 variants, a modification occurring during ER-Golgi transport, are in line with the results of the cell surface biotinylation and cystein derivatization, demonstrating that PSEN1 maintains its topology throughout the biosynthetic pathway.

This nine TMD model is consistent with the different functions assigned to PSEN1 (Fig. 2). Most notably, it conserves the juxtaposed position of two conserved aspartate residues [D257 and D385 (numbering of human PSEN1)] in TMD6 and 7 that serve as active site residues for the catalytic role of PSEN in γ-secretase cleavage (Section IV.B.2). Moreover, the well-documented binding of β-catenin to the hydrophilic loop domain, implicating PSEN1 in β-catenin turnover, is also conserved (Section IV.B.3). Furthermore, the luminal orientation of the C-terminus is compatible with its binding to the TMD of APP, telencephalin (TLN), NCT, and APH-1, since the extreme C-terminus most likely protrudes in the lipid bilayer for interaction (Annaert *et al.*, 2001; Bergman *et al.*, 2004b; Kaether *et al.*, 2004; Shiraishi *et al.*, 2006). This hypothesis is corroborated by the finding that antibodies directed against this absolute C-terminal region cannot immunoprecipitate the PSEN1-CTF in conditions where the integrity of the complex is preserved (Fraering *et al.*, 2004a). Following the identification of a second binding domain for APP in the first TMD of PSEN1, a ring structure topology for PSEN1 was proposed, assuming that this domains acts in concert with the C-terminus for binding APP (Annaert *et al.*, 2001).

However, this new 9 TMD topology urges reevaluation of (1) two-hybrid interaction studies assuming a cytosolic orientation of the C-terminus and (2) the validity of C-terminal reporter constructs to explore, e.g., functions of PSEN1 in trafficking and interaction with other γ-secretase complex members (Section IV.F.2) (Kaether *et al.*, 2004). Taken together, we believe that this new nine TMD topology most faithfully mimics the *in vivo* situation and will persist until ultimate proof will be provided by structural biologists. However, given the hydrophobic nature of PSEN1, this task will not be easy to achieve.

PSENs are not posttranslationally modified by glycosylation, but PSEN1 and PSEN2 can be differentially phosphorylated (Walter *et al.*, 1998), a modification that might regulate PSEN-CTF levels (Kirschenbaum *et al.*, 2001; Lau *et al.*, 2002), PSEN association with β-catenin (Kirschenbaum *et al.*, 2000), and PSEN cleavage by caspases during apoptosis (Fluhrer *et al.*, 2004; Walter *et al.*, 1999). However, the major posttranslational modification of PSEN is its endoproteolysis in HR7, resulting in a stable ~30-kDa NTF and a stable ~20-kDa CTF (Fig. 2) (Kim *et al.*, 1997; Thinakaran *et al.*, 1996). This cleavage is thought to occur cotranslationally or very early after biosynthesis (Annaert *et al.*, 1999; Zhang *et al.*, 1998) and is assumed to be autocatalytic or mediated by an unknown "presenilinase." Intriguingly, one study suggested that signal peptide peptidase (SPP, also called IMP1), one of the PSEN homologues, can execute intramembranous cleavage in TMD9

of PSEN holoprotein after overexpression of both proteins (Section IV.F.1) (Moliaka *et al.*, 2004). Moreover, the domain around this cleavage site strictly resembles the hydrophobic domain around the endoproteolysis site. Therefore, endoproteolytic cleavage of PSEN within HR7 may be an example of intramembranous proteolysis by another member of the PSEN family or by PSEN itself.

Although endoproteolysis is not an absolute requirement for γ-secretase activity, it is necessary for the formation of stable active complexes (Capell *et al.*, 1998; Levitan *et al.*, 2001a; Steiner *et al.*, 1999). Indeed, excess FL-PSEN (e.g., following overexpression) is not integrated in the complex and is therefore rapidly degraded by the proteasome (Steiner *et al.*, 1998). The noncovalent PSEN-NTF/CTF heterodimer is the biologically active form of PSEN, since coexpression of PSEN1-NTF and PSEN1-CTF, chimeric PSEN1-NTF/PSEN2-CTF, or PSEN2-NTF/PSEN1-CTF can restore γ-secretase complex formation and activity in *PSEN*-deficient mammalian cells (Laudon *et al.*, 2004b; Stromberg *et al.*, 2005). Furthermore, several amino acid residues and motifs seem to be critical for endoproteolysis, e.g., residues within TMD1, Y288, and other residues in HR7, D257 and D385 in TMD6 and TMD7, the conserved C-terminal PALP motif, and the C-terminal end of PSEN1 (Brunkan *et al.*, 2005; Kaether *et al.*, 2004; Laudon *et al.*, 2004a; Tomita *et al.*, 2001; Wolfe *et al.*, 1999). In many cases, however, mutation of these residues abrogates both endoproteolysis and catalytic activity. In addition, endoproteolysis of PSEN requires interaction with other members of the γ-secretase complex such as PEN-2, and the conserved motif (Trp–)Asn–Phe in the proximal two-thirds of TMD4 of PSEN1 is responsible for this binding (Section IV.D.2) (Fig. 2) (Kim and Sisodia, 2005b; Watanabe *et al.*, 2005). Other interacting proteins, such as ubiquilin-1 and -2, also seem to be involved in the endoproteolytic process (Massey *et al.*, 2005; Prokop *et al.*, 2005).

## 2. Presenilin, a Diaspartyl Protease

In recent years, much evidence has been collected showing that γ-secretase is an intramembrane aspartyl protease with the conserved Asp D257 and D385 constituting its catalytic core. Not only these Asp residues, but also their flanking regions in TMD6 and 7 are highly conserved or even identical among PSEN members, including the family of PSEN homologues (Section IV.B.1) (Ponting *et al.*, 2002). Furthermore, they share homology with the putative active site of bacterial type 4 prepilin proteases, which are also polytopic aspartyl proteases (Steiner *et al.*, 2000).

Mutation of either of these Asp residues in PSEN1 greatly reduces γ-secretase processing of APP and Notch1, and such mutations in both PSEN1 and PSEN2 even completely abolish Aβ production (Berezovska *et al.*, 2000; Kimberly *et al.*, 2000; Wolfe *et al.*, 1999). However, some discussion

persisted on the contribution of each Asp residue to γ-secretase activity, since D257 mutations only impaired Notch cleavage, while D385 mutants blocked both APP and Notch processing (Capell *et al.*, 2000a; Kim *et al.*, 2001; Morihara *et al.*, 2000). Most likely, this apparent discrepancy is methodological in nature, because the former experiments were carried out in a wild-type *PSEN* background using transient overexpression. A reevaluation of the effects of the Asp mutants on PSEN function in $PSEN1^{-/-}PSEN2^{-/-}$ cells finally confirmed the involvement of both residues in the catalytic site of γ-secretase (Nyabi *et al.*, 2003). Moreover, Asp mutants rescued PEN-2 stability, NCT maturation, and complex formation, suggesting that these residues are critical for the catalytic function of PSEN, but not for its other roles (Section IV.B.3).

In support of this catalytic role, transition state analogue γ-secretase inhibitors designed to bind to the active site of an aspartyl protease were shown to bind directly to the NTFs and CTFs of PSEN1 and PSEN2 (Berezovska *et al.*, 2000; Esler *et al.*, 2000; Li *et al.*, 2000b; Seiffert *et al.*, 2000). In addition, mutation of the Asp residues abrogated inhibitor binding, while replacement of the nearby tyrosines altered γ-secretase cleavage specificity (Wrigley *et al.*, 2004). Taken together, these data assign the aspartates and the conserved residues around them as the active site of the γ-secretase complex.

Importantly, this catalytic site of PSEN1 formed by D257 and D385 is spatially separated from its substrate binding site. This was first demonstrated biochemically by the identification of the APP binding domains in PSEN1 (Section IV.B.1) (Annaert *et al.*, 2001). Further support came from the observation that active site-binding transition state-specific γ-secretase inhibitors did not affect binding of PSEN1 to APP-CTFs (Berezovska *et al.*, 2003; Esler *et al.*, 2002; Pitsi and Octave, 2004; Tian *et al.*, 2002; Xia *et al.*, 2000). Moreover, under saturating conditions, the substrates APP and Notch were shown to compete for proteolysis but not for binding to PSEN (Schroeter *et al.*, 2003). Finally, docking of APP on PSEN1 seemed to be NCT dependent (Berezovska *et al.*, 2003), in agreement with the recent finding that the NCT ectodomain functions as a substrate receptor (Section IV.F.1). It was suggested that the substrate docking site was located at the interface between the PSEN-NTF and -CTF, in close proximity to the active site (Kornilova *et al.*, 2005). The latter finding does not necessarily contradict the ring structure topology we proposed originally, since the substrate might pass through both PSEN1 subunits to the middle of the ring to access the active site. Alternatively, the active site may reside at the interface between two PSEN1 molecules composing a dimer. In this case, one PSEN1 molecule might bind the substrate and offer it to the second PSEN1 molecule (Schroeter *et al.*, 2003). This hypothesis is supported by the finding that different dimeric associations between PSEN1 fragments are possible (Hebert *et al.*, 2003b; Herl *et al.*, 2006). Furthermore, the active site conformation of γ-secretase, which is dependent on the C-terminal PAL motif

(Wang *et al.*, 2006a), is similar to that of SPP, whose catalytically active species appears to be a homodimer (Section IV.F.1) (Nyborg *et al.*, 2004).

### 3. Presenilin, "Hubbing" for More Functions

Evidence is accumulating that certain PSEN1 (and probably PSEN2) domains fulfill functions that are different from and independent of its catalytic role in γ-secretase activity (Thinakaran and Parent, 2004). Most convincing is the binding of β-catenin to the hydrophilic loop of PSEN1, but not PSEN2 (Fig. 2) (Kang *et al.*, 2002; Soriano *et al.*, 2001) and its associated role as a negative regulator of the Wnt/β-catenin signaling pathway. PSEN1 functions as a scaffold that rapidly couples β-catenin phosphorylation through two sequential kinase activities independent of the canonical Wnt-mediated signal transduction pathway. Consequently, in the absence of PSEN, the stepwise phosphorylation of β-catenin is disconnected, resulting in increased β-catenin stability *in vitro* and *in vivo* (Kang *et al.*, 2002). Indeed, mice models with targeted inactivation of *PSEN1* in the skin display a gain of β-catenin signaling, resulting in epidermal skin tumors (Soriano *et al.*, 2001; Xia *et al.*, 2001). Interestingly, it has recently been suggested that the interaction between PSEN1 and β-catenin depends on cadherins, which also mediate the effects of PSEN1 on β-catenin stability (Serban *et al.*, 2005).

A second γ-secretase-independent role for PSEN1 emerged from the delay in turnover of neuron-specific TLN (ICAM-5) observed in $PSEN1^{-/-}$ hippocampal neurons (Esselens *et al.*, 2004; Raemaekers *et al.*, 2005). TLN accumulated in large nonacidified membrane-bound organelles that did not colocalize with markers of the endosomal/lysosomal degradative route. Instead, TLN recruited several conjugation complexes involved in autophagic vacuole formation, such as the Apg12/Apg5 and LC3 proteins. These findings led to the conclusion that *PSEN1* deficiency affects the turnover of TLN (and probably also other proteins) by impairing the capability of autophagic-like vacuoles to fuse with lysosomes, resulting in their accumulation. A similar phenotype of accumulating degradative organelles is seen in $PSEN1^{-/-}$ cortical neurons, although in this case synuclein proteins are translocated to these organelles (Wilson *et al.*, 2004). Interestingly, α-synuclein-positive organelles are also found in Lewy bodies, suggesting that similar mechanisms may form the basis of several neurodegenerative diseases. In agreement with this new role of PSEN1, activation of the endosomal/lysosomal system as well as alterations in autophagic degradation, leading to autophagic vacuoles containing APP, APP-CTFβ, PSEN1, and NCT, have recently been observed in the brain of AD patients (Nixon, 2005; Yu *et al.*, 2005).

Interestingly, Wilson and co-workers noticed that the synuclein accumulation was accompanied by a dysregulation of calcium channels. This is supported by the recent discovery of a γ-secretase indepentent function of PSENs as passive ER calcium leak channels (Tu *et al.*, 2006). Other groups have also reported a role for PSENs in capacitative calcium entry, a refilling mechanism for depleted

intracellular calcium stores, although the observed effects were in this case γ-secretase dependent (Yoo *et al.*, 2000). Similarly, PSENs can modulate phosphoinositide-mediated calcium signaling through the production of the AICD (Leissring *et al.*, 2002).

Third, PSEN plays a role in the intracellular transport and subcellular localization of several proteins, but it is not yet clear whether this function is always independent of its catalytic role in γ-secretase activity. For instance, recruitment of phospholipase D1 (PLD1) to the Golgi/TGN was recently shown to depend on its interaction with the loop domain of PSEN1 (Fig. 2) (Cai *et al.*, 2006a). In addition, several membrane proteins depend on PSENs for proper transport, maturation, and localization, including the TrkA receptor, tyrosinase, and APP (Cai *et al.*, 2003; Naruse *et al.*, 1998; Rechards *et al.*, 2006; Wang *et al.*, 2006b; Wrigley *et al.*, 2005), but also other γ-secretase components like NCT and PEN-2 (Section IV.F.3) (Bergman *et al.*, 2004a; Chen *et al.*, 2003; Herreman *et al.*, 2003; Wang *et al.*, 2004). However, it is not unlikely that in some cases the observed mislocalizations following *PSEN* deficiency are of indirect origin. They might, for instance, be caused by changes in caveolin-1 expression in *PSEN1* deficient cells, resulting in a loss of caveolae at the cell surface (Wood *et al.*, 2005). Recently, PSEN, NCT and APH-1 were shown to have a γ-secretase independent role in the control of the intracellular distribution of GSK3β and a PKC at intracellular junctions in *Drosophila*, a function that is also essential for prevention of tau hyperphosphorylation (Doglio *et al.*, 2006).

Finally, a γ-secretase-independent role of PSEN in somitogenesis was recently suggested (Section IV.E) (Huppert *et al.*, 2005). Furthermore, several physiological functions have been assigned to PSEN1 or PSEN2, including roles in skeletal development, neurite outgrowth and synaptic plasticity, apoptosis, and tumorigenesis (Thinakaran and Parent, 2004). However, it remains to be investigated whether any of these functions is directly related to one of the many proteins that have been documented to interact with PSEN1 or PSEN2 (Fig. 2). Three proteins are known to selectively interact with PSEN2 but not PSEN1: calmyrin (Stabler *et al.*, 1999), sorcin (Pack-Chung *et al.*, 2000), and FLH2 (Tanahashi and Tabira, 2000). Interaction of the latter with PSEN2-NTF can lead to activation of PDGF receptor expression, thereby implicating PSEN2 in PDGF-induced Akt/ERK signaling (Kang *et al.*, 2005). These observations suggest that in the future more PSEN2-specific functions will be discovered, further emphasizing the differences between the two homologues rather than their common role in γ-secretase-mediated cleavage.

## C. Nicastrin, a First Companion

### 1. Identifying Nicastrin as a True γ-Secretase Component

NCT orthologues are found in mammals, *C. elegans* (APH-2), and *Drosophila*, whereas a weakly similar protein exists in *Arabidopsis* (www.ensembl.org).

NCT can partially substitute for APH-2 in *C. elegans*, showing a conservation of function between these proteins (Levitan *et al.*, 2001b). In rat, a 62-amino acid-long alternative splice variant of NCT can be found, which lacks exon 3 and is preferentially expressed in neurons (Confaloni *et al.*, 2005).

The human gene encoding NCT (*NCT*) maps to chromosome 1q22–23. Although this region shows genetic linkage and/or allelic association with late onset AD, only a few studies reported an increased risk for AD in patients with certain haplotypes of the *NCT* gene, while others did not (Bertram and Tanzi, 2004) (www.alzgene.org). So far, two missense substitutions of conserved residues in the mouse *NCT* sequence have been identified (aa 21 and aa 678) that influence γ-secretase cleavage at the Notch S3 site but not APP cleavage (Rozmahel *et al.*, 2002).

*In vivo*, NCT plays a central role in PSEN-mediated processing of APP and Notch/GLP-1, and it can bind both substrates. The protein is found in high-molecular-weight complexes with PSEN1 or PSEN2 (Chen *et al.*, 2001; Goutte *et al.*, 2000; Yu *et al.*, 2000). Moreover, NCT is retained on an immobilized active site-directed γ-secretase inhibitor, and anti-NCT antibodies can immunoprecipitate γ-secretase activity from detergent-solubilized microsomes, providing biochemical evidence that NCT is a true γ-secretase member (Esler *et al.*, 2002).

## 2. Nicastrin's Role Slowly Revealed

The human *NCT* gene encodes a type I transmembrane glycoprotein consisting of 709 amino acids, with a short cytoplasmic domain (17 amino acids), which is dispensable for complex assembly and activity, and a large ectodomain with a putative signal peptide sequence, several N-glycosylation sites, and four regularly spaced cysteins suggesting disulfide bonds (Fig. 3A) (Capell *et al.*, 2003; Yu *et al.*, 2000).

After its synthesis in the ER, NCT becomes complex glycosylated and sialylated during its transport through the Golgi. However, complex glycosylation is not an absolute requirement for binding to PSEN1, reaching the cell surface, or even γ-secretase activity (Herreman *et al.*, 2003). On the other hand, NCT ectodomain integrity is required for correct folding and thus for NCT function. Indeed, the ectodomain undergoes major conformational changes, most likely during early transport. These changes are essential for interaction with PSEN and PEN-2 (but not APH-1), γ-secretase complex assembly, and activity (Section IV.F.2) (Olry *et al.*, 2005; Shirotani *et al.*, 2003).

Furthermore, the ectodomain contains a highly conserved DYIGS sequence (aa 336–340 in human NCT). This motif is situated within a larger domain (aa 261–502) that shows sequence similarity to the aminopeptidase/transferrin receptor superfamily, commonly referred to as the DYIGS and peptidase homologous region or DAP domain, although no peptidase

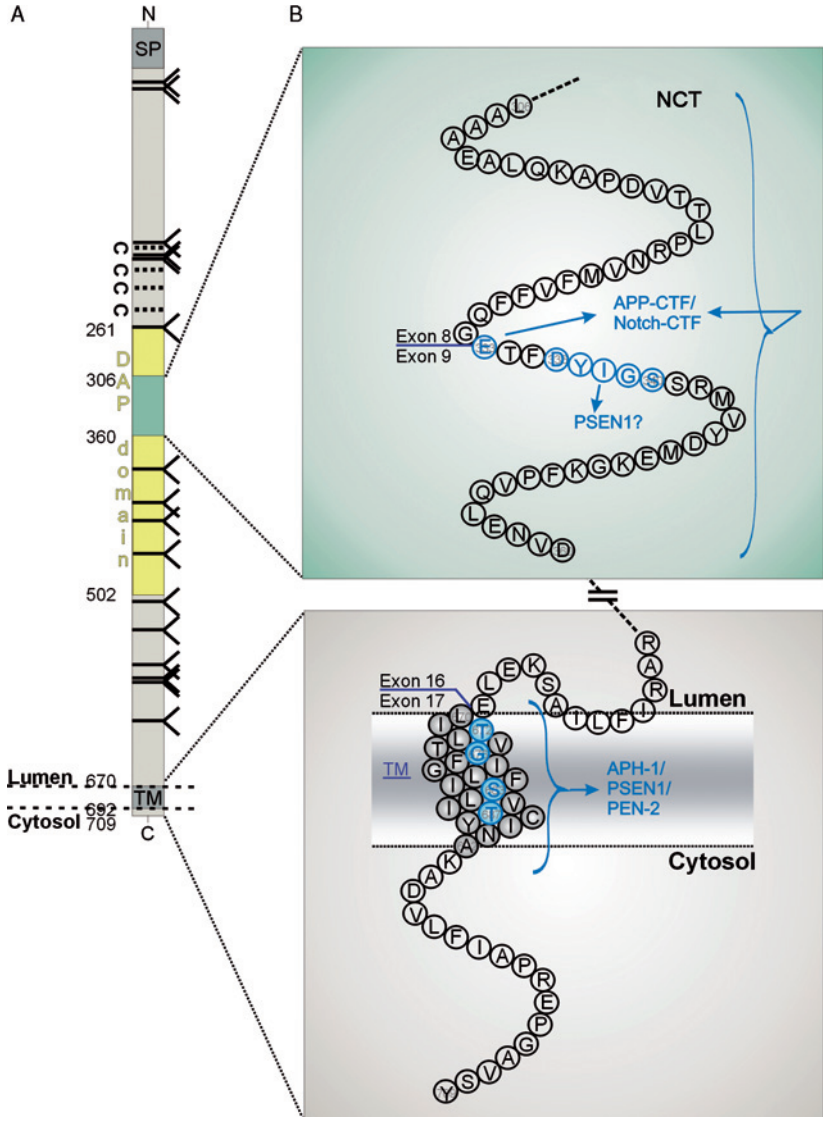



FIG. 3 Predicted membrane topology and functional domains of human NCT. (A) Overall domain structure of the type I transmembrane protein NCT. Amino acid numbering of domain boundaries is shown on the left side of the bar. Glycosylation sites (—⋌) and conserved cysteines (C) are marked. The yellow box indicates the DAP domain containing the highly conserved region from aa 306 to aa 360 (green). SP, signal peptide; TM, transmembrane domain. (B) Detailed amino acid representation of the highly conserved region (aa 306–360) and the TM and IC domains of human NCT. Exon boundaries are indicated and amino acid numbering is shown inside circles. The conserved region, containing the DYIGS sequence,

activity appears to be associated with NCT to date (Fig. 3) (Fagan *et al.*, 2001; Fergani *et al.*, 2001). However, recent studies assign important functions to this domain. Missense and deletion mutations in the DYIGS motif respectively increase and reduce Aβ secretion, without affecting substrate binding, while Notch S3 cleavage is differentially modulated (Chen *et al.*, 2001). In addition, the DAP domain, and especially the Glu-333 residue herein, was recently shown to function as a γ-secretase substrate receptor (Section IV. F.1) (Shah *et al.*, 2005). However, the NCT ectodomain is not essential for γ-secretase assembly and activity.

The maturation process, and hence the trafficking of NCT, occurs slowly as compared to other proteins in the secretory pathway, suggesting that NCT may be actively retained in pre-Golgi compartments. Since NCT has no known retention or retrieval sequences (Capell *et al.*, 2005), regulation of retrieval may occur indirectly, i.e., through the interaction with other proteins such as ER-Golgi SNAREs or even other γ-secretase components (Section IV.F.2). A region of human NCT encompassing the last 50 residues of the ectodomain, the TMD and the cytoplasmic domain, is important for mediating interactions with human PSEN1, APH-1, and PEN-2. In particular, the hydrophilic residues T672, G676, S683, and T687 appear to be critical for this interaction and for γ-secretase complex assembly, consistent with the general knowledge that the bulk of the γ-secretase complex resides within the membrane (Fig. 3B) (Capell *et al.*, 2003; Morais *et al.*, 2003). Even in the absence of PSEN and PEN-2, the TMD mediates the binding of NCT to APH-1, leading to the formation of an NCT/APH-1 subcomplex (Section IV.F.2).

## D. Completing the Picture: APH-1 and PEN-2

### 1. Adding a Flavor of Variety to γ-Secretase

The human genome contains only one *PEN-2* gene, located on chromosome 19q13 near the *ApoE* gene. In contrast, two orthologous APH-1 proteins are found, APH-1A and –1B, and their genes, *APH-1A* and *APH-1B,* are located on chromosome 1q21, in a genomic region associated with late onset AD, and on chromosome 15q22, respectively (Francis *et al.*, 2002; Kehoe *et al.*, 1999). So far, no clear linkage of *APH-1A/B* and *PEN-2* polymorphisms with

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and E333 in particular, may function as a receptor for substrates like APP-CTF and Notch-CTF (blue bracket). The DYIGS motif is thought to play a role in the interaction with PSEN1 (blue). The TM domain, and especially a hydrophilic patch composed of T672, G676, S683, and T687, is important for interaction with APH-1, PSEN1, and PEN-2 (blue).

a higher risk for AD has been demonstrated (www.alzgene.org). Mammalian APH-1A has at least two splice variants, differing in the length of their open reading frame (ORF): mAPH-1A$^{L}$ [with a longer (L) ORF of seven exons encoding 265 amino acids] and mAph1A$^{S}$ [with a shorter (S) ORF of six exons encoding 247 residues] (Fig. 4A). mAPH-1B has six exons and encodes 257 amino acids. The APH-1A variants show ~56% amino acid identity to APH-1B (Gu *et al.*, 2003; Lee *et al.*, 2002). A gene duplication of *APH-1B* in rodents gave rise to a third gene, *APH-1C*, so both genes, found in tandem (±20 kb apart) on chromosome 9, are highly similar (96.3% at the nucleotide level) (Coolen *et al.*, 2005; Hebert *et al.*, 2004). Furthermore, APH-1 and PEN-2 orthologues are also identified in *Drosophila* (dAPH-1/dPEN-2), *C. elegans*, zebrafish, *Xenopus,* and other phyla (www.ensembl.org).

Soon after the genetic linkage of *APH-1* and *PEN-2* to PSEN-dependent γ-secretase processing, biochemical evidence emerged for their physical association with this complex in mammalian cells (Kimberly *et al.*, 2003; Steiner *et al.*, 2002).

## 2. Small in (Molecular) Weight But Not Less Important

***APH-1*** is a hydrophobic seven TMD protein with a proposed topology essentially identical to that of seven TMD receptors, with the N-terminus and C-terminus facing the lumen and cytosol, respectively (Fig. 4A) (Fortna *et al.*, 2004). Both APH-1A and APH-1B are expressed in almost all tissues, and APH-1A$^{S}$ is 1.5–3 times more abundantly expressed than APH-1A$^{L}$ (Saito and Araki, 2005). Exogenously overexpressed APH-1 appears to undergo at least three distinct endoproteolytic events, but this finding could not be confirmed at endogenous levels (Fortna *et al.*, 2004; Gu *et al.*, 2003). Furthermore, no other posttranslational modifications, such as glycosylation, were observed (Goutte *et al.*, 2002).

Several studies in *C. elegans* (Goutte *et al.*, 2002), *Drosophila* (Niimura *et al.*, 2005), and mammals (Edbauer *et al.*, 2004; Lee *et al.*, 2004; Saito *et al.*, 2005a) identified a conserved glycine in the fourth TMD of APH-1 (Gly-122 in mAPH-1A) that is critically important for the function of this protein. In mAPH-1A, Gly-122 forms a membrane helix–helix GXXXGXXXG motif together with Gly-126 and Gly-130. This motif is essential for the interaction with PSEN and PEN-2, but not with NCT. Mutating Gly-122 leads to retention of the APH-1/NCT subcomplex in the ER, confirming or indicating that (1) the interaction with PSEN is important for transport of APH-1 and NCT to the Golgi (Section IV.F.2) and (2) the GXXXG motif plays a role in γ-secretase complex assembly. Furthermore, this motif may also stabilize APH-1 itself through intramolecular associations between its TMDs. The TMDs in PSEN and PEN-2 mediating the binding to this APH-1 motif have not yet been identified, nor have the TMD(s) in APH-1 that bind to NCT.

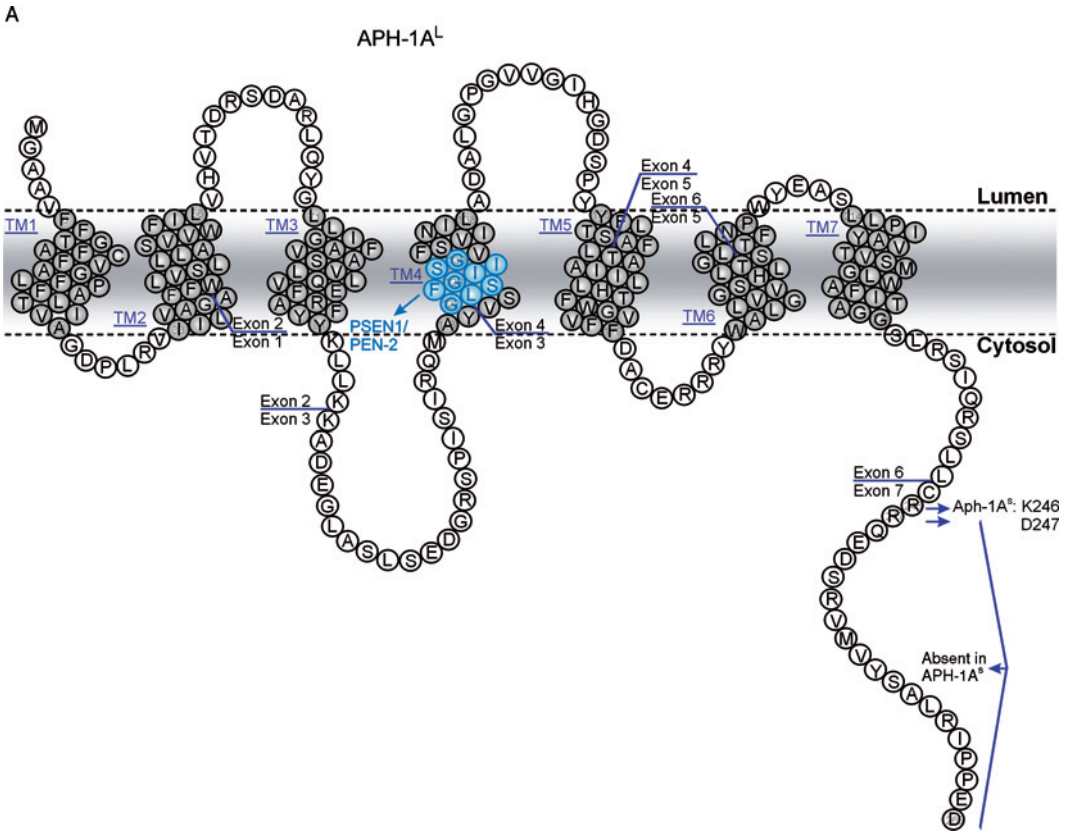


FIG. 4 (*continued*)

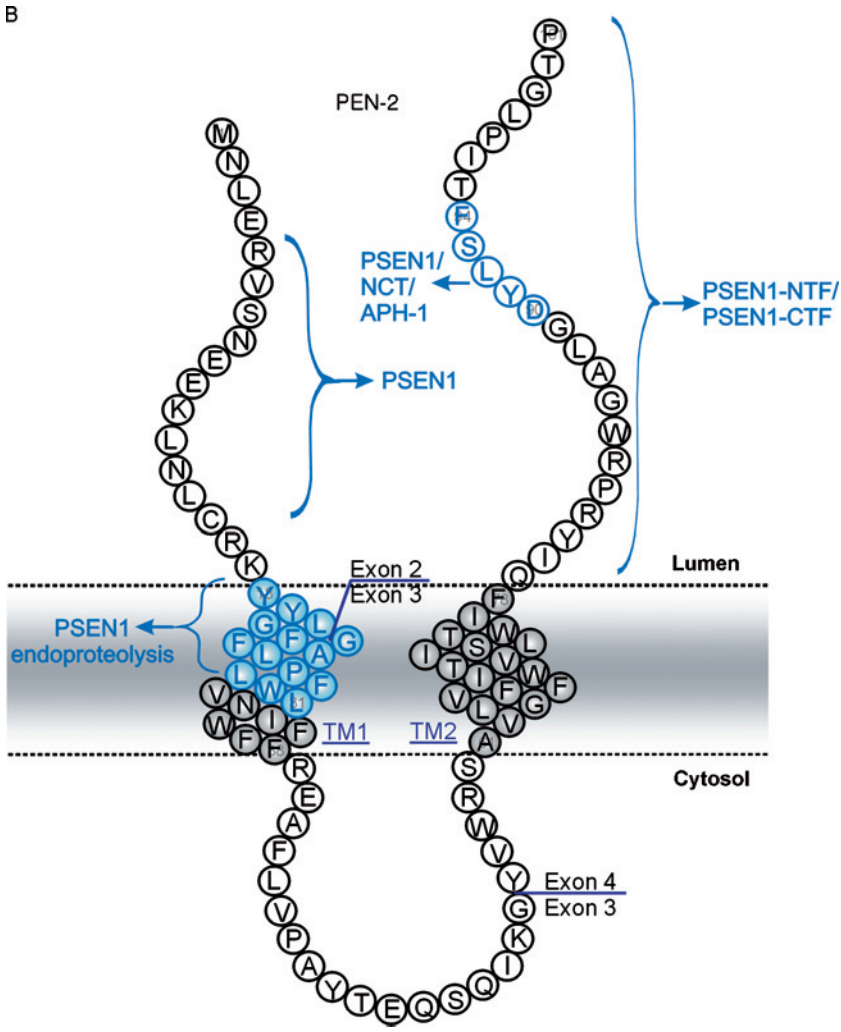


FIG. 4 Predicted membrane topology of human APH-1A$^{L}$ and human PEN-2, showing amino acid sequence, amino acid numbering inside circles, and exon boundaries. Interaction domains with other γ-secretase complex components are indicated in blue. (A) Detailed amino acid representation of the predicted seven TMD topology of human APH-1A$^{L}$. Aph-1A$^{S}$ contains only 247 amino acids and the last two residues are K246 and D247 instead of R246 and R247. The membrane helix–helix GXXXGXXXG motif in the fourth TMD, containing the conserved G122, as well as G126 and G130, is important for interaction with PSEN1 and PEN-2 (blue). (B) Detailed amino acid representation of the membrane topology of the hairpin membrane protein human PEN-2. The N-terminal domain is important for interactions with PSEN1 (blue bracket), whereas the proximal two-thirds of TMD1 (blue) have a function in PSEN1 endoproteolysis. The hydrophilic C-terminal part is required for stabilization of the PSEN1-NTF and PSEN1-CTF formed after endoproteolysis (blue bracket). In particular, the highly conserved DYLSF motif (blue) may play a role in binding of PEN-2 to other components of the γ-secretase complex.

Mammalian ***PEN-2*** encodes a hairpin membrane protein of 101 amino acids and has two predicted TMDs, while both its N- and C-terminus face the lumen (Fig. 4B) (Bergman *et al.*, 2004a; Crystal *et al.*, 2003; Francis *et al.*, 2002). RNAi-mediated knockdown of *PEN-2* does not affect APH-1 and APP levels, but impairs NCT maturation, PSEN1 endoproteolysis, and FL-PSEN stabilization. Consequently, γ-secretase complex assembly is also impaired, leading to a significant reduction of Aβ secretion (Steiner *et al.*, 2002; Takasugi *et al.*, 2003; Luo *et al.*, 2003a). However, PEN-2 overexpression promotes the production of PSEN1 fragments (Hu and Fortini, 2003; S. H. Kim *et al.*, 2003). Moreover, PEN-2 is said to enhance γ-secretase cleavage after PSEN heterodimer formation, possibly by enhancing the affinity or the accessibility of the substrate to the catalytic site (Shiraishi *et al.*, 2004). Taken together, these findings suggest independent roles for PEN-2 in PSEN endoproteolysis (Section IV.B.1) and in γ-secretase complex assembly, apparently orchestrated by different domains within the protein (Fig. 4B). Indeed, the N-terminal domain of PEN-2 may mediate interaction with PSEN1, since introducing a glycosylation consensus motif in this region prevents its association with PSEN1 (Crystal *et al.*, 2003). In addition, a conserved DYLSF motif (aa 90–94) in the C-terminus may be involved in the interaction with other γ-secretase components (Hasegawa *et al.*, 2004). Importantly, the integrity of the hydrophilic C-terminus, both in length and in overall sequence, is important for stabilizing the PSEN-NTF and -CTF after endoproteolysis, but not for endoproteolysis itself. Furthermore, the length of this region is also important for NCT maturation and γ-secretase activity (Hasegawa *et al.*, 2004; Kim and Sisodia, 2005a; Prokop *et al.*, 2004, 2005). Finally, the proximal two-thirds of TMD1 seem to be functionally important for endoproteolysis of PSEN1 holoprotein and the generation of PSEN1 fragments (Kim and Sisodia, 2005a).

The further identification and delineation of the putative binding domains between γ-secretase complex members will become a crucial element in understanding (the sequence of) complex assembly.

## E. Genetic Deficiency of γ-Secretase Subunits

Although the list of γ-secretase substrates is still growing (Section IV.F.1), the best studied and probably also the most important substrates are APP and especially Notch. This crucial role of the γ-secretase complex in Notch and APP signaling has been confirmed by studies in *C. elegans*, *Drosophila,* and mouse (Sections IV.A/C.1/D.1) (Chung and Struhl, 2001; Hu *et al.*, 2002; Levitan *et al.*, 2001b; Lopez-Schier and St Johnston, 2002). Genetic inactivation or downregulation of one of the γ-secretase complex components inevitably causes defects in γ-secretase processing of APP and Notch (Section IV.F.3)

(Annaert and De Strooper, 2002; Struhl and Greenwald, 2001) and a phenotype that resembles a *Notch*-deficient phenotype (Huppert *et al.*, 2000; Krebs *et al.*, 2000). Indeed, *PSEN1*$^{-/-}$ or *PSEN1*$^{-/-}$*PSEN2*$^{-/-}$ (Marjaux *et al.*, 2004), *NCT*$^{-/-}$ (Li *et al.*, 2003a,b), or *APH-1A*$^{-/-}$ (Ma *et al.*, 2005; Serneels *et al.*, 2005) mice are embryonic lethal and show disturbed somite segmentation, angiogenic vascular morphogenesis deficits in the yolk sac, kinks in the neural tube, and distention of the pericardial sac.

However, differences between the knockout models of the different γ-secretase complex members can also be discerned. For instance, *PSEN1*$^{-/-}$*PSEN2*$^{-/-}$ embryos die at E9.5 and *NCT*$^{-/-}$ and *APH-1A*$^{-/-}$ embryos at E10.5, while *PSEN1*$^{-/-}$ embryos show only perinatal lethality (E17-P1), and *PSEN2*$^{-/-}$, *APH-1B*$^{-/-}$, *APH-1C*$^{-/-}$, and the combined *APH-1BC*$^{-/-}$ mice (which can be considered as a model for total *APH-1B* loss in humans) are viable and fertile. These findings indicate that PSEN1 and APH-1A are essential and that PSEN2, APH-1B, and APH-1C are redundant for normal Notch signaling during mammalian embryological development. The *APH-1B*$^{-/-}$ and *APH-1C*$^{-/-}$ mice are even less affected than the *PSEN2*$^{-/-}$ mice, which suffer from lung hemorrhages and mild pulmonary fibrosis in adulthood. However, *APH-1BC*$^{-/-}$ deficiency affects steady-state levels of PSEN and PEN-2 and causes a mild but significant reduction in APP processing in selective regions of the adult brain, especially in brainstem and olfactory bulb, while absence of *PSEN2* does not detectably alter processing of APP. This effect of *APH-1B/C* inactivation on APP processing combined with the absence of major effects on Notch processing opens a window for the development of less toxic drugs that specifically target this γ-secretase subunit combination.

Furthermore, body axis extension and mesoderm segmentation are more severely affected in *PSEN1*$^{-/-}$*PSEN2*$^{-/-}$ and *NCT*$^{-/-}$ embryos than in *APH-1A*$^{-/-}$ mice, reflecting the residual level of γ-secretase activity in *APH-1A*-deficient embryos because of the presence of APH-1B and APH-1C, which is sufficient to maintain at least partially the Notch-driven segmentation. However, the exact role of the γ-secretase complexes containing APH-1B and APH-1C isoforms and the point during development when they are active, if any, remains to be investigated.

Moreover, the phenotype of *PSEN1*$^{-/-}$*PSEN2*$^{-/-}$ mice is more severe than that of single *Notch*-deficient mice. This might reflect the fact that in *PSEN* double-deficient mice not one but all four Notch signaling pathways are inactivated (Mizutani *et al.*, 2001; Saxena *et al.*, 2001).

However, **PSEN** was recently shown to have a **γ-secretase independent role** in somitogenesis. Indeed, *PSEN1*$^{-/-}$*PSEN2*$^{-/-}$ mice have no somites, whereas *Notch1*$^{-/-}$*Notch2*$^{-/-}$ embryos form cervical somites, and *NCT*$^{-/-}$ and *APH-1A*$^{-/-}$-deficient embryos that lack γ-secretase activity also have anterior somites (Huppert *et al.*, 2005). Therefore, the loss of this

γ-secretase-independent role of PSEN in somitogenesis might contribute to the severity of the $PSEN1^{-/-}PSEN2^{-/-}$ phenotype.

On the other hand, some **PSEN-independent Notch signaling** is suggested to occur as well in the earliest stages of somitogenesis in $PSEN1^{-/-}PSEN2^{-/-}$ embryos (Huppert *et al.*, 2005), confirming earlier observations in mice and cell cultures (Berechid *et al.*, 2002; Takahashi *et al.*, 2000). This residual Notch signaling might explain why *PSEN* double-deficient mice can develop until E9.5.

Furthermore, **PSEN-dependent signaling pathways other than the Notch** signaling pathway, e.g., the Wnt/β-catenin pathway, might also contribute to the severity of the $PSEN1^{-/-}PSEN2^{-/-}$ phenotype. Indeed, $PSEN1^{-/-}$ mice develop a cortical dysplasia resembling human type 2 lissencephaly, associated with the disappearance of Cajal-Retzius cells (Hartmann *et al.*, 1999), a phenotype that is also seen in $APP^{-/-}/APLP\text{-}1^{-/-}/APLP\text{-}2^{-/-}$ triple knockout mice (Section II.B). This might indicate that not only loss of Notch signaling, but also a loss of APP signaling contributes to the phenotype observed in *PSEN*-deficient mice.

To our knowledge, no ***PEN-2*** knockout mice are generated to date. Interestingly, knockdown of *PEN-2* in zebrafish induces p53-dependent apoptosis, suggesting a role for PEN-2, and especially its cytosolic loop domain, in neuronal cell survival and protection from apoptosis *in vivo* (Campbell *et al.*, 2006; Zetterberg *et al.*, 2006).

## F. The γ-Secretase Complex

### 1. A Case of Regulated Intramembrane Proteolysis

γ-Secretase cuts its substrates within a TMD. This cleavage is hence an example of regulated intramembrane proteolysis (RIP), a mechanism conserved from bacteria to higher vertebrates (Brown *et al.*, 2000). Besides γ-secretase, several other proteases govern RIP, now tentatively named intramembrane-cleaving proteases (I-CLiPs). All known I-CLiPs are hydrophobic multipass membrane proteins whose catalytic residues lie within predicted TMDs (Annaert and De Strooper, 2002; Wolfe and Kopan, 2004). γ-Secretase is unique among them because of its tetrameric nature with one component, PSEN, being a diaspartyl protease. So in this respect it differs from SPP, which may act alone, although it is homologous to PSEN (Section IV.B.2). Cleavage by SPP is important in immune system surveillance. The first identified I-CLiP, however, was site-2 protease (S2P), which has an HEXXH motif characteristic of zinc metalloproteases. This enzyme mediates RIP of SREBP (sterol regulatory element binding protein) after a first proteolytic cleavage by S1P (Rawson *et al.*, 1997). This cutting of

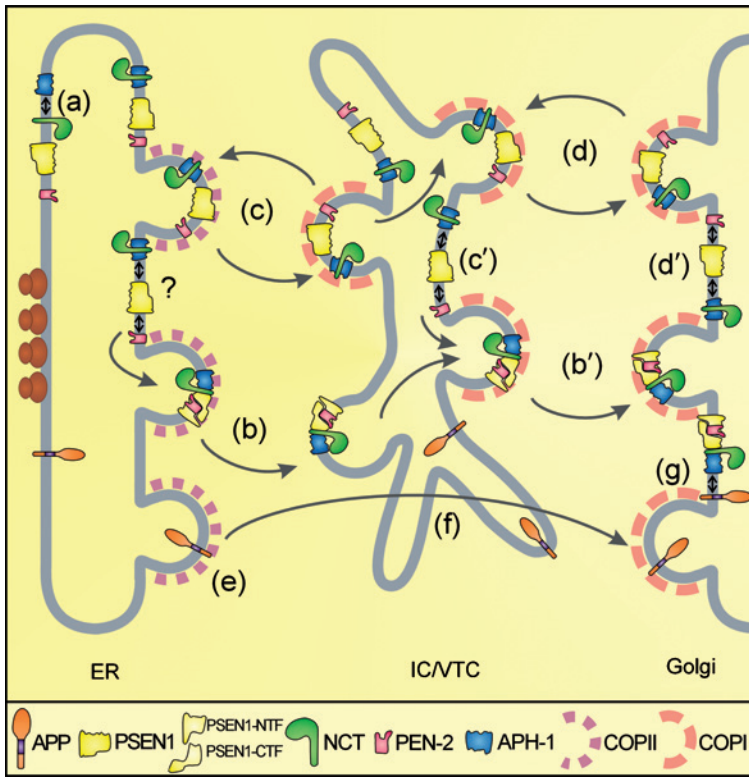


FIG. 5 Hypothetical model coupling γ-secretase complex assembly to ER-Golgi trafficking and ER quality control. Newly synthetized γ-secretase components are cotranslationally inserted into the ER membrane (ribosomes are represented in brown). The assembly of a subcomplex containing immature NCT and APH-1 most likely occurs in the ER (a). It is not yet clear in which subcellular compartment assembly of this subcomplex with FL-PSEN and PEN-2 takes place. PSEN and PEN-2 could bind either subsequently or as a PSEN/PEN-2 subcomplex to the immature NCT/APH-1 subcomplex, and this interaction leads to full complex formation and endoproteolysis of PSEN. Full complex formation in the ER itself (b) may indicate that this is a prerequisite for ER export via COPII-coated vesicles. Afterward, the full complex is transported to the vesicular tubular complexes (VTC) or the intermediate compartment (IC) and further via COPI-coated vesicles to the cis-Golgi cisternae (b′). Alternatively (?), the NCT/APH-1 subcomplex, FL-PSEN, and PEN-2 (individually or as a subcomplex) might separately exit the ER and be transported to the IC/VTC (c), where they might assemble (c′) and be transported to the Golgi (b′). If full complex assembly is not reached in the IC/VTC, the NCT/APH-1 subcomplex, FL-PSEN, and PEN-2 may be transported via COPI-coated organelles to cis-Golgi cisternae (d), where they either assemble into a full complex (d′) or are actively retrieved to the IC and/or the ER via COPI-coated retrograde organelles as part of the secondary ER quality control system in assembling multimeric protein complexes (d). The ER export of APP (indicated in orange, with the Aβ sequence in pink) is uncoupled from PSEN1 export, but also requires COPII coats (e). However, the maturation and turnover of APP have much higher

SREBP controls cellular cholesterol homeostasis. Finally, Rhomboid proteins are I-CLiPs that define a family of serine proteases mainly involved in activating membrane-tethered EGFR ligands (Urban *et al.*, 2002), although remote Rhomboid-like members reside in mitochondria where they can cleave OPA-1 or its orthologues (Cipolat *et al.*, 2006; Herlan *et al.*, 2003; McQuibban *et al.*, 2003).

A first general characteristic of I-CliPs, with Rhomboid as an exception, is the requirement for a preceding proteolytic cleavage by a classical membrane-bound protease that removes a large part of the ectodomain or another regulatory part of the substrate. This primary cleavage is often regulated, either by control of the subcellular localization of the substrate, like SREBP cleavage by S1P, or by ligand binding to the substrate, as seen for the Notch receptor.

A second common feature is the coupling of RIP to the regulated transport of proteases/substrates and the requirement for additional factors for this regulation. For instance, sterols induce binding of Insig-1 to SCAP, the SREBP escort protein. This interaction prevents the exit of the SCAP–SREBP complex from the ER and hence SREBP processing (Sun *et al.*, 2005; Yang *et al.*, 2002). Similarly, Spitz needs the ER export factor Star to become activated by Rhomboid cleavage (Lee *et al.*, 2001).

In the case of γ-secretase-mediated RIP of APP, several regulatory mechanisms can be distinguished (Fig. 5). First, the recent finding that ER export of APP and PSEN1 is differentially regulated suggests separate unknown cargo receptors or mechanisms for recruitment into COPII coated transport organelles (Kim *et al.*, 2005b). Second, cellular quality control involved in the control of γ-secretase complex assembly between the ER and Golgi might also be a major transport regulator (Section IV.F.2).

The substrates of I-CLiPs are typically folded into an α-helix that needs to bend or unwind to make the amide bonds susceptible to hydrolysis. Therefore, the presence of helix-disrupting residues within the TMD of these substrates is a general strategy for intramembrane proteolysis and provides a means of substrate specificity (Lemberg and Martoglio, 2002; Urban and Freeman, 2003; Wolfe and Kopan, 2004; Ye *et al.*, 2000). Moreover, the presence of helix-breaking residues in the center of TMD9 is also required for SPP-mediated intramembrane cleavage of the PSEN holoprotein, the only multipass transmembrane protein identified as a substrate for RIP to date (Section IV.B.1) (Moliaka *et al.*, 2004).

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kinetics than, for instance, NCT, indicating that APP behaves as a typical membrane protein of the secretory pathway (f). Several studies have suggested a role for PSEN in the transport and maturation of APP. Hence, it cannot be ruled out that APP associates with the γ-secretase complex as early as the Golgi complex (g).

However, γ-secretase substrates do not appear to contain such helix-disrupting residues, and this might be the reason why γ-secretase is able to process so many different substrates. Indeed, there are only two known prerequisites for γ-secretase substrates: they should be type I membrane proteins and the bulk of the extracellular domain has to be shed from the full-length precursor protein (Struhl and Adachi, 2000). Therefore, except for the three APP family members APP, APLP-1, and APLP-2 (Section II.B), many other γ-secretase substrates have been identified (De Strooper, 2003; Kopan and Ilagan, 2004), such as the four known Notch receptors, CD44, E/N-cadherin, ErbB-4, syndecan 3, LRP (Lleo *et al.*, 2005), Nectin-1, deleted in colorectal cancer (DCC) (Parent *et al.*, 2005), the Notch ligands Delta and Jagged, growth hormone receptor (Cowan *et al.*, 2005), the p75 neurotrophin receptor [p75(NTR)] (Frade, 2005; Zampieri *et al.*, 2005), the voltage-gated sodium channel β2 subunit (Kim *et al.*, 2005a), tyrosinase (Wang *et al.*, 2006b), the MHC class I protein HLA-A2 (Carey *et al.*, 2006), sorLA (Section III.B.3) (Bohm *et al.*, 2006) and ephrinB2 (Georgakopoulos *et al.*, 2006).

This list of γ-secretase substrates will rapidly grow in the next few years. So far, in the case of Notch, N-cadherin, sorLA, ErbB-4, and p75 (NTR), the involvement of the released cytoplasmic fragments in downstream transcriptional modulation been documented (Kenchappa *et al.*, 2006; Marambaud *et al.*, 2003; Sardi *et al.*, 2006; Selkoe and Kopan, 2003), while this is still under debate for the AICD fragment (Section II.C.2). Alternatively, γ-secretase might act as a "membrane-proteasome," employing RIP as a means to abrogate the physiological roles of its respective unprocessed substrates (Kopan and Ilagan, 2004).

Because of the absence of helix-disrupting residues in the TMD of γ-secretase substrates, there is no sequence specificity near its proteolysis site, so γ-secretase needs another strategy to obtain substrate specificity. Probably the ring structure topology of PSEN provides a cavity within the lipid bilayer, facilitating the access of water molecules to the catalytic site and the breaking of peptide bonds. Recently, substrate recognition for the γ-secretase complex was shown to be determined by the interaction between the ectodomain of NCT and the short extracellular N-termini of type I membrane proteins (Section IV.C.2) (Shah *et al.*, 2005). The conserved Glu-333 and other residues of the NCT DAP domain form a substrate binding pocket that can recognize and interact with putative γ-secretase substrates only if they are placed at a correct spatial distance within the two-dimensional bilayer and if their binding sites are sterically compatible. So although the NCT ectodomain can recognize many short extracellular domains of type I membrane proteins, the binding properties of NCT to these N-termini can be differentially regulated by the length and amino acid composition of these substrates. Therefore, this interaction may be the primary determinant of substrate specificity of γ-secretase. This substrate-recognition function of the NCT ectodomain is typical for an aminopeptidase, but so far, and despite

the sequence homology of DAP with aminopeptidase domains, no activity has been found to be associated with it (Section IV.C.2).

Following this recognition of γ-secretase substrates by the NCT ectodomain, the captured substrates are probably aligned and docked in the lipid bilayer at the substrate-docking site of PSEN and presented to the catalytic core for intramembrane cleavage (Section IV.B.2).

### 2. Assembly and Maturation of the γ-Secretase Complex: A Balanced Play

The correct functioning of the γ-secretase complex depends not only on the regulation of its activity and on its general biophysical properties, but also on correct assembly. This assembly, which is believed to be highly regulated, involves at least three major aspects, i.e., the sequence of assembly, the subcellular localization of the assembly process, and the stoichiometry of the interacting components. Although none of these three facets is fully understood, the available data may already allow us to identify "steppingstones" toward complex assembly (Fig. 5).

Blue native (BN) PAGE analysis of membrane extracts using different detergents reveals mature complexes next to subcomplexes of, e.g., NCT/APH-1/PSEN1-CTF, NCT/APH-1, and PSEN1-NTF/PEN-2 (Fraering *et al.*, 2004a), but it is not clear whether these subcomplexes truly exist or are generated due to differential detergent resistance. However, a stable subcomplex of immature NCT and APH-1 seems to be formed very early in the biosynthetic pathway, as demonstrated by step- and pairwise coexpression of γ-secretase components, and this is probably the first step in complex assembly (Hu and Fortini, 2003; Lee *et al.*, 2002; Morais *et al.*, 2003). This intermediate complex is indeed more resistant to detergent dissociation, suggesting a direct interaction between NCT and APH-1 (Gu *et al.*, 2003; LaVoie *et al.*, 2003). The immature NCT/APH-1 complex is further stabilized by subsequent associations with other γ-secretase complex members (Morais *et al.*, 2003), although it is debated whether PSEN binds to this heterodimeric complex alone or in a subcomplex with PEN-2. In the first view, FL-PSEN binds to the immature NCT/APH-1 subcomplex, which functions as a scaffold for further assembly and stabilizes the PSEN holoprotein. Subsequently, PEN-2 associates with this trimeric intermediate, followed by PSEN endoproteolysis and stabilization of the resulting PSEN fragments (Section IV.D.2) (Gu *et al.*, 2003; Iwatsubo, 2004; LaVoie *et al.*, 2003; Niimura *et al.*, 2005). Full assembly is accompanied by a conformational switch of the NCT ectodomain, followed by its trafficking through and complex glycosylation in the Golgi and final formation of a fully mature and active complex (Section IV.C.2) (Capell *et al.*, 2005; Shirotani *et al.*, 2003). The alternative view is based on studies supporting the existence of a PSEN1/PEN-2 intermediate complex next to the

NCT/APH-1 complex (Fraering *et al.*, 2004a; Wang *et al.*, 2004; Zhang *et al.*, 2005b). The fact that PEN-2 is required for PSEN endoproteolysis and fragment stabilization (Section IV.D.2) indicates that such an intermediate should be formed before or during cleavage of PSEN holoprotein. Taken together with the observation that endogenous FL-PSEN1 is enriched in nuclear envelope and rough ER (Annaert *et al.*, 1999), this PSEN/PEN-2 subcomplex, if present, may be formed very shortly after biosynthesis in the ER.

From the data available so far, one tends to conclude that γ-secretase assembly occurs prior to exit from the ER and that further maturation and activation take place in the Golgi or beyond. This conclusion suffers from overinterpretation, since it is largely based on the use of KKxx motifs in NCT and on the immature glycosylation status of NCT. However, a dilysine motif is a Golgi-to-ER retrieval motif and glycoproteins like NCT become endoglycosidase H-resistant only in cis-Golgi cisternae. Therefore, regulation of complex assembly should not necessarily be restricted to the ER, but might occupy ER-to-Golgi recycling as well. Indeed, the recycling between early compartments is part of the cellular quality control, and this mechanism turns out to be more and more important in the assembly of heteromultimeric membrane proteins such as MHC class II and potassium channels (Michelsen *et al.*, 2005). In this system, arginine-based motifs keep improperly assembled subunits in the ER by retrieval from the Golgi, until they become masked by multisubunit assembly. Although such retrieval motifs are not apparent in γ-secretase components, similar but yet unknown mechanisms might exist (Fig. 5) (Section IV.F.1). Interestingly, PSEN1 is preferentially enriched over COPI-coated areas in the intermediate compartment, arguing for an active recycling mechanism (Rechards *et al.*, 2003). In support of this, Kaether and co-workers (2004) identified a hydrophobic stretch in the C-terminus of PSEN1 that is apparently required for the retention of unassembled PSEN1 in the ER. However, the nature of this retention signal should be reinvestigated in light of the new PSEN1 topology (Section IV.B.1). Alternatively, recycling between early compartments can be mediated by interaction of individual γ-secretase complex components or subcomplexes with other membrane proteins bearing motifs operating in Golgi-to-ER retrieval, such as SNARE proteins or membrane proteins like BAP31/29, ERGIC-53/p58, and RER1p (Section IV.C.2) (Annaert *et al.*, 1997; Sato *et al.*, 2003). In this case, (sub)complex assembly abrogates their association, allowing the full complex to escape from the early compartments. Interestingly, packaging and ER export of PSEN1 and APP seem to be uncoupled, indicating that separate cargo-sorting mechanisms are indeed operating as early as the exit from the ER (Section IV.F.1) (Kim *et al.*, 2005b).

Although high-molecular-weight γ-secretase complexes ranging from ~250 kDa up to 2000 kDa, depending on the methodology used, have been reported (Section IV.A), the sum of the apparent molecular weights of all four γ-secretase members results in a theoretical mature complex of only

~220 kDa. Therefore, it is likely that the active complex is multimeric in nature. Indeed, more recent studies employing BN-PAGE or size exclusion chromatography have correlated γ-secretase activity with dimeric or tetrameric complexes of ~670 kDa, ~800 kDa, and ~900 kDa, potentially including docked substrates (Evin *et al.*, 2005; Gu *et al.*, 2004; Wrigley *et al.*, 2005). However, common separation technologies appear to result in a severe loss of γ-secretase complex activity, suggesting that additional cofactors are eluted away during several successive isolation steps. Such cofactor candidates are most likely of a nonproteinaceous nature, since addition of a whole brain lipid extract can compensate for the loss in activity (Wrigley *et al.*, 2005). Accordingly, addition or depletion of cholesterol can significantly increase or lower γ-secretase activity. These findings corroborate the earlier observations demonstrating that sphingomyelin and phosphatidylcholine can dramatically improve enzyme activity and that the lipid-mediated conformation of both γ-secretase and its substrate plays an important role in the production of the neurotoxic Aβ42 and Aβ43 peptides [which in turn were shown to regulate sphingomyelin and cholesterol levels (Section II.C.3)] (Fraering *et al.*, 2004b). Conversely, it cannot be ruled out that the observed oligo- or tetrameric nature of the largely inactive complexes is generated only after extraction, due to the removal of the critical lipid environment. Taken together, these findings emphasize the importance of a proper lipid microenvironment for active γ-secretase, which is not unexpected given the presence of 19 TMDs in the monomeric complex. Hence, to obtain the exact size of the complex, which can still be monomeric, future strategies should aim at identifying the minimal (phospho) lipids required for restoring full activity. Recently, the first attempts in 3D reconstruction of the γ-secretase complex revealed a low-density central cavity, potentially representing an aqueous intramembrane chamber for proteolysis, and at least 2 openings to this chamber for access and release of substrates and their cleavage products (Lazarov *et al.*, 2006; Ogura *et al.*, 2006).

In addition, other factors have been proposed to influence γ-secretase activity. The only candidate protein that may be an additional cofactor or even a regulatory subunit of the complex, namely CD147 (also referred to as basigin or EMMPRIN), (Zhou *et al.*, 2005) and TMP21 (Chen *et al.*, 2006). Although the association of both proteins with a high-molecular-weight γ-secretase complex still requires confirmation, they seem to rather down-modulate γ-site cleavage. In addition, ERK1/2 appears to negatively regulate γ-secretase activity at endogenous levels by phosphorylating NCT (Kim *et al.*, 2006). Furthermore, ATP was recently shown to selectively activate γ-secretase-mediated cleavage of APP and not Notch by binding to a nucleotide-binding site probably situated on the PSEN1-CTF. This binding may induce a conformational change, allowing the substrate to enter the catalytic site (Fraering *et al.*, 2005; Netzer *et al.*, 2003).

Finally, PLD1, a phospholipid-modifying enzyme, was recently shown to inhibit γ-secretase activity by disrupting the interactions between the γ-secretase complex members. This role of PLD1 is independent of its catalytic

activity. Potentially, the interaction of PLD1 with PSEN1 elicits a conformational change in PSEN1, which prevents its association with the other complex components (Cai *et al.*, 2006a). This inhibition of Aβ generation elicited by PLD1 is independent of its stimulating effect on intracellular APP trafficking from the TGN, which requires catalytically active PLD1 (Cai *et al.*, 2006b).

In conclusion, reconstituting γ-secretase activity will require the recombination of the purified complex together with its proper lipid microenvironment and additional cofactors. Although this is a major challenge, obtaining this information will not only allow more accurate size measurements, but it is also the next hurdle that needs to be tackled in the race to crystallization of the γ-secretase complex.

With respect to this reconstitution of γ-secretase activity, also worth mentioning is the diversity in complexes and their substrate-cleaving activities. Indeed, already six distinct proteolytically active γ-secretase complexes can be formed by combining one of the APH-1 isoforms with either PSEN1 or PSEN2 (Gu *et al.*, 2004; Ma *et al.*, 2005). Given the ubiquitous cell and tissue distribution, different complexes coexist in the same cell. However, some variation exists in the expression of APH-1 proteins and NCT, and this tissue-specific distribution of certain complexes might reflect differential activities toward various γ-secretase substrates (Hebert *et al.*, 2004; Ilaya *et al.*, 2004; Shirotani *et al.*, 2004). Similarly, PSEN1-bearing complexes exhibit more APP-C99 processing than PSEN2-containing complexes (Bentahir *et al.*, 2006; Lai *et al.*, 2003; Mastrangelo *et al.*, 2005). This variability in composition and activity of γ-secretase complexes opens a therapeutic window for the development of inhibitors that can selectively manipulate γ-secretase activities and subsequent substrate processing. However, most likely, a complete knowledge of the molecular architecture of individual complexes, including structural information, is required to achieve this goal.

## 3. γ-Secretase Activity: Localization Matters!

Many groups have invested much time and effort in locating γ-secretase complex members and activity using transient overexpression of these components. These studies have taught us that single subunit overexpression leads to nonphysiological accumulation of this component in the ER, because the other cofactors become rate-limiting, as postulated earlier (Section IV.A) (Thinakaran *et al.*, 1997). Consequently, only studies using balanced expression levels of all four components, either endogenous or cooverexpressed, can be considered relevant. So from the subcellular localization experiments done before the discovery of NCT, APH-1, and PEN-2, only the limited number studying endogenous PSEN1 levels can be taken into account. For historical reasons, the subcellular distribution of PSEN1 is known in most detail. In brain and fibroblasts, endogenous PSEN1 is predominantly located in ER, intermediate compartment, and cis-Golgi (Annaert *et al.*, 1999; Pasternak *et al.*, 2004; Rechards *et al.*, 2003; Yu *et al.*, 1998). This observation fuelled the "spatial paradox," pointing out a major

discrepancy between PSEN1 localization in early biosynthetic compartments where essentially no γ-secretase activity resides, and Aβ production in post-Golgi compartments (Section IV.A) (Annaert and De Strooper, 1999; Cupers *et al.*, 2001a; Maltese *et al.*, 2001).

Two lines of research essentially contributed to solving this spatial paradox. First, several studies, including proteomic and advanced imaging approaches, revealed the presence of small amounts of endogenous γ-secretase components in the TGN (Baulac *et al.*, 2003; Siman and Velji, 2003), at the cell surface (Berezovska *et al.*, 2003; Chyung *et al.*, 2005; Herreman *et al.*, 2003; Kaether *et al.*, 2002, 2006; Ray *et al.*, 1999) including lipid rafts (Section IV.F.4), in endosomes/lysosomes (Kaether *et al.*, 2006; Lah and Levey, 2000; Pasternak *et al.*, 2003), and in autophagic vacuoles (Section IV.B.3) (Yu *et al.*, 2005). Recently, a fraction of Aβ peptides was also found in multivesicular bodies and exosomes, but it is not yet clear whether γ-secretase cleavage itself takes place in these compartments (Rajendran *et al.*, 2006).

Second, active γ-secretase complexes may be formed gradually as PSEN1 and NCT mature through conformational changes and complex glycosylation in the Golgi (Section IV.F.2). This suggests that individual components can traffic beyond the Golgi only when properly assembled, explaining their very low levels in post-Golgi compartments. This theory was confirmed recently in cell lines cooverexpressing all four complex members, with a concomitant increase in γ-secretase activity and NCT maturation and a redistribution of overexpressed PSEN1 to the cell surface (Wrigley *et al.*, 2005). Similarly, genetic deficiency of one of the γ-secretase complex members usually results in aberrant distributions, decreased levels, and incomplete maturation of other components or subcomplexes. Indeed, in $NCT^{-/-}$ and $APH\text{-}1A^{-/-}$ fibroblasts, γ-secretase complex assembly is impaired, PEN-2 levels are lowered, and PSEN endoproteolysis is decreased, leading to an accumulation of FL-PSEN (Li *et al.*, 2003a,b; Zhang *et al.*, 2005b). Furthermore, NCT glycosylation is disturbed in *PSEN* and *APH-1A*-deficient cells (Edbauer *et al.*, 2002; Herreman *et al.*, 2003; Ma *et al.*, 2005; Serneels *et al.*, 2005). Since no active γ-secretase complex is formed, APP processing is severely compromised, resulting in an abolished secretion of Aβ and AICD and an accumulation of APP-CTFs and FL-APP. These findings underscore the mutual dependency of all γ-secretase complex members for their stability, transport, and localization (Section IV.B.3).

## 4. Lipid Rafts, Key to Regulating APP RIP?

Because of therapeutic interest, originating from epidemiological studies, in intervening with the cellular cholesterol balance to modulate APP processing, the localization of γ-secretase components (or secretases in general) in lipid rafts and caveolae is being thoroughly investigated. The biochemical separation of these components in lipid raft fractions may indeed form the cell biological basis in cells for the observed requirement of lipids and cholesterol for

γ-secretase activity (Section IV.F.2) (Urano *et al.*, 2005; Vetrivel *et al.*, 2004; Wada *et al.*, 2003; Wahrle *et al.*, 2002). In addition to cholesterol, this raft localization is also dependent on protein isoprenylation (Urano *et al.*, 2005).

However, in our opinion there is one major caveat that is biophysical in nature. Lipid rafts are defined as very small highly ordered and densily packed assemblies of cholesterol and sphingolipids (Simons and Ikonen, 1997), so there might be little room left to include complexes with 19 TMDs. The problem becomes even larger if an oligomeric nature of the active complex is assumed (Section IV.F.2). Therefore, it is not unlikely that the γ-secretase complex is not present inside lipid rafts, but is rather closely associated with the rims of raft domains. This concept, although not yet proven, offers an explanation for the differential distribution of γ-secretase substrates in rafts fractions (e.g., APP) versus nonraft domains (e.g., Notch, Jagged2, DCC, N-cadherin) (Vetrivel *et al.*, 2005).

The fact that BACE1 is a lipid raft component (Section III.B.3) (Kametaka *et al.*, 2003; Marlow *et al.*, 2003; Riddell *et al.*, 2001; Tun *et al.*, 2002) led to a debate on whether cleavage of APP by β- and γ-secretase takes place inside or outside lipid rafts (Kaether and Haass, 2004).

Two competing hypotheses exist about the regulation of APP shedding. The first hypothesis claims that under normal physiological conditions, a minor fraction of APP is cleaved by BACE1 inside lipid rafts. Cholesterol depletion disrupts lipid rafts and disturbs the association of BACE1 and/or APP with these domains, thereby lowering β-cleavage and Aβ production (Cordy *et al.*, 2003; Ehehalt *et al.*, 2003). Since ADAM10 is generally believed to reside outside lipid rafts, the shift of APP to nonraft domains promotes the nonamyloidogenic pathway (Kojro *et al.*, 2001). In contrast, a second hypothesis proposes that BACE1 cleavage of APP occurs preferentially outside lipid rafts. In this case, moderate reduction of membrane cholesterol levels also disrupts the association of BACE1 with lipid rafts, causing a shift to nonraft domains. Here, BACE1 encounters more substrate, resulting in more APP cleavage and Aβ production. The same effect is seen in hippocampal membranes from AD patients. So the neuronal lipid rafts may act as membrane ''basins'' from which BACE1 can exit or enter to initiate or arrest APP cleavage in nearby nonraft domains (Abad-Rodriguez *et al.*, 2004). Recently, the cholesterol-synthesizing enzyme seladin-1, which is downregulated in affected neurons of AD brain, was shown to play a key role in this process. This enzyme stimulates brain cholesterol levels and formation of detergent-resistant microdomains, thereby recruiting BACE1 into these domains, leading to a reduction of both Aβ40 and Aβ42 levels *in vitro* and *in vivo* (Crameri *et al.*, 2006).

Evidence supporting both theories has been found in experiments on cell cultures, animal models, and human clinical trials (Kaether and Haass, 2004; Puglielli *et al.*, 2003b; Wolozin, 2004). Several factors have been suggested to

influence the conflicting observations on Aβ generation in both hypotheses, such as the extent of membrane cholesterol reduction (Abad-Rodriguez *et al.*, 2004), changes in lipid raft resident Aβ clearing enzymes such as plasminogen/plasmin (Ledesma *et al.*, 2003) due to altered seladin-1 expression (Crameri *et al.*, 2006), differences in the blood-brain barrier penetrating capacity of cholesterol-lowering drugs, and cholesterol-independent effects of these drugs on isoprenoids or other lipids influencing α-, β-, or γ-secretase activity (Cole *et al.*, 2005; Kalvodova *et al.*, 2005). However, it is clear that further research is required, preferentially with respect to the aging brain. Interestingly, Kern and co-workers (2006) recently reported an age-associated general decline of PSEN1 and NCT levels, resulting in decreased γ-secretase activity concomitant with increased cholesterol levels in senescent cells. Moreover, the distribution of PSEN1 and BACE1 in lipid rafts was also altered, probably accounting for the observed changes in APP processing.

## V. Possible Contribution of Cell Biology to Therapy

In the past two decades, molecular cell biology has made a major contribution to unraveling the secrets of AD. As a result, many therapies targeting the initial players in AD pathogenesis, instead of treating the later symptoms, are currently under development. However, many questions remain and answering them will definitely reveal new and hopefully better therapeutic targets.

Since AD is characterized by an imbalance between the production and degradation of Aβ, these toxic peptides are the first and major drug target. Consequently, a variety of strategies have been designed to either inhibit the production of the neurotoxic Aβ species or to enhance the clearance of these peptides.

In the past few years, it has become increasingly evident that not the soluble Aβ peptides themselves but rather their oligomeric successors are the candidate initiators of the pathogenic cascade (Section I). Therefore, these earliest effectors of synaptic compromise in AD are considered a major drug target. Several methods have been developed to either inhibit their formation or to neutralize these oligomers in the brain, such as administration of metal-complexing compounds (Ritchie *et al.*, 2004; Rogers and Lahiri, 2004) or other aggregation blockers (Citron, 2004; Tanzi *et al.*, 2004) and active or passive Aβ immunization strategies (Broytman and Malter, 2004; Geylis and Steinitz, 2006; Janus, 2003; Nitsch, 2004). The latter strategies were shown to be effective in reducing cognitive dysfunction (Hartman *et al.*, 2005; Lee *et al.*, 2006; Walsh *et al.*, 2005a,b; Wilcock *et al.*, 2004). Moreover, Aβ immunization can also exert its effects at a later stage in the pathogenic process by clearing amyloid plaques in the brain of AD patients. However, it is necessary

to be cautious in treating humans in this way as several side effects were observed in the first clinical trials.

In addition, the degradation of Aβ can be enhanced by upregulating the proteases or mechanisms responsible for its clearance, such as insulin-degrading enzyme (IDE), neprilysin, plasmin, and peroxisome proliferator-activated receptor γ (PPARγ) (Camacho *et al.*, 2004; Crameri *et al.*, 2006; Selkoe, 2001).

However, many cell biological questions concerning Aβ aggregation and degradation still remain. Although receptors such as LRP have been shown to mediate the uptake of Aβ (complexed with apoE) (Harris-White and Frautschy, 2005), it is not yet clear whether these receptors can also recognize oligomeric Aβ species. Therefore, any cell surface receptors specifically recognizing and mediating the uptake of these toxic oligomeric Aβ urgently need to be identified and their use as potential drug targets needs to be explored. In addition, pharmacological target candidates for the prevention and treatment of AD might arise from studies on Aβ degradation and clearance, as shown by the discovery of somatostatin receptors, which can upregulate Aβ proteolysis in the brain catalyzed by neprilysin (Saito *et al.*, 2005b).

In addition to targeting Aβ once it is produced, another obvious strategy is the inhibition of Aβ production itself, thereby intervening even more upstream in the pathological cascade. Aβ production might be prevented by inhibiting the Aβ-generating enzymes β- or γ-secretase (Beher and Graham, 2005; Churcher and Beher, 2005; Thompson *et al.*, 2005; Tomita and Iwatsubo, 2004), although it has increasingly been proposed that α-secretase may be a potential therapeutic target for AD (Lichtenthaler and Haass, 2004). Indeed, stimulating **α-secretase** activity, thereby shifting APP processing from the amyloidogenic pathway to the nonamyloidogenic pathway, can decrease Aβ production and amyloid plaque formation and improve memory deficits *in vitro* and *in vivo* (Etcheberrigaray *et al.*, 2004; Postina *et al.*, 2004; Caccamo *et al.*, 2006; Rezai-Zadeh *et al.*, 2005). However, further research is needed to identify more precisely the ADAMs that have α-secretase activity and especially those that are active in the brain. In this way, the design of drugs aimed specifically at increasing nonamyloidogenic processing of APP but not other α-secretase substrates will be facilitated.

Selective inhibition of **BACE1** activity has been shown to reduce Aβ generation and to rescue cognitive abnormalities in transgenic mice (Ohno *et al.*, 2004, 2006; Luo *et al.*, 2003b). Recently, new strategies to selectively inhibit BACE1 activity were designed, such as peptidomimetic noncleavable substrate analogues and BACE1 RNAi (Kao *et al.*, 2004; Nawrot, 2004; Singer *et al.*, 2005). In addition, passive or active immunization therapies were developed, targeting the β-secretase cleavage site of APP and not the enzyme itself with monoclonal antibodies or intrabodies (Arbel *et al.*, 2005; Paganetti *et al.*, 2005). However, recent findings showing a complex phenotype (smaller stature and electrophysiological, behavioral, and cognitive alterations) and

an enhanced lethality for BACE1 knockout mice, challenge the general idea of BACE1 as a safe drug target (Section III.B.2). Therefore, the effects of BACE1 inhibition on the growing list of BACE1 substrates should be carefully considered.

Similarly, inhibition of **γ-secretase** cleavage of APP can alter the processing of its other substrates (Section IV.F.1), leading to significant adverse effects in adults, e.g., in the hematopoietic/ immunological system, in the gastrointestinal system, and in the skin, most likely caused by blocking Notch signaling (Annaert and De Strooper, 2002; Harrison *et al.*, 2004; Milano *et al.*, 2004; Tournoy *et al.*, 2004; Wong *et al.*, 2004). Moreover, *PSEN1* conditional knockout mice, in which *PSEN1* inactivation is restricted to the adult forebrain, show subtle but significant deficits in long-term spatial memory and a reduction in neurogenesis (Feng *et al.*, 2001; Yu *et al.*, 2001).

Therefore, the major hurdle in the design of γ-secretase inhibitors is to target specifically the proteolysis of APP-CTFs without affecting the processing of Notch or other substrates. However, a therapeutic window seems to exist, since many studies have indicated differences in the cleavage process of APP and Notch. For instance, mutations in NCT and PSEN can differentially affect γ-secretase cleavage of APP and Notch (Sections IV.C.1 and 2) (Walker *et al.*, 2005). Moreover, certain γ-secretase inhibitors can indeed specifically target APP cleavage, e.g. the GSK-3 inhibitor lithium (Phiel *et al.*, 2003) and certain nonsteriodal antiinflammatory drugs (NSAIDs), which specifically lower Aβ42 production (Weggen *et al.*, 2003a,b). Nevertheless, the true factors that determine γ-secretase substrate specificity are still largely unknown and are most likely related to substrate dimerization, recognition, and docking to the enzyme complex. However, some new findings in cell biology might help us to further understand these mechanisms and to design new strategies specifically inhibiting Aβ production. For example, the recently discovered nucleotide-binding site in the γ-secretase complex, which can bind ATP leading to a selective activation of APP processing, might represent an attractive drug target (Section IV.F.2). Furthermore, the receptor role of the NCT ectodomain towards various substrates (Section IV.F.1) and the role of different γ-secretase complexes containing different combinations of an APH-1 isoform with either PSEN1 or PSEN2 (Sections IV.E and F.2) in determining substrate specificity should be investigated in more detail. In addition, structural information about the γ-secretase complex including its proper lipid microenvironment and its additional cofactors is urgently needed (Section IV.F.2). This knowledge is expected to result in a major breakthrough in the design of specific inhibitors. The structure of the complex will also answer other burning questions such as the stoichiometry and the proposed ring structure topology of the γ-secretase complex.

The regulation of trafficking and subcellular localization of the different secretases versus their substrates also deserves more scrutiny (Section IV.F.3).

A good example are the two co-existing but conflicting hypotheses on the effects of cholesterol on Aβ generation that are currently leading us to opposite conclusions when it comes to deciding on therapies aimed at lowering cholesterol in the central nervous system (Section IV.F.4). Related to this, the differential distribution of APP-CTFs and other substrates as well as the association of γ-secretase complexes with lipid raft and nonraft raft domains suggests other means to determine substrate specificity and should therefore be further investigated.

A completely different therapeutic approach may target more upstream (trafficking) events, for instance, the consecutive steps of assembly and activitation of the (different) γ-secretase complex(es). Indeed, based on what we know on assembly and localization of individual γ-secretase components, we believe that assembly of the complex occurs in early compartments and may involve yet unidentified quality control mechanisms for multimeric assembly (Section IV.F.2). Moreover, it is not unlikely that even different sorting determinants exist for the assembly of the various γ-secretase complexes.

It will be a major task for molecular cell biologists to determine the subcellular compartment(s) in which the different γ-secretase complexes are assembled, where the respective active complexes reside, and when the different substrates associate with them. We predict that this strategy will open future avenues for interfering with APP processing and specifically Aβ production.

In conclusion, it is clear that discoveries in cell biology in the past two decades have had and will have a great impact on the development of therapies for AD. However, some major questions have to be answered first. By tackling these next hurdles, we will gain fundamental insight into the complex molecular cell biology of AD, which is an absolute requirement in the quest for efficient therapies for this devastating disorder.

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