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COMMENTARY

Proteolytic processing and cell biological functions of the amyloid precursor protein

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SUMMARY

Recent research has identified some key players involved in the proteolytic processing of amyloid precursor protein (APP) to amyloid β-peptide, the principal component of the amyloid plaques in Alzheimer patients. Interesting parallels exists with the proteolysis of other proteins involved in cell differentiation, cholesterol homeostasis and stress responses. Since the cytoplasmic domain of APP is anchored to a complex protein network that might function in axonal elongation, dendritic arborisation and neuronal cell migration, the proteolysis of APP might be critically involved in intracellular signalling events.

Key words: Amyloid precursor protein, Secretase, BACE, Fe65, Presenilin, ADAM

INTRODUCTION

In the past two years enormous progress has been made towards understanding of the cell biology of Alzheimer's disease (AD). Insights from the cell biology field have boosted AD research, and ideas originally raised in the AD field are now further explored by cell biologists. The interest in the cell biology of amyloid precursor protein (APP) originates from the observation some 15 years ago that the main component of amyloid plaques, a hallmark of AD, is generated by proteolysis of this larger precursor protein (Glenner and Wong, 1984; Haass et al., 1992b; Kang et al., 1987; Masters et al., 1985; Weidemann et al., 1989). This has provoked a real avalanche of research that has progressively increased our understanding of the molecular mechanisms involved in the proteolytic processing of APP. Analogies between the processing of APP, and the processing of Notch and the transcription factor SREBP (sterol-regulatory-element binding protein), and, more recently, the involvement of the presenilins in the unfolded-protein-response (UPR) pathway have led to new molecular concepts of signal transduction mechanisms.

ALZHEIMER'S DISEASE

The brains of patients suffering from AD are characterised by the progressive deposition of protein fragments in amyloid plaques and by the appearance of neurofibrillary tangles. The neurofibrillary tangles are intraneuronal lesions consisting of 10-nm-thick paired helical filaments. The main component of these filaments is the hyperphosphorylated form of the microtubule-binding protein Tau. The amyloid plaques are deposits of amyloid beta (Aβ) peptides organised in 7-10-nm-thick fibrils intermixed with non-fibrillar forms of the peptide. The mature plaques contain in addition degenerating axons and dendrites, and are surrounded and invaded by variable numbers of microglia and reactive astrocytes. This indicates that there is an inflammatory component in the neurodegenerative process.

Genetic studies have identified missense mutations in the *APP* gene (Ancolio et al., 1999; Chartier-Harlin et al., 1991; Eckman et al., 1997; Goate et al., 1991; Hendriks et al., 1992; Mullan et al., 1992; Murrell et al., 1991) and in the genes that encode presenilins (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995) that cause some rare familial forms of AD. The *APP* gene on chromosome 21 encodes the amyloid precursor protein (APP; Kang et al., 1987), a type I integral membrane protein that is proteolytically cleaved to different fragments, including the amyloid beta (Aβ) peptides of the amyloid plaques. Most *APP* mutations that cause AD increase the proteolytic processing of APP, increasing production of the Aβ peptide (Cai et al., 1993; Citron et al., 1992; Haass et al., 1994; Suzuki et al., 1994a). Significantly, the AD-causing missense mutations in the genes on chromosome 14 and 1, which encode presenilin 1 and presenilin 2, respectively, also affect APP processing (Borchelt et al., 1996; Citron et al., 1997; Duff et al., 1996; Scheuner et al., 1996). They increase the production of a 42-residue form of Aβ peptide that is particularly prone to precipitation and aggregation (Lansbury, 1997). Thus, abnormal processing of APP and increased generation of Aβ appear to be the central events in the pathogenesis of the hereditary forms of AD. Since the pathological lesions in the non-familial form of AD are similar to those found in the hereditary forms, it is logical to assume that abnormal processing of APP also plays an important role in the pathogenesis of the more frequent, sporadic

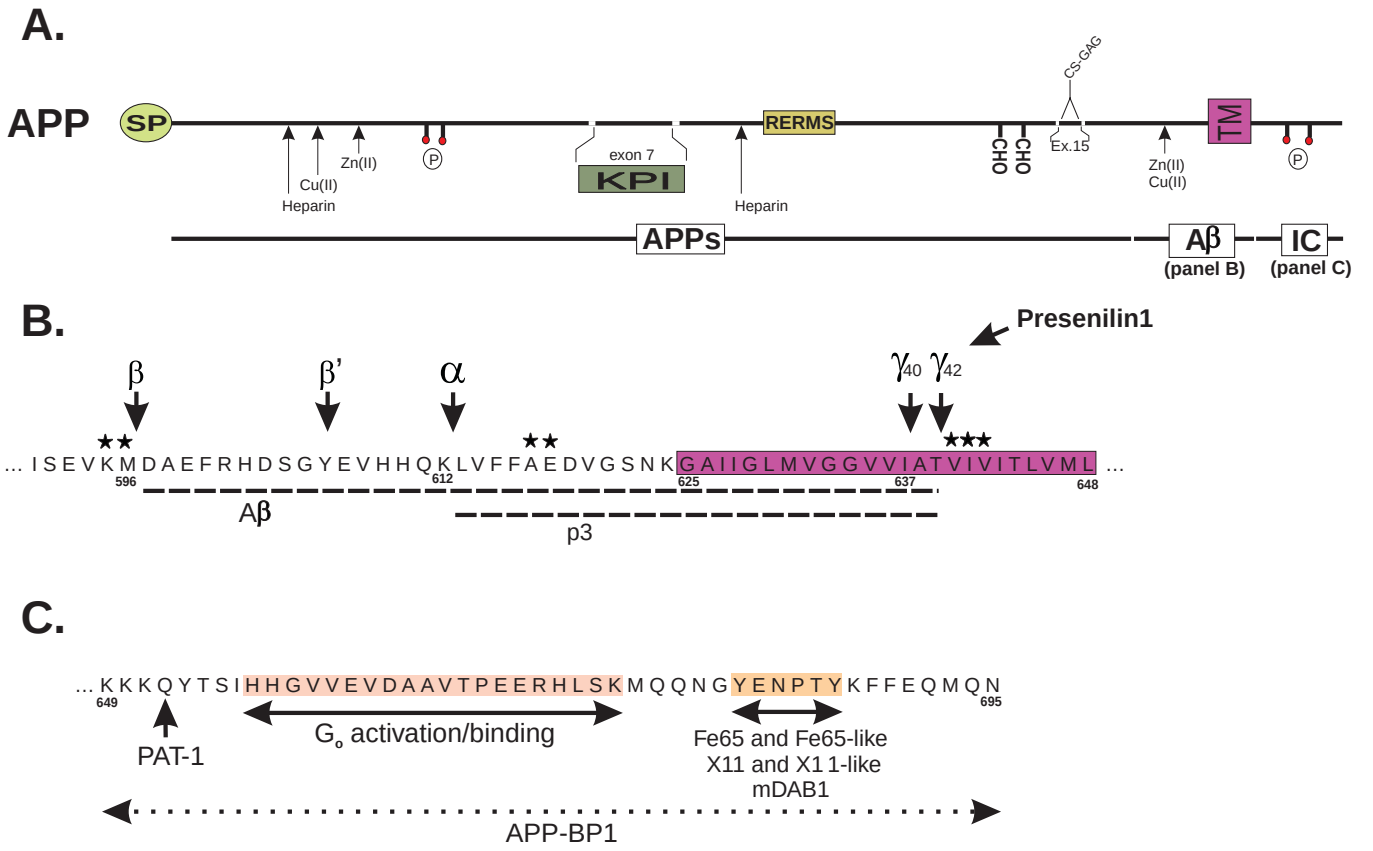

Fig. 1. Domain structure of the amyloid precursor protein APP695. Panel A depicts the overall structure including the relative positions of the heparin, metal-binding, phosphorylation (P) and glycosylation sites (CHO). Two alternatively spliced variants, containing a Kunitz-type protease inhibitor (KPI) consensus sequence or a chondroitin sulphate glycosaminoglycan attachment site (CS-GAG), are also indicated. SP: Signal peptide; TM: transmembrane region; Aβ: amyloid β-peptide; IC: intracellular domain; APPs: soluble APP. (B and C) The amino acid sequences of the regions encompassing the amyloid β-peptide sequence and the intracellular domain, respectively. (B) BACE cleaves APP after Met596 (β) and Tyr606 (β′), whereas ADAM-10 (best candidate α-secretase) processes APP within the amyloid β-peptide sequence (α), thereby generating the p3 peptide. γ-Secretase cleavage in the transmembrane region (TM) involves presenilin 1 and generates amyloid β-peptides of mainly 40 and 42 amino acid residues long (γ40 and γ42). The asterisks indicate the locations of mutations causing familial AD. (C) Protein interactions involving the cytoplasmic tail domain of APP. Fe65/Fe65-like as well as X11/X11-like proteins and mDAB1 bind to residues on the YENPTY sequence of APP, whereas the His657-Lys676 sequence is involved in the binding to G_o. The most proximal tyrosine residue (Tyr653) is critical for basolateral sorting of APP in epithelial cells and for binding of PAT-1. The exact binding site of APP-BP1 remains to be identified.

form of the disease. Note that mutations in the gene that encodes Tau are not associated with AD (Lee and Trojanowski, 1999). They cause another type of dementia: frontotemporal dementia with parkinsonism (FTDP-17). This disease is characterised by widespread deposition of tangles and by the absence of amyloid plaques (Hutton et al., 1998; Spillantini et al., 1998).

The roles of Tau, APP, the presenilins and additional proteins, such as apolipoprotein E and α2-macroglobulin, in the pathogenesis of hereditary and sporadic AD have been elaborated in recent reviews (Haass and De Strooper, 1999; Hardy, 1996; Lee and Trojanowski, 1999; Price and Sisodia, 1998; Selkoe, 1999; Tanzi, 1999), and we therefore focus here on recent findings concerning the more fundamental cell biology of APP.

THE APP FAMILY

The mammalian APP family contains three members: APP and the APP-like proteins, APLP1 and APLP2 (Sprecher et al., 1993; Wasco et al., 1992, 1993). Homologues have been identified in *Drosophila* (Luo et al., 1992), *C. elegans* (Daigle and Li, 1993) and *Xenopus* (Okado and Okamoto, 1992). The APP family members are type I integral membrane proteins that have relatively large extracellular domains and short intracellular domains. The mammalian proteins are relatively ubiquitously expressed. The *APP* gene contains 19 exons, of which exons 7, 8 and 15 can be alternatively spliced. All possible splice variants have been detected by RT-PCR in tissues. Some of the variants (Fig. 1) contain a Kunitz-type proteinase inhibitor domain (Kitaguchi et al., 1988; Ponte et al., 1988; Sandbrink et al., 1994; Tanzi et al., 1988) and function in blood coagulation. Classical N-glycosylation and O-glycosylation occur during transit through the endoplasmic reticulum (ER) and the Golgi apparatus (Weidemann et al., 1989). The splicing of exon 15 creates a chondroitin-sulphate-glycosaminoglycan-attachment site, which is used in astrocytes but not in neurons. The resulting high-molecular-mass APP has been called appican (Pangalos et al., 1995; Shioi et al., 1995). Addition of sulphate and phosphate in

the late Golgi compartment and at the cell surface further increases the structural complexity of APP and APP-like proteins (Hung and Selkoe, 1994; Suzuki et al., 1994b; Walter et al., 1997; Weidemann et al., 1989). Holo-APP is proteolytically cleaved by α-, or β-secretases, releasing the 100-120-kDa ectodomain of APP (soluble APP or APPs) and 10-12-kDa membrane bound APP C-terminal fragments. An additional cleavage in the transmembrane domain of APP by γ-secretase results in the secretion of the 4-kDa Aβ peptide (from the APP C-terminal fragment generated by the β-secretase) and the p3 fragment (from the APP C-terminal fragment generated by the α-secretase, Fig. 1). The Aβ peptide produced in that way in neuronal and glial cells from the brain is believed to precipitate in the plaques of AD patients. The biosynthesis and proteolytic processing of APLP1 and APLP 2 has received much less attention, but both APP homologues are also modified by alternative splicing and glycosylation, and proteolytic processing also releases their soluble ectodomains (Lo et al., 1995; Lyckman et al., 1998; Naruse et al., 1998; Nitsch et al., 1996; Paliga et al., 1997; Slunt et al., 1994; Thinakaran and Sisodia, 1994).

PROTEOLYTIC PROCESSING OF APP

The principal proteolytic cleavage of APP is performed by a protease designated α-secretase. The α-secretase cleaves between Lys16 and Leu17 (residues 612 and 613 of APP) in the sequence that produces for the Aβ peptide itself (Fig. 1), and therefore precludes generation of Aβ peptide. Initially it was believed that this cleavage is the physiological one (Esch et al., 1990; Sisodia et al., 1990), and that it prevents amyloidogenesis in AD. In reality this is an oversimplification, because only a fraction of the total pool of APP is cleaved by α-secretase in most cell types, leaving most of the APP protein intact. Furthermore, β- and γ-secretase processing of APP (at the N- and C-termini of the Aβ sequence) also occur under physiological conditions; this indicates that all fragments of APP, including the Aβ peptide, are part of normal physiology (Haass et al., 1992b; Seubert et al., 1992).

α-SECRETASE

The α-secretase processing of APP is somewhat similar to the processing of a series of other integral membrane proteins, including the growth factors TGF-α and TNF-α, the cell adhesion molecule L-selectin, and the growth factor co-receptor syndecan (Werb and Yan, 1998). The processing of all these proteins, including APP, consists of a constitutive component and a regulated component that can be activated via protein kinase C (PKC) (Buxbaum et al., 1993; LeBlanc et al., 1998; Nitsch et al., 1992) and other second messenger cascades (Mills and Reiner, 1999). The protease responsible for TNF-α release has been identified (Black et al., 1997; Moss et al., 1997). Called tumor necrosis factor-α converting enzyme (TACE), it is a member of the ADAM family (a disintegrin and metalloproteinase; for a review see Schlondorff and Blobel, 1999). The TACE-knockout mice display a complex lethal phenotype and ectodomain-shedding defects for many proteins, including TGF-α, the TNF receptor and L-selectin (Peschon et al., 1998). Fibroblasts derived from the TACE-knockout mice show defects in APP secretion, but only in PKC-stimulated secretion (Buxbaum et al., 1998). TACE thus plays a role only in the regulatory component of the α-secretase processing of APP in fibroblasts.

Very recently, two other members of the ADAM family have also been implicated in α-secretase processing of APP: ADAM10 (Lammich et al., 1999) and MDC9 (also known as meltrin γ; Koike et al., 1999). Co-transfection of ADAM10 or MDC9 with APP results in increased (constitutive and regulated) secretion of APPs. ADAM10 furthermore specifically cleaves synthetic substrates that contain the α-secretase-cleavage site, and a dominant negative form of ADAM10 interfered with endogenous α-secretase processing of APP (Lammich et al., 1999). The presence of ADAM10 at the cell surface but also in the Golgi apparatus and possibly in surface-destined transport vesicles (Lammich et al., 1999) is in line with previous reports providing (indirect) evidence for α-secretase processing of APP in these subcellular compartments (De Strooper et al., 1993; Haass et al., 1995a,b; Kuentzel et al., 1993; Sambamurti et al., 1992; Sisodia, 1992). Before concluding, however, that ADAM10 or any other metalloproteinase is really the α-secretase, we clearly need further research. Transgenic mice in whom the different candidate enzymes have been inactivated will allow us to explore in further detail the biological significance of the findings.

The net result of proteolytic cleavage of APP and endocytosis of the remaining holo-APP (see below) is the rapid removal of any cell-surface-expressed APP. The estimated half-life for surface-expressed APP is indeed less than ten minutes (Koo et al., 1996); consequently, only minor amounts of APP (as compared to the total cellular pool) are detected at the cell surface (Kuentzel et al., 1993; Sambamurti et al., 1992). The cleavage of APP by α-secretase results in the generation of APPs, which might have biological functions in growth regulation and neuroprotection, and, in the case of forms containing the Kunitz proteinase inhibitor domain, in blood coagulation (Van Nostrand et al., 1990). The C-terminal, 83-residue APP fragments (C83) remaining in the cell membrane have a relative long half-life and can be detected to different extents in metabolically labeled cells (Oltersdorf et al., 1990).

β-SECRETASE

The β-secretase cleaves APP at the N terminus of the Aβ peptide sequence and is the first prerequisite for generation of Aβ peptides. It is reassuring that four independent approaches all led to the identification of the same candidate β-secretase (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999): BACE (beta-site APP-cleaving enzyme). BACE-1 is a type I integral membrane protein that has a putative prodomain and contains two $D^T/_SG^T/_S$ motifs in its extracellular domain. These motifs are the classical signature of the catalytic domain of aspartyl proteinases. Transfecting BACE-1 into cells increases β-secretase processing of APP, whereas inhibition of BACE-1 by antisense oligonucleotides decreases β-secretase activity. Importantly, the purified ectodomain of BACE-1, containing the aspartyl protease consensus sequence, cleaves synthetic peptides that mimick the β-secretase-clipping site in APP. The enzyme functions optimally at low pH, is not inhibited by the classical aspartyl

protease inhibitor pepstatin and is highly expressed in brain. Finally, BACE-1 displays a higher affinity for APP forms that contain the so-called Swedish type of missense mutation ($K_{595}M_{596}$ to $N_{595}L_{596}$). This mutation causes familial AD and for a long time has been known to enhance β-secretase processing of APP (Cai et al., 1993; Citron et al., 1992, 1994). Genome searches indicate that at least one other human BACE homologue exists, which suggests that BACE represents a new class of transmembrane aspartyl proteinases. Remarkably, BACE-1 cleaves at two different positions in APP: the classical β-secretase-clipping site (Asp1, see Fig. 1), releasing the N terminus of the Aβ peptide, but also at Glu11 (Fig. 1), releasing a shorter form of the Aβ peptide previously identified in plaques (Masters et al., 1985; Naslund et al., 1994) and K293 cells (Haass et al., 1992b). This cleavage site was also detected in neuronal cultures, where it was considered to be an α-secretase site (Simons et al., 1996).

Preliminary results indicate that BACE-1 is located in the Golgi apparatus and endosomes (Vassar et al., 1999). Previous experiments had shown that surface-iodinated APP is a precursor to the Aβ peptide and that the generation of this peptide is inhibited when the endocytosis signal sequence, YENP, in the cytoplasmic tail of APP is removed or when clathrin-coat assembly, and therefore endocytosis, is inhibited by culturing of cells in low-potassium conditions (Koo and Squazzo, 1994; Perez et al., 1999). A slight delay in the secretion of Aβ peptide compared with the secretion of soluble APP in the cell culture medium, further supports the conclusion that the Aβ peptide is mainly generated in the endosomes after endocytosis of APP holoprotein from the cell surface (Perez et al., 1996). Interestingly, opposite results were obtained with APP containing the Swedish mutation, which makes it a better substrate for β-secretase (see Fig. 1): Aβ peptide generation is independent of the endocytosis signal, and the kinetics of production parallel those for APPs generation (Martin et al., 1995; Perez et al., 1996; Thinakaran et al., 1996). β-Secretase therefore appears to act on this mutant APP in the Golgi apparatus and the surface-directed transport vesicles (Haass et al., 1995b; Stephens and Austen, 1996). β-Secretase must therefore be present in the same compartments as α-secretase, and, in the competition for APP substrate, α-secretase must have higher affinity for wild-type APP whereas β-secretase must have a higher affinity for the Swedish-type APP. In some cell types (e.g. MDCK cells) heterogeneity at the β-secretase-cleavage site has been observed: peptides starting at Val3, Ile6 and other residues (Haass et al., 1995a) are generated. The possibility that, apart from BACE-1, other enzymes function as β-secretases should therefore not be ruled out. Whether BACE-1 is responsible for the low level of β-secretase activity observed in the ER and intermediate compartment of neurons, which Chyung et al. (1997) suggest is responsible for the generation of an intracellular pool of Aβ peptide, remains to be demonstrated.

Lowering of the cholesterol concentration inhibits β-secretase processing of APP (Frears et al., 1999; Simons et al., 1998). Under the experimental conditions used, APP appeared no longer to be incorporated into 'rafts', membrane microdomains consisting of proteins, glycosphingholipids and cholesterol. This suggests that rafts, or at least a correct microdomain structure of the internal membranes, is an important determinant for the correct exposure of APP to BACE (Simons et al., 1998).

γ-SECRETASE

From a cell biological point of view, the γ-secretase cleavage, which cleaves the C-terminal end of the Aβ peptide (Fig. 1), is extremely intriguing. The transmembrane domain of APP is predicted to extend from Gly625 to Leu648 and to end just before the triplet Lys649-Lys651, which implies that γ-secretase cleaves in the hydrophobic environment of the cell membrane. This concept is provocative, since water is needed to hydrolyse peptide bonds. However, although the molecular mechanisms involved remain unclear, proteolysis in or close to the transmembrane domain of integral membrane proteins and release of the cytoplasmic domain appears to be pivotal in several signalling pathways. The best-studied example is the SREBP/SCAP regulation of cholesterol homeostasis (Brown and Goldstein, 1997). SREBP is a hairpin-like, membrane-bound transcription factor. Under low-cholesterol conditions, a site 1 protease (S1P) of the subtilisin family of proteases cleaves its luminal loop domain (Sakai et al., 1998). Subsequent cleavage by a hydrophobic site-2 zinc metalloprotease (S2P; Rawson et al., 1997) in the transmembrane domain occurs by default, releasing the cytoplasmic N-terminal domain of SREBP (Fig. 2). This domain is a DNA-binding protein that translocates to the nucleus to activate transcription of genes such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase, HMG CoA reductase and other enzymes that are involved in cholesterol biosynthesis. A cholesterol-sensing protein, SCAP (SREBP-cleavage-activating protein), regulates the first cleavage by S1P. SCAP is a multitransmembrane-domain-containing protein that resides in the ER, like SREBP. Upon lowering of cholesterol, SCAP escorts SREBP from the ER to a post-ER compartment in which S1P is operating (DeBose-Boyd et al., 1999; Nohturfft et al., 1999). SCAP appears thus to be a 'transport chaperone'. The proposed mechanism therefore couples vesicular transport to proteolysis and signal transduction (DeBose-Boyd et al., 1999).

It should be pointed out from the beginning that none of the proteins involved in SREBP processing is likely to be involved in APP processing (De Strooper et al., 1999; Ross et al., 1998). However, one can draw analogies between SREBP/SCAP and APP/presenilin (Brown and Goldstein, 1997; Chan and Jan, 1999; De Strooper et al., 1998). The presenilins (Haass and De Strooper, 1999), like SCAP, contain eight transmembrane domains. They are also located mainly in the membranes of the ER, the intermediate compartment and the cis-Golgi apparatus (Annaert et al., 1999), although a small amount of the presenilin pool might actually be associated with the cell membrane (Georgakopoulos et al., 1999; Ray et al., 1999). In neurons derived from presenilin-1-knockout mice, C-terminal fragments of APP generated by α- and β-secretase accumulate in an undefined cellular compartment, and release of amyloid β-peptide and p3 is inhibited. This suggests a selective role for the presenilins in γ-secretase processing of APP (De Strooper et al., 1998). As mentioned already, missense mutations of the *presenilin* genes are an important cause of familial AD (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995). All the mutations investigated affect γ-secretase processing of APP, increasing slightly the relative amount of Aβ peptides ending at residue 42 (639 of APP; Borchelt et al., 1996; Citron et al., 1997; Scheuner et al., 1996). The hypothesis that

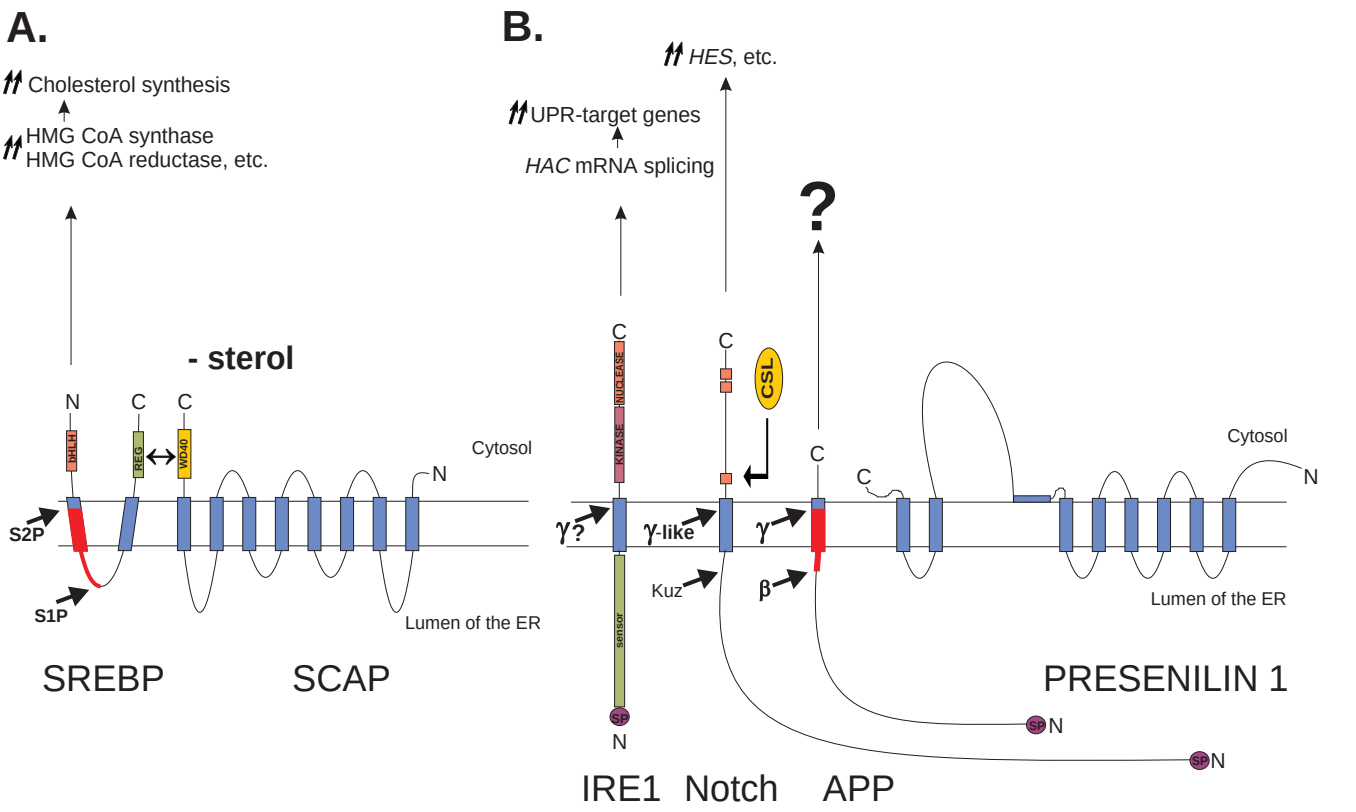

Fig. 2. Established and putative targets for SCAP and presenilin 1. (A) In the absence of sterols, SCAP activates cleavage of SREBP by S1P through its interaction with the regulatory domain in SREBP (REG). The subsequent triggering of S2P activity results in the release of the N-terminal domain bearing a transcription factor of the basic-helix-loop-helix (bHLH) type, which in turn upregulates genes involved in cholesterol synthesis. (B) Comparison of the proteolytic processing in APP, Notch and Ire1. Extracellular cleavage of APP by β-secretase and Notch by ADAM-10 (Kuz) or TACE precedes intramembranous cleavage by γ-(like) secretase activities. It remains to be investigated whether γ-secretase-like activity is also involved in the processing of Ire1, an upstream component of the UPR pathway. At least the intracellular domain of Ire1 seems to be translocated in a presenilin-1-dependent way to the nucleus, where it induces upregulation of UPR-target genes. In the case of Notch, the intracellular domain can bind to members of the CSL family of transcription factors. After cleavage this complex ultimately ends up in the nucleus, where it induces target genes such as HES. The analogy with APP processing is striking, although no evidence is available that the APP intracellular domain is involved in an analogous signalling cascade.

presenilins themselves could be proteases responsible for γ-secretase cleavage of APP (De Strooper et al., 1998; Wolfe et al., 1999a) gained considerable support from the observation that substitution of either one of two critical aspartate residues in transmembrane domains 6 and 7 of presenilin by other amino acid residues (even conservative substitutions such as glutamate) results in a 'dominant negative' effect on APP processing (Wolfe et al., 1999c). Amyloid β-peptide release becomes inhibited, and accumulation of α- and β-secretase-cleaved APP fragments is observed; this is similar to the situation in cells derived from presenilin-1-knockout mice (De Strooper et al., 1998; Steiner et al., 1999; Wolfe et al., 1999c). APP processing can indeed be inhibited by aspartyl protease inhibitors (Wolfe et al., 1999b), and the aspartyl residues in the presenilins have been conserved in evolution, which suggests that they have an important function (Brockhaus et al., 1998; Leimer et al., 1999, Wolfe et al., 1999a).

Although the 'presenilin is γ secretase' hypothesis is attractive, some caution is required. First, direct proof that presenilins are proteases is still lacking. The interpretation that the aspartate residues are part of a catalytic domain is entirely speculative. To test this possibility, transition-state-specific γ-secretase inhibitors should be established and crosslinked to presenilin. In the absence of such data it remains possible that the dominant negative effects observed with the aspartate mutants of presenilin are a consequence of structural alterations in the presenilins that interfere with, for instance, binding to an unidentified protein. Furthermore, the subcellular localisation of the presenilins and the compartments in which γ-secretase activity is believed to exist do not overlap completely (Annaert et al., 1999). This 'spatial paradox' remains to be resolved (Annaert and De Strooper, 1999). Finally, refined analysis of the specificity of γ-secretase processing, using APP mutants, suggests that several proteases are involved in this process (Murphy et al., 1999).

The alternative interpretation is then that the presenilins are only important cofactors in the cleavage of transmembrane proteins such as APP (De Strooper et al., 1998), APLP-1 (Naruse et al., 1998), Notch (De Strooper et al., 1999) and Ire1p (Niwa et al., 1999; see Fig. 2 and below). Their function could theoretically be to create a microdomain in the membrane that, for instance, allows entry of the water molecules necessary for hydrolysis by γ-secretase. A sorting chaperone function like that described for SCAP, allowing

transport of APP, Notch and other substrates from the ER to a more downstream compartment in which γ-secretase resides, is also possible. Finally, the presenilins might be involved in a step necessary for transport or maturation of the γ-secretases themselves. The enrichment of presenilins in the intermediate compartment between the ER and the cis-Golgi is at least compatible with such possibilities (Annaert et al., 1999). Cell-free reconstitution assays investigating the early steps of ER transport could help us to analyse this problem in further detail. It should be mentioned that presenilins could be multifunctional proteins, responsible for the correct insertion of a series of proteins in multimolecular complexes (microdomains), on the one hand, and catalyzing the cleavage of the transmembrane domains of a subset of proteins of these complexes on the other hand.

As mentioned already, Notch processing is also dependent on presenilins. The link between Notch and presenilins was originally made in *C. elegans* (Baumeister et al., 1997; Levitan and Greenwald, 1995). Presenilin-1-knockout mice, furthermore, show some signs of deficient Notch signalling (Hartmann et al., 1999; Shen et al., 1997; Wong et al., 1997). Only when the presenilin 2 gene is also inactivated, does the full Notch-deficiency phenotype become more obvious in mice (Donoviel et al., 1999; Herreman et al., 1999). Notch-1 is a large type 1 integral membrane protein involved in complex cell-fate decisions (Artavanis-Tsakonas et al., 1999). Following ligand binding, its cytoplasmic domain is released by proteolysis at a site in or close to the transmembrane domain (Fig. 2 and Schroeter et al., 1998). The cytoplasmic domain is a signalling factor, which is transported in complex with members of the CSL (CBF1, Su(H), Lag-1) family of DNA-binding proteins to the nucleus. This brings genes involved in myogenesis, neuronal differentiation and hematopoiesis under the control of Notch (Artavanis-Tsakonas et al., 1999). The deficient Notch signalling (De Strooper et al., 1999; Donoviel et al., 1999; Herreman et al., 1999; Song et al., 1999) and the classical Notch phenotypes in presenilin-deficient *Drosophila* (Struhl and Greenwald, 1999; Ye et al., 1999) and *C. elegans* (Baumeister et al., 1997; Levitan and Greenwald, 1995) are now explained at the molecular level by the need for presenilins to cleave the cytoplasmic domain of Notch (De Strooper et al., 1999; Song et al., 1999; Struhl and Greenwald, 1999). That a γ-secretase-like activity is involved in Notch processing is further corroborated by the fact that known γ-secretase inhibitors also inhibit Notch processing (De Strooper et al., 1999).

Cells that express presenilin mutants found in AD patients not only show abnormal processing of APP but also display disturbed stress responses and apoptosis when treated with the glycosylation inhibitor tunicamycin or with the calcium ionophore A23187 (Katayama et al., 1999). The induction of a series of chaperones, e.g. GRP78/Bip, GRP94 and PDI (protein disulphide isomerase), is compromised in these cells (Katayama et al., 1999) and also in cells derived from presenilin-1-deficient mice (Niwa et al., 1999). The expression of these chaperones is controlled by the unfolded-protein response (UPR) signalling pathway. The molecular switch involved is Ire1p, a type I integral membrane protein like APP (Fig. 2). Its N-terminal domain is oriented towards the lumen of the ER and is believed to act as a detector of unfolded proteins.

In response to the accumulation of unfolded proteins in the ER (e.g. after tunicamycin treatment), Ire1p oligomerizes and becomes (auto)phosphorylated. The cytoplasmic domain of Ire1p contains a serine/threonine kinase domain and an endoribonuclease domain at the C-terminal end (Fig. 2). This domain is, at least in yeast, involved in a unique splicing event, the excision of an inhibitory 252-nucleotide intron from the *Hac1* mRNA, which allows translation of the mRNA into active Hac1p protein. Hac1p is a transcription factor that binds to the UPR elements in the promoters of genes involved in the UPR and thereby controls transcription of the relevant chaperones. Although the mammalian homologue of Hac1p has not been identified yet, mammalian Ire1p can splice the yeast *Hac1* mRNA. Mammalian Ire1p therefore probably operates in a similar fashion to its yeast counterpart.

Niwa et al. (1999) could demonstrate that during the UPR the cytoplasmic part of Ire1p translocates to the nucleus. This could suggest that this domain is specifically cleaved in response to induction of the UPR. In fibroblasts derived from presenilin-1-deficient mice, the nuclear translocation of the cytoplasmic domain of Ire1p in response to induction of the UPR appeared to be strongly decreased (Niwa et al., 1999). Given the role of presenilin 1 in the processing of Notch and APP, the authors proposed that presenilin is also needed for proteolysis of Ire1p. Several questions remain to be answered. Direct evidence for proteolysis of Ire1p and for the effect of presenilins thereon is not available, and the proposed involvement of proteolysis in this signal transduction pathway remains therefore largely speculation. Furthermore, a mammalian homologue of Hac1p remains to be identified. Finally, it is unclear to what extent the presenilin deficiency in transgenic mice affects the UPR in vivo. Presenilin-1-knockout mice survive beyond embryonic day 16 (Hartmann et al., 1999; Shen et al., 1997; Wong et al., 1997), presenilin-2-knockout mice are virtually healthy (Herreman et al., 1999; Steiner et al., 1999) and the double-knockout mice (Donoviel et al., 1999; Herreman et al., 1999) survive until embryonic day 9. The major phenotypical alterations can be explained by partially or completely deficient Notch signalling in these mice, and it is unclear to what extent a disturbed UPR further contributes to this phenotype.

In conclusion, interesting parallels between the different pathways – i.e. Notch signalling, Ire1p signalling and APP processing – are emerging (Fig. 2). Presenilin appears to play an important role in a series of proteolytic processes that control the release of the intracellular domains of these proteins (Fig. 2). The established roles of the intracellular domains of Notch and Ire1p, and of the N-terminal part of SREBP, in signal transduction suggest that the intracellular domain of APP is also involved in analogous activities. However, the intracellular APP fragment generated by γ-secretase processing remains highly elusive, and no studies have yet been published that analyse its fate.

LYSOSOMAL PROCESSING OF AMYLOID PRECURSOR PROTEIN

In PC12 and in 293 cells, >70% of newly synthesised APP is degraded (Caporaso et al., 1992; Knops et al., 1992). This degradation is partially inhibited by chloroquine or ammonium chloride, which suggests that lysosomes are involved. This was corroborated by experiments using lysosomal proteinase inhibitors, by antibody-uptake experiments and by the isolation

of lysosomal fractions in the presence and absence of leupeptin, all of which demonstrated that APP is metabolised in lysosomes and that an array of C-terminal derivatives is produced (Estus et al., 1992; Golde et al., 1992; Siman et al., 1993; Haass et al., 1992a; Yamazaki et al., 1996). Parts of these fragments are potentially amyloidogenic because they contain the Aβ peptide sequence. It is not clear whether these fragments are indeed further processed towards Aβ peptides and can contribute to the amyloidogenesis in AD-affected brain.

An unsettled issue is whether all the APP that ends up in the lysosomes must first travel over the cell surface. Indirect observations and arguments (De Strooper et al., 1993; Kuentzel et al., 1993; Lai et al., 1995) suggest that newly synthesised APP could be transported directly from the *trans*-Golgi network to lysosomes via a clathrin-mediated pathway similar to that for the mannose-6-phosphate receptors and lysosome-associated membrane glycoproteins (Traub and Kornfeld, 1997).

APP PROTEOLYSIS DURING APOPTOSIS

An important question in AD research is to what extent apoptotic processes are part of the pathogenetic mechanism. Apoptotic cells become relatively rapidly removed from the brain, and it is therefore very difficult to estimate the contribution of apoptosis to the neurodegenerative process in vivo. Many cell biological studies in vitro, however, suggest that the presenilins and APP are substrates for caspases, and that mutations associated with AD enhance the pro-apoptotic activities (Barinaga, 1998; Chui et al., 1999; Gervais et al., 1999; Giambarella et al., 1997; Mattson et al., 1998; Vito et al., 1996; Wolozin et al., 1996). Interestingly, the Aβ peptide and the apoptosis hypotheses for the pathogenesis of AD need not be considered mutually exclusive. Whereas high concentrations of aggregated Aβ peptide are directly toxic to neurons (Pike et al., 1993; Yankner et al., 1990), which explains the neurodegeneration in the more advanced stages of AD, low concentrations of (soluble) Aβ peptide apparently increase the vulnerability of neurons to apoptosis by downregulating Bcl-2 and upregulating Bax expression (Paradis et al., 1996).

Recently three groups demonstrated that APP is itself a substrate for the caspases; it is cleaved at Asp664-Ala665 in its cytoplasmic domain (Gervais et al., 1999; Pellegrini et al., 1999; Weidemann et al., 1999). It is unclear whether caspase-3, caspase-6, or caspase-8 is the main mediator of this cleavage (Gervais et al., 1999; Pellegrini et al., 1999; Weidemann et al., 1999). Gervais et al. (1999) generated a synthetic mutant of APP with a stop codon at this cleavage site to mimic the product of apoptotic cleavage. Expression of this mutant in cells yielded fivefold more Aβ peptide compared with wild-type APP, providing the molecular basis for a positive feedback loop in the pathogenetic process and raising the possibility that upregulation of certain caspases precedes Aβ peptide secretion (Gervais et al., 1999; LeBlanc, 1995). Some of the findings in this study remain, however, controversial (Haass, 1999). As will be discussed below, several proteins interact with the cytoplasmic tail of APP. It has been suggested that abnormal activation of a G_o trimeric complex that binds to this domain causes apoptosis in the COS-cell-derived cell clone NK1 and in a neuroblastoma-derived cell line (Giambarella et al., 1997; Nishimoto et al., 1993; Okamoto et al., 1995; Yamatsuji et al., 1996a,b).

APP FUNCTION

The functions of APP and its homologues in vivo remain poorly understood. The overall structure of the protein suggests APP functions as a receptor or growth factor (Rossjohn et al., 1999). As was already mentioned above, APP has also been implied in pro- and anti-apoptotic functions (Yamatsuji et al., 1996b; Wolozin et al., 1996; Xu et al., 1999), which does not contradict the growth factor/receptor hypothesis for APP. However, APP-knockout mice display only subtle neurological deficits, such as decreased locomotor activity and forelimb grip strength (Muller et al., 1994; Zheng et al., 1995), probably because the functions of APP, APLP-1 and APLP-2 are redundant (von Koch et al., 1997). Magara et al. (1999) observed an enhanced deficit in corpus callosum formation in those APP-deficient mice strains that display a predisposing genetic background. Also, neurons derived from the APP-deficient mice display deficient survival and neurite outgrowth (Perez et al., 1997), which suggests that APP contributes to neuron viability, axonogenesis and dendritic arborization.

The search for ligands or receptors that interact with the large ectodomain of APP has not been very successful – for reasons that are not yet clear. Several functional subdomains (Fig. 1) have, however, been identified – for example, the RERMS sequence that appears to have growth-promoting properties (Ninomiya et al., 1993), and the two heparin-binding domains that are responsible for binding to the glycan moieties of proteoglycans, such as glypican (Williamson et al., 1996). The physiological role of these binding interactions remains to be elucidated. Best studied are the Cu(II)- and Zn(II)-binding activities of APP. The Zn(II) binding is assumed to play mainly a structural role (Bush et al., 1993), whereas APP is able to catalyse a reduction of Cu(II) to Cu(I) (Multhaup et al., 1996).

The large, promiscuous LRP receptor is thus far the only known receptor that binds to APPs containing the Kunitz-type proteinase inhibitor sequence (Kounnas et al., 1995; Knauer et al., 1996). LRP is also responsible for the clearance of other protease-protease-inhibitor complexes, such as α2-macroglobulin and tissue plaminogen activator inhibitor type 1 (Krieger and Herz, 1994). However, it is mainly the APP695 form that lacks the Kunitz-type inhibitor domain that is expressed in neurons, and therefore the major receptor for APP in brain remains elusive. Obviously, more research should be invested in the identification of putative receptors and ligands for APP. For the time being, and in the absence of clear, defined molecular interactions, the interpretation of the many cell biological studies implicating APP in functions such as cell growth, cell adhesion and neuritic outgrowth remains mostly speculation.

In contrast, important progress has been made in identifying key proteins that interact with the short cytoplasmic domain of APP (summarised in Fig. 1C and Fig. 3). Two-hybrid screening assays have been very rewarding in that regard and have led to the discovery of two families of adapter proteins that appear to interact with the tyrosine-containing motifs in this domain. The Fe65 protein can be considered as the prototype in this regard (Russo et al., 1998). Fe65 is a multimodular protein containing a WW motif and two phosphotyrosine-interaction domains (PID). Blaikie et al. (1994) originally defined the PID in proteins such as Shc: it binds to motifs such as the NPXY clathrin-coated-pit endocytosis signal, provided that they

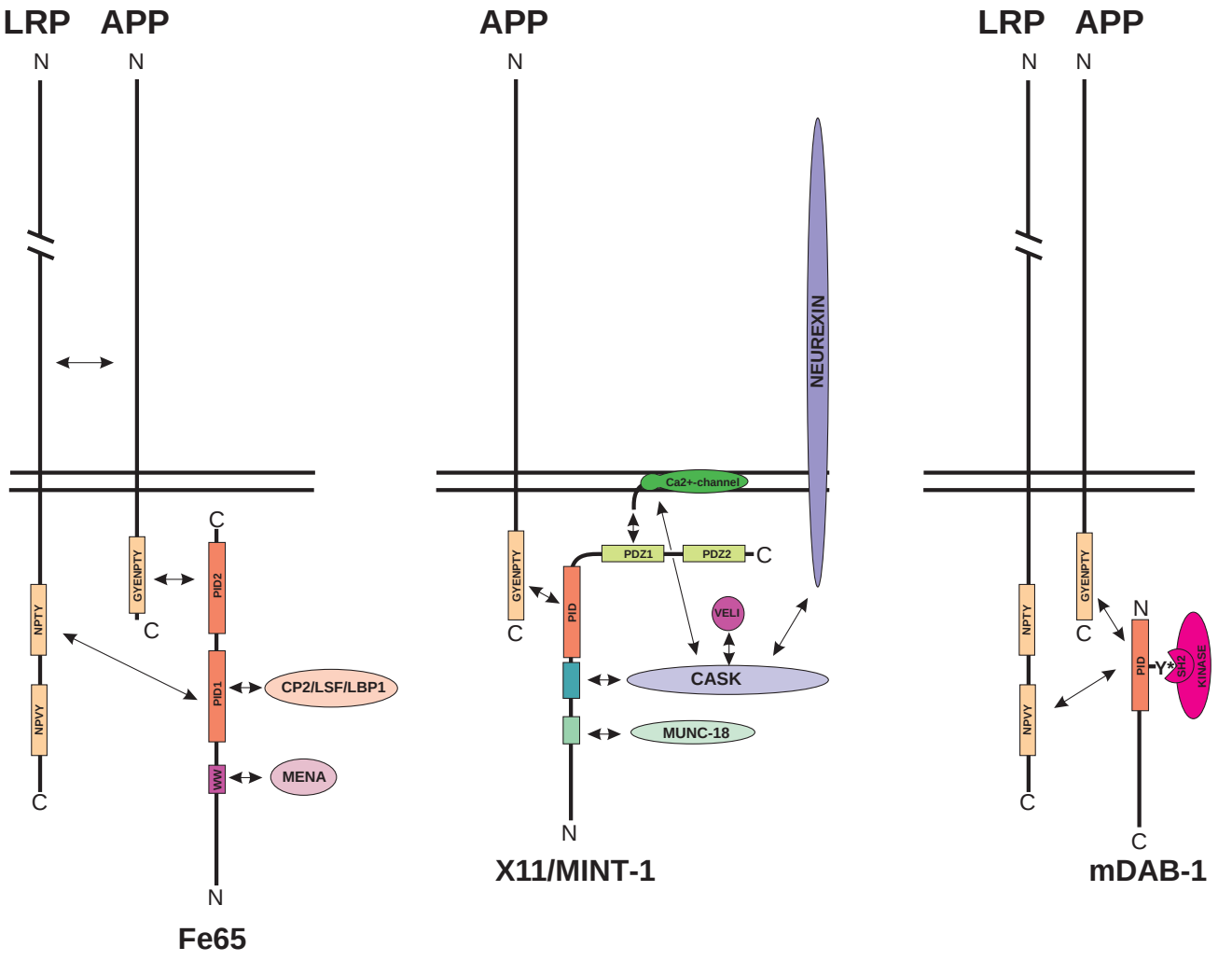

Fig. 3. Comparison of the molecular networks involving the APP cytoplasmic domain. In the first network, the phosphotyrosine-interaction domains (PIDs) of Fe65 are involved in the binding to the NPTY motif of LRP (PID1) and the GYENPTY motif of APP (PID2). PID1 also interacts with the transcription factor CP2/LSF/LBP1, which could suggest a link between γ-secretase proteolysis of APP, release of the cytoplasmic domain and nuclear signalling. On the other hand, the WW domain of Fe65 binds to Mena, linking APP to, for instance, actin remodelling. Second, the GYENPTY sequence in APP also serves as a signal for the binding of the PID domain of X11. The N terminus of X11 contains binding sites for Munc-18 and CASK. The C-terminal association of the voltage-gated Ca^{2+}-channel with the PDZ1 domain of X11 and the SH3 domain of CASK and the binding of CASK to VELI and neurexin connect APP with synapse function and cell adhesion.Third, the PID of mDAB-1 binds to the NPXY domain in LRP and APP. In its phosphorylated state (Y*) , this PID domain can recruit kinases (e.g. Abl, Fyn and Src), linking APP to downstream phosphorylation-dependent signalling pathways.

contain a phosphorylated tyrosine residue. Only the second PID in Fe65 is involved in APP binding, leaving the other PID and the WW domain for other binding partners (Fiore et al., 1995; Guenette et al., 1996; Zambrano et al., 1997). Surprisingly, phosphorylation of the tyrosines in the GYENPTY sequence in the cytoplasmic tail of APP is not necessary for the interaction with the second Fe65 PID (Borg et al., 1996). The PIDs in Fe65 are indeed significantly divergent from those originally described in Shc and other proteins (Russo et al., 1998).

A second APP-binding protein, X11, also binds through a PID to the GYENPTY motif in the cytosolic APP tail (Borg et al., 1996; McLoughlin and Miller, 1996; Tanahashi and Tabira, 1999; Tomita et al., 1999). The crystal structure of this interaction has been elucidated (Zhang et al., 1997). Although the requirements for binding of Fe65 and X11 to the GYENPTY motif differ slightly (Borg et al., 1996), they are sufficiently overlapping to make it conceivable that these proteins compete for binding to APP (Fig. 3). Interestingly, opposite effects on APP processing have been observed after co-expression of APP with either Fe65 or X11. Fe65 overexpression increased the amount of cell-surface-associated APP, as well as the processing of APP to APPs and Aβ peptide (Sabo et al., 1999). By contrast, X11 decreased processing and cellular retention of APP (Borg et al., 1998b; Sastre et al., 1998; Tomita et al., 1999). Thus, the functional balance between the two proteins is important for regulation of APP metabolism and possibly APP function. This balance could be further modified by a third protein, mDAB1 (the mammalian homologue of Disabled), that binds to the same region (Homayouni et al., 1999; Trommsdorff et al., 1998). mDAB1 contains only one PID but, after phosphorylation, can recruit

kinases such as Abl, Fyn or Src by binding to their SH2 domains. Interestingly, mDAB1-knockout mice show neuronal migration disorders similar to those evident in reeler mice (Howell et al., 1997).

The additional PID and the WW domain in Fe65, and the two PDZ domains in X11, link APP to other proteins (Fig. 3). Mena, the mammalian homologue of the *Drosophila* Enabled protein, binds to the WW motif in Fe65 (Ermekova et al., 1997). Ena and DAB have opposite effects on the Abl-deficient phenotype in *Drosophila* and are involved in axogenesis (Gertler et al., 1995). It is probably significant that their mammalian homologues can become recruited into the network surrounding APP. Typically Mena and its homologue VASP are concentrated in focal adhesions, and are found in areas of actin remodelling, such as lammellipodia and the tips of axonal growth cone filapodia (Lanier et al., 1999). The Mena and mDAB interactions could therefore provide the molecular link between APP and the cytoskeleton and be involved in the proposed functions of APP in cell adhesion, growth cone outgrowth and axon guidance (Ermekova et al., 1998).

The non-APP-binding PID of Fe65 binds to the transcription factor CP2/LSF/LBP1 (Zambrano et al., 1998). Whether this transcription factor provides the proposed link between proteolytic cleavage of APP and signalling (as was suggested above) remains to be established. This PID also links APP to the LRP family of lipoprotein receptors (Trommsdorff et al., 1998) – this time at the cytoplasmic side of the membrane. Interesting studies have implicated two members of this family in binding to reelin, an extracellular matrix protein important for the correct neuronal migration during cortical development, and to mDAB, which is also involved in this neuronal migration. Apparently, a protein complex at the cytoplasmic side of the cell membrane links APP with the LRP family of proteins and is potentially important for control of important neuronal migration processes (Hiesberger et al., 1999). A similar disorder is seen in mice that have an inactivated presenilin 1 gene (Hartmann et al., 1999). Intriguingly, although not yet well understood, mice that lack either reelin or reelin receptors of this LRP family, also show hyperphosphorylation of Tau (Hiesberger et al., 1999). These observations might be the tip of an iceberg of an underlying molecular network linking the cell biology of APP and Tau. These initial and still superficial insights could suggest that all important molecular players involved in the pathogenesis of AD operate in related signalling pathways.

Molecular partners of X11 have also been identified (Fig. 3). Munc18, a protein thought to be involved in synaptic vesicle exocytosis binds to a region in the N-terminal half of X11α. X11 is therefore also known as the Munc18-interacting protein 1 or MINT-1 (McLoughlin et al., 1999). X11α is in addition part of a heterotrimeric complex with the mammalian homologues of *C. elegans* Lin2 (CASK) and Lin7 (VELI) (Borg et al., 1998a, 1999; Butz et al., 1998). This complex is located at the presynaptic membrane and is linked through CASK to the neurexins. One of the PDZ domains of X11α also links this complex to a presynaptic voltage-gated calcium channel (Maximov et al., 1999). We can expect that further screening will ultimately identify other ligands that interact with the second PDZ domain of X11α. Obviously, this is only the beginning of the enormous task of deciphering the network surrounding the cytoplasmic domain of APP. In addition, two other mammalian homologues of Fe65 and X11 are known, which complicates the work even further (Borg et al., 1998a; Duilio et al., 1998; Guenette et al., 1996; McLoughlin and Miller, 1996; Tomita et al., 1999), especially since they bind not only to the cytoplasmic tail of APP but also to the cytoplasmic tails of APLP1 and APLP2 – at least in vitro.

Proteins that bind to other parts of the APP tail have also been identified. For instance, Nishimoto and co-workers demonstrated some time ago that the GTP-binding protein G_o binds to the His657-Lys676 sequence in the APP tail (Nishimoto et al., 1993). They further provided evidence that APP activates G_o after stimulation with an anti-APP antibody (Okamoto et al., 1995). AD-associated mutation in APP constitutively activated G_o and could induce apoptosis via the Gβγ subunit (Giambarella et al., 1997; Okamoto et al., 1996; Yamatsuji et al., 1996a,b). Bruillet et al. (1999) confirmed the interaction of APP with G_o in primary cultured neurons. Although these results are potentially very important for our understanding of the function of APP, further investigations are now needed to clarify the physiological function of these interactions.

An interesting protein that provides a potential link between APP and microtubules is PAT1 (protein interacting with the APP tail 1; Zheng et al., 1998). The binding of this novel protein requires Tyr653 in the APP tail (Fig. 1), which is also critical for the basolateral sorting of APP in epithelial MDCK cells (De Strooper et al., 1995a,b; Haass et al., 1995a). Further functional analysis to delineate more precisely the function of PAT1 in APP transport is now required. Finally, Chow et al. (1996) identified an APP-binding protein 1 (APP-BP1) by screening a cDNA expression library with the C-terminal region of APP, but functional analysis of this interaction is lacking at this moment.

CONCLUSION AND PERSPECTIVES

Recently, and especially because of the progressive unravelling of the cytoplasmic protein network surrounding the C-terminal tail of APP, some insight into the cell biological function of this protein is emerging. Claims that APP is involved in synapse formation, axonal and dendritic outgrowth and neuronal survival seem at least indirectly supported by the finding that APP is part of a huge protein complex that has important functions in neuronal cell migration and synaptic transmission. In addition, a lot of insight has been gained into the molecular processes controlling APP proteolysis. However, although APP is probably one of the most studied proteins in molecular cell biology, the roles of the different proteolytic fragments remain largely hidden in a fog of speculation. Only the further dissection of the molecular signalling pathways interacting with the APP cytoplasmic domain and of those involved in the recognition of the ectodomain of APP can help us to reveal the functions of APP.

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