γ-Secretase activity requires the presenilin-dependent trafficking of nicastrin through the Golgi apparatus but not its complex glycosylation

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Summary
Nicastrin and presenilin are two major components of the γ-secretase complex, which executes the intramembrane proteolysis of type I integral membrane proteins such as the amyloid precursor protein (APP) and Notch. Nicastrin is synthesized in fibroblasts and neurons as an endoglycosidase-H-sensitive glycosylated precursor protein (immature nicastrin) and is then modified by complex glycosylation in the Golgi apparatus and by sialylation in the trans-Golgi network (mature nicastrin). These modifications are not observed with exogenously overexpressed nicastrin. Under normal cell culture conditions, only mature nicastrin is expressed at the cell surface and binds to the presenilin heterodimers. Mature nicastrin has a half-life of more than 24 hours. In the absence of presenilin 1 and 2, nicastrin remains entirely endoglycosidase H sensitive, is retained in the endoplasmic reticulum and is slowly degraded. Single presenilin 1 or presenilin 2 deficiency affects glycosylation of nicastrin to a lesser extent than the combined presenilin deficiencies, suggesting a correlation between either the transport of nicastrin out of the endoplasmic reticulum or the concomitant complex glycosylation of nicastrin, and γ-secretase activity. However, when complex glycosylation of nicastrin was inhibited using mannosidase I inhibitors, γ-secretase cleavage of APP or Notch was not inhibited and the immature nicastrin still associates with presenilin and appears at the cell surface. Complex glycosylation of nicastrin is therefore not needed for γ-secretase activity. Because the trafficking of nicastrin to the Golgi apparatus is dependent on presenilins, our data point to a central role of presenilin in nicastrin maturation/localization, which could help to partially resolve the ‘spatial paradox’.

Key words: Nicastrin, Presenilin, γ-Secretase, Alzheimer’s disease, Glycosylation

Introduction
γ-Secretase activity is responsible for the cleavage of the transmembrane domain of the amyloid precursor protein (APP), releasing the amyloid peptide Aβ and the APP intracellular domain. Aβ is a major component of the amyloid plaques characteristic of Alzheimer’s disease. The γ-secretase molecular machinery consists at least of presenilins (PSs) (Herreman et al., 2000; Zhang et al., 2000), nicastrin (Chung and Struhl, 2001; Hu et al., 2002; Lopez-Schier and St Johnston, 2002; Yu et al., 2000), pen-2 (Francis et al., 2002; Steiner et al., 2002) and the aph proteins (Francis et al., 2002; Goutte et al., 2002). It has been the subject of intense research not only because of its therapeutic potential for Alzheimer’s disease, but also because of the wide variety of biological processes in which regulated intramembrane proteolysis by γ-secretase has been implicated (Annaert and De Strooper, 2002; Kopan and Goate, 2000; Selkoe, 1999; Sisodia and St George-Hyslop, 2002; Steiner and Haass, 2000). Indeed, a similar, PS-dependent proteolytic process is involved in the release of the cytoplasmic domains of an increasing range of type I integral membrane protein receptors, such as Notch (De Strooper et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999), low density lipoprotein receptor-related protein (LRP) (May et al., 2002), ErbB-4 (Ni et al., 2001), E-cadherin (Marambaud et al., 2002) and probably others. Why the cytoplasmic domain of all of these receptors is shed is not yet fully understood. However, in the case of Notch, it is established that the released intracellular domain combines with DNA-binding proteins of the CSL family to generate a transcriptionally active complex that regulates gene expression (Kopan and Goate, 2000). For APP, the situation is less clear, although the analogy with Notch suggests that the intracellular domain of APP could be involved in gene regulation as well (Annaert and De Strooper, 1999). Recent experimental evidence in favor of this hypothesis (Cao and Sudhof, 2001; Cupers et al., 2001b; Gao and Pimplikar, 2001; Kimberly et al., 2001; Leissring et al., 2002) is indirect and genes whose expression is regulated under physiological conditions by the APP intracellular domain have been only gradually identified (Baek et al., 2002). It is also possible that γ-secretase is involved in the release and disassembly of the...
cytoplasmic protein complexes that are hooked up to the intracellular domain of APP and other proteins like LRP and the cadherins. Such disassembly could be part of a cellular response to extracellular stimuli, a possibility that remains to be further explored.

It is clear that γ-secretase processing is a prototype example of a very general biological phenomenon and further understanding of its molecular cell biology is therefore of major importance. Questions concerning the specificity and regulation of the cleavage process or the subcellular compartment in which the γ-secretase operates need to be addressed in more detail. In the current manuscript, we focus on the relationship between two established γ-secretase components: PS and nicastrin. The absolute requirement for PS in the γ-secretase process was established some years ago (De Strooper et al., 1998; Herreman et al., 2000; Zhang et al., 2000) and has not convincingly been challenged since then (Nyabi et al., 2002). An important issue is whether the two aspartate residues in transmembrane domains 6 and 7 constitute the active catalytic site of the γ-secretase complex (Wolfe et al., 1999). This claim found considerable support both in the observation that several known γ-secretase inhibitors bind to PS directly (Esler et al., 2000; Li et al., 2000b; Seifert et al., 2000) and in the recent identification of proteolytically active, remote homologues of PS (Pontig et al., 2002; Weihofen et al., 2002). The fact that substrates like Notch and APP are co-precipitated with PS (Esler et al., 2002; Ray et al., 1999b) seems to contradict the hypothesis that PS mediates their cleavage. However, this can probably be explained by the intramolecular separation between the substrate-binding site and the putative catalytic site in PS (Annaert et al., 2001), implying that binding and cleavage are two separate events and that a conformational change is needed to bring the substrate towards the catalytic site. This could explain why substrate and putative protease remain in complex for a certain period of time (Annaert et al., 2001). In any case, PSs are not capable of cleaving APP substrates without additional cofactors. For instance, while PSs are abundantly present in the endoplasmic reticulum (ER), the intermediate compartment and the cis-Golgi (Annaert et al., 1999; Culvenor et al., 1997; Lah et al., 1997), they do not efficiently cleave an APP substrate that is specifically retained in these compartments (Cupers et al., 2001a; Malteze et al., 2001). Only after addition of brefeldin A are the APP substrates proteolysed, indicating that other proteins or specific post-translational modifications of the γ-secretase complex in the Golgi apparatus are needed to trigger this proteolytic activity (Cupers et al., 2001a). This dissociation between PS subcellular localization and γ-secretase activity has been previously called the ‘spatial paradox’ (Annaert and De Strooper, 1999). Nicastrin was shown to co-purify biochemically with PS (Yu et al., 2000) and to interact with the γ-secretase substrates APP (Yu et al., 2000) and Notch (Chen et al., 2001). By contrast, the crucial functional role of nicastrin in γ-secretase cleavage of Notch has been convincingly demonstrated recently by genetic and biochemical means in Drosophila (Chung and Struhl, 2001; Hu et al., 2002; Lopez-Schier and St Johnston, 2002). These experiments also indicated that nicastrin is needed to stabilize PS protein expression (Hu et al., 2002; Lopez-Schier and St Johnston, 2002). Although the experiments indicated at first glance that the effects of nicastrin inactivation on Notch cleavage and signalling could be indirect via the destabilizing effect on PSs, other possibilities are not excluded. Indeed, normal PS expression was maintained in nicastrin-deficient cells under certain conditions, while Notch signalling remained perturbed (Lopez-Schier and St Johnston, 2002).

In the current manuscript, we have investigated the subcellular distribution and the post-translational modifications of nicastrin in neurons and fibroblasts. We have studied the effects of PS deficiency on the cell biology of nicastrin and have analyzed the role of nicastrin glycosylation and PS association in γ-secretase activity.

Materials and Methods

Cell culture

Mouse embryonic fibroblasts (MEFs) were derived from PS1/PS2-deficient mouse embryos and their littermate controls (Herreman et al., 1999; Herreman et al., 2000). Tissues were digested by collagenase and seeded into culture flasks. Outgrowing cells were immortalized by transfection with the plasmid pMSSVLT, driving expression of the large T antigen. Cultures were maintained in DMEM/F12 containing 10% fetal calf serum (FCS). Primary cultures of mixed cortical neurons derived from wild-type and PS1-knockout littersmates were generated as before (De Strooper et al., 1998). In brief, total brain of 14-day-old embryos was dissected in Hank’s Balanced Salt medium, trypsinized and plated on dishes (Nunc) pre-coated with poly-L-lysine (Sigma). Cultures were maintained in neurobasal medium with B27 supplement and 5 μM cytosine arabinoside was added to prevent glial cell proliferation. Deoxymannojirimycin (MNJ, 0.2 mg/ml, Sigma) or kifunensine (1 μg/ml, Calbiochem), to block mannosidase I, were added to the cultures as indicated.

HEK293 cells stably transfected with APP695/Sw were kindly provided by C. Haass (Adolf-Butenandt-Institut, Munich, Germany). HEK293 cells were transiently transfected with plasmids encoding mNotchΔE or NICD using fuzgene (Roche), according to the instructions of the manufacturer.

Antibodies

A new antibody, B59.2, was raised against the C-terminal 15 amino acids of nicastrin coupled to keyhole limpet hemocyanin (Imject, Pierce). Rabbit polyclonal antibody (pAb) B12.6 against APP (De Strooper et al., 1995), B12.9 against PS1 N-terminal fragment (NTF) (De Strooper et al., 1997) and B32.1 against PS1 C-terminal fragment (CTF) (Annaert et al., 1999) have been characterized before. Antibodies against calnexin and human PS1 CTF (mAb 5.2) were kindly provided by A. Helenius (University of Zurich, Switzerland) and B. Cordell (Scios, Sunnyvale, California). Antibodies against BIP and nicastrin were obtained from Stressgen and Chemicon. Monoclonal antibody (mAb) WO2, recognizing the C-terminus of the Aβ sequence was from Abeta GmbH (Heidelberg). For some applications, pAb B19.2 and B59.2 were biotinylated according to the instructions of the manufacturer (Pierce).

Deglycosylation of nicastrin

MEFs/neurons were harvested in PBS supplemented with 5 mM EDTA, 1 μg/ml pepstatin A and 100 U/ml aprotinin. After centrifugation (800 g, 10 minutes), cells were lysed in either 100 mM phosphate buffer pH 5.7 for endoglycosidase H (endoH)- or O-glycosidase treatment or in phosphate buffer pH 7.4 for N-glycosidase F treatment. Phosphate buffer was supplemented with 0.1% SDS, 0.5% Triton X-100, 0.5% β-mercapto-ethanol and protease inhibitors to optimize enzyme activities. In the case of neuraminidase treatment (1 mU/20 μl, Roche) cells were lysed in 20 mM phosphate buffer.
pH 7.4. The cell lysate was incubated on ice for 20 minutes and centrifuged at 20,800 g for 15 minutes. After 10 minutes incubation at 70°C, cell lysates were incubated overnight with endoH (1 U/20 µl Roche), N-glycosidase F (1 U/20 µl Roche) or O-glycosidase (1 mM/20 µl Roche). Samples were separated by SDS-PAGE on pre-cast 7% Tris-Acetate gels (Nupage, Invitrogen, Life Technologies) and blotted. Affinity-purified nicastrin pAb B59.2 was used at a dilution of 1/3000 in blocking buffer (Tris-buffered saline, 0.1% Tween, 5% nonfat dry milk). For detection, HRP-coupled secondary antibodies (BioRad) were used followed by chemiluminescence detection (Renaissance, Perkin Elmer).

Surface biotinylation
For cell-surface biotinylation, cells at 80% confluency were incubated in PBS pH 8-8.5 containing 0.5 mg/ml NHS-SS-biotin (Pierce) for 30 minutes at 4°C on a rocking platform. After quenching with 100 mM glycine and 0.5% BSA, cells were extracted in DIP buffer (1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS in 150 mM NaCl and 50 mM Tris-HCl pH 7.4). After centrifugation (20,800 g, 10 minutes), cleared cell extracts were incubated overnight (4°C) with streptavidin beads (Pierce). Bound material was eluted with 25 µl Nupage sample buffer (Invitrogen), electrophoresed on pre-cast 4-12% Bis-Tris Nupage gels in MOPS running buffer and processed for western blotting using pAb B59.2.

Pulse-chase experiments
MEFs at 80% confluency were metabolically labeled for 15 minutes with methionine-free DMEM medium supplemented with 100 µCi/ml [35S]-methionine. After labeling, cells were washed once and incubated for the indicated time periods in DMEM/F12 with 1% FCS. Next, cells were extracted in 250 mM sucrose, 5 mM Tris-HCl (pH 7.4), 1 mM EGTA and 1% Triton X-100 in the presence of protease inhibitors, and cleared by centrifugation (20,800 g, 15 minutes). Labeled protein was immunoprecipitated using 30 µl protein G sepharose and specific antibodies against PS1, APP and nicastrin. Immunoprecipitates were finally eluted in 25 µl Nupage sample buffer containing 1% β-mercapto-ethyl alcohol and electrophoresed on 7% Tris-Acetate Nupage gels in Tris-Acetate running buffer (Invitrogen). Radiolabeled bands were visualized by phosphorimaging and ImageQuant 4.1 software (Molecular Dynamics).

Co-immunoprecipitation experiments
Cell pellets were resuspended and homogenized in 250 mM sucrose, 5 mM Tris-HCl (pH 7.4) and 1 mM EGTA supplemented with protease inhibitors using a ball-bearing cell cracker (10 passages, clearance 10 µm). After low-speed centrifugation (800 g, 10 minutes), the postnuclear supernatant was ultracentrifuged (100,000 g, 1 hour) and the membrane pellet resuspended in 10 mM Tris and 1mM EDTA containing 0.5% CHAPS, and incubated at 4°C for 1 hour. For immunoprecipitation, the cleared extract (100,000 g, 1 hour) were incubated overnight (4°C) with protein G sepharose and specific antibodies as indicated. Immunoprecipitates were solubilized in 25 µl Nupage sample buffer (Invitrogen), electrophoresed on 4-12% Nupage Bis-Tris Nupage gels in MOPS running buffer and processed for western blotting.

Immunofluorescence experiments
PS1- and 2-deficient MEFs were rescued with human PS1 using the retroviral system for stable transduction (Clontech). By western blot analysis, expressed human PS1 is fully endoproteolytically processed and restores both APP processing and nicastrin maturation (G.V.G. and W.A., unpublished). This cell line allowed us to apply triple immunofluorescent staining using mouse mAb 5.2 against human PS1, rabbit pAb against calnexin, and guinea-pig pAb against nicastrin (Chemicon). Briefly, cells were grown to 30-50% confluency on glass coverslips in DMEM/F12 containing 10% FCS, washed twice in Dulbecco’s PBS and fixed for 10 minutes in ice-cold methanol and for 2 minutes in acetone. Cells were subsequently washed three times with Dulbecco’s PBS and blocked for 2 hours in blocking buffer (Dulbecco’s PBS containing 2% BSA, 2% FCS, 0.2 % fish gelatine and 5% of normal rabbit, goat and guinea-pig whole serum). Fixed cells were incubated with the three primary antibodies diluted in blocking buffer for 1 hour. After washing in PBS, immune complexes were visualized using alexa-488, -555 and -647 conjugated antibodies (Molecular Probes). Finally, cover slips were mounted with moviol and analyzed on a Biorad MRC1024 confocal microscope equipped with a Nikon Axiophot inverted microscope and a 60x plan apochromat (Nikon) oil-immersion objective. The different fluorochromes were sequentially captured using LaserSharp 3.0 and finally processed in Adobe Photoshop 5.0.2 (Adobe, CA).

Cell free γ-secretase assay
Microsomal membranes, prepared as described above, were washed twice in 0.02% saponin and finally resuspended in Tris-EDTA containing 0.5% CHAPS, and incubated at 4°C for 1 hour. Cleared extracts (100,000 g, 1 hour) were incubated overnight (37°C) with recombinant APP C100-flag (Li et al., 2000a). Finally, de novo formed Aβ was analyzed by SDS-PAGE on 10% Bis-Tris Nupage gels in MES running buffer and followed by western blotting using mAb WO2.

Results
Mature nicastrin contains a mixture of high-mannose and complex oligosaccharide side chains
Western blot analysis of total cell extracts from wild-type MEFs using an antibody raised against the C-terminus of nicastrin revealed two bands running with apparent Mr~130 kDa and ~110 kDa (Fig. 1A, left panel). EndoH digestion reduced the apparent Mr of the upper band by ~15 kDa, and of the lower band by ~40 kDa, indicating that both proteins contained one or more high-mannose N-linked oligosaccharides. When both proteins were treated with N-glycosidase F to remove all N-linked oligosaccharide chains, they co-migrated in SDS-PAGE at the same Mr~110 kDa. Western blot analysis of total cell extracts from wild-type MEFs using an antibody raised against the C-terminus of nicastrin revealed two bands running with apparent Mr~155 kDa and ~100 kDa (Fig. 1A, left panel). EndoH digestion reduced the apparent Mr of the upper band by ~15 kDa, and of the lower band by ~40 kDa, indicating that both proteins contained one or more high-mannose N-linked oligosaccharides. When both proteins were treated with N-glycosidase F to remove all N-linked oligosaccharide chains, they co-migrated in SDS-PAGE at the same Mr~110 kDa. Pulse-chase experiments (see below, Fig. 6) further demonstrated that the Mr~110 kDa protein is gradually turned over to the higher Mr~130 kDa protein, indicating that the form with low Mr is the high-mannose oligosaccharide modified precursor form of nicastrin as it is synthesized in the ER. The higher Mr~110 kDa form is mature nicastrin containing a mixed population of endoH-sensitive and endoH-resistant oligosaccharides, as they are generated during trafficking through the Golgi apparatus. Interestingly, this mature form is largely, or completely, absent in PS1- and PS1- and 2-deficient MEFs, respectively (insets in Fig. 1A, and see below).

The specific binding of lectins to carbohydrate moieties was used to analyze the glycosylation pattern of nicastrin in wild-type MEFs in further detail (Fig. 1B). The immature glycosylated nicastrin reacted strongly with Galanthus nivalis agglutinin (GNA), indicating the presence of terminally linked mannose sugar residues, in agreement with its endoH sensitivity. The mature nicastrin also reacted weakly with this lectin, confirming the presence of at least one high-mannose oligosaccharide on mature nicastrin as well. The mature
Fig. 1. Glycosylation modifications of nicastrin and effect of PS deficiencies. (A) Western blot analysis of total cell extracts from wild-type, PS1–/–, PS2–/– and PS1–/–PS2–/– MEFs using an antibody raised to the C-terminus of nicastrin (B59.2). Cell extracts were subject to digestion with endoH (H) and N-glycosidase F (F); −20°C and 37°C represent control samples (no addition of enzyme) kept at −20°C and 37°C. Note the partial endoH resistance of mature nicastrin (NCTm) in wild-type and in PS2–/– MEFs, and in PS1–/– MEFs to a lesser extent (overexposed part of the blot below). In PS1–/–PS2–/– MEFs, only endoglycosidase-sensitive immature nicastrin (NCTi) is observed (see also overexposed inset). (B) Lectin binding to nicastrin. Nicastrin was immunoprecipitated from total cell extracts of wild-type MEFs and blotted using the indicated lectins. The immature glycosylated nicastrin reacted strongly with Galanthus nivalis agglutinin (GNA), indicating the presence of terminally linked mannose residues. NCTm also reacted weakly with this lectin. Furthermore, NCTm reacted with Maackia amurensis agglutinin (MAA) and Datura stramonium agglutinin (DSA), indicating the presence of sialic acid residues and complex and hybrid N-glycan structures, respectively. No reaction was observed with peanut agglutinin, specific for O-glycan modifications. Control glycoproteins demonstrated the specificity of the lectins. (C) Western blot analysis of total cell extracts from wild-type and PS1–/– neurons. C, control (no enzyme); H, endoH digestion; F, N-glycosidase F digestion; N, neuraminidase digestion; O, O-glycosidase digestion. Notice the partial sensitivity to endoH and the complete sensitivity to N-glycosidase F of neuronal nicastrin. A small shift in mobility is also observed after neuraminidase digestion. (D) Western blot analysis of total cell extracts from wild-type and APP–/– MEFs. C, control (no enzyme); H, endoH digestion; F, N-glycosidase F digestion. No differences between wild-type and APP–/– MEFs could be observed.

The nicastrin protein also reacted with the lectins Datura stramonium agglutinin (DSA), which indicates the presence of complex and hybrid N-glycan structures. Finally, the reactivity of the lectin Maackia amurensis agglutinin (MAA) indicated that sialic acid residues are added to the oligosaccharide side chains of mature nicastrin. No reaction was observed with the lectin peanut agglutinin (PNA), suggesting the absence of O-glycan modifications. Control glycoproteins demonstrated the specificity of the lectins. (C) Western blot analysis of total cell extracts from wild-type and PS1–/– neurons. C, control (no enzyme); H, endoH digestion; F, N-glycosidase F digestion; N, neuraminidase digestion; O, O-glycosidase digestion. Notice the partial sensitivity to endoH and the complete sensitivity to N-glycosidase F of neuronal nicastrin. A small shift in mobility is also observed after neuraminidase digestion. (D) Western blot analysis of total cell extracts from wild-type and APP–/– MEFs. C, control (no enzyme); H, endoH digestion; F, N-glycosidase F digestion. No differences between wild-type and APP–/– MEFs could be observed.

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PS1 interacts with mature nicastrin and is required for its complex maturation

PS1 was immune precipitated from (metabolically labeled) MEFs using PS1 NTF- and CTF-specific antibodies. Mainly the mature form of nicastrin and only a very small amount of immature nicastrin co-precipitated with the PS complex (Fig. 2A). To analyze to what extent the oligosaccharide modifications of nicastrin were involved in the interaction with PS1, we treated MEFs with MJN, a mannosidase type I inhibitor. This treatment inhibits the trimming of the high-mannose oligosaccharides upon arrival in the cis-Golgi, and therefore prevents their further maturation to complex oligosaccharides in the distal Golgi compartments. As expected, this treatment resulted in fully endoH-sensitive nicastrin (Fig. 2B). Surprisingly, under these conditions, immature nicastrin still co-precipitated well with PS1, indicating that the carbohydrate modifications themselves are not required for the binding to PS (Fig. 2C).

We next investigated whether the absence of PS or APP [which both bind to nicastrin (Esler et al., 2002; Yu et al., 2000)] affected the expression of nicastrin (Fig. 1A,D). In the
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complete absence of PS (PS1−/−PS2−/− MEFs), only the lower immature endoH-sensitive nicastrin band is observed (Fig. 1A, four right-hand lanes). Interestingly, it appears that PS1 contributes significantly to the maturation of nicastrin. Indeed, in single PS2−/− MEFs, we found similar amounts of mature nicastrin as in wild-type MEFs whereas, in PS1−/− MEFs, only some mature glycosylated nicastrin was visible after prolonged exposure (Fig. 1A, inset showing overexposed parts of the blots). When analyzed in APP-deficient cells (Fig. 1D), no effect on the expression or glycosylation pattern of nicastrin was observed. Also, PS1 expression was unaffected in APP-knockout cells (not shown).

Finally, in PS1-deficient neurons, one single nicastrin band is observed, as in wild-type neurons (Fig. 1C). However, after endoH treatment, most of the nicastrin protein migrates at 70 kDa (Fig. 1C, right panel, lane 2), indicating that it contains only glycosyl residues of the high-mannose, immature type. This confirms that PS1 is also needed in neurons for an efficient maturation of nicastrin. Since we are not able to generate PS1 and 2 double-deficient neurons owing to the early lethality of the embryos (Herreman et al., 1999), it is not possible to confirm that the residual maturation of nicastrin in the PS1-deficient neurons was, as in fibroblasts, indeed dependent on PS2.

Subcellular localization of endogenous nicastrin

We next investigated whether the major defects in glycosylation caused by PS deficiency also affected the subcellular localization of nicastrin. We first confirmed that nicastrin could reach the cell surface by performing surface biotinylation experiments (Fig. 3). The specificity of this finding is clear from the fact that immature nicastrin was not biotinylated in these experiments, either in wild-type or in PS-deficient cells (Fig. 3). Also, at the immunocytochemical level, the absence of PS expression causes changes in the distribution of nicastrin. In wild-type cells, only a limited overlap of nicastrin with the ER marker protein BIP is observed (Fig. 4, top), with important amounts of nicastrin distributed in vesicular structures in the cytoplasm. In PS1−/−PS2−/− MEFs, nicastrin distribution was far more restricted and the overlap with BIP was much more prominent (Fig. 4, bottom and compare insets in merged pictures).

To define more precisely to what extent nicastrin co-distributed with PS1, we triple labeled PS1−/−PS2−/− MEFs stably transduced with human PS1 using antibodies against endogenous calnexin, human PS1 and endogenous nicastrin. The calnexin antibodies showed the typical reticular structure of the ER (Fig. 5, insets in bottom panel). As described before (Annaert et al., 1999), the subcellular distribution of PS1 largely overlaps with calnexin, but also the non-ER structures (such as the intermediate compartment) are immunostained. The overall pattern of nicastrin is in general different from both PS1 and calnexin staining, displaying a more perinuclear dot-like pattern. However, some discrete structures show co-localization of nicastrin with PS1 and/or with calnexin (Fig. 5, insets in merged pictures).

Turnover of nicastrin is slow in wild-type and PS-deficient cells

The turnover and maturation of nicastrin was further analyzed in wild-type and PS1- and 2-deficient MEFs by metabolic pulse labeling (15 minutes) followed by different chase periods (Fig. 6). At the end of the pulse, only immature nicastrin was clearly visible in the wild-type fibroblasts (Fig. 6A, upper panel). After

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**Fig. 2.** Binding of mature nicastrin to PS. (A) PS1 NTF, PS1 CTF and nicastrin were immunoprecipitated from total cell extracts of wild-type MEFs and blotted with B59.2 (α-nicastrin). As a control, pre-immune serum (PI) was used for immunoprecipitation. Both PS1 NTFs and CTFs interact preferentially with the mature form of nicastrin (NCTm).

(B) Wild-type MEFs were treated for 24 or 48 hours with MNJ (0.2 mg/ml). This treatment inhibits mannosidase 1 and therefore the full maturation of the glycosylation of nicastrin. Only endoH-sensitive nicastrin is found. (C) Wild-type MEFs were treated for 48 hours with MNJ (0.2 mg/ml). PS1 NTF and NCT were immunoprecipitated from total cell extracts and blotted with B59.2 (α-NCT). As a control, PI was used for immunoprecipitation. Note that deglycosylation of nicastrin does not inhibit interaction with PS1.

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**Fig. 3.** Surface expression of nicastrin is disturbed in PS-deficient cells. Wild-type (WT) and PS1−/−PS2−/− MEFs (KO) were surface biotinylated. Biotinylated proteins were precipitated using streptavidin beads and immunoblotted with B59.2 (α-nicastrin).

Western blot analysis of total cell extracts is shown as a control. Note that only in WT MEFs is mature nicastrin labeled.
6 hours, most of the protein was chased into the mature nicastrin and little precursor remained visible. The generation of mature nicastrin is relatively slow, reaching a maximum after 3 hours of chase. The mature nicastrin was remarkably stable during the next 24 hours, indicating an extremely slow degradation of the mature protein. This is not an artefact of the cell culture system, since APP for instance becomes fully glycosylated and is already degraded after 3 hours of chase (Fig. 6B, upper panel).

In PS1−/−PS2−/− MEFs, the precursor was synthesized to a similar extent as in the wild-type cells (Fig. 6A, lower panel). However, even after prolonged chase periods, no mature nicastrin was observed, confirming that PS is absolutely required for the maturation of nicastrin. The pool of immature nicastrin was remarkably slowly degraded in the PS-deficient cells (Fig. 6A, lower panel) indicating that PSs are not needed for the stability of nicastrin. Again, the global pattern of maturation and degradation of APP was fast in the PS1- and 2-deficient MEFs (Fig. 6B, lower panel).

As mentioned above, similar pulse-chase experiments performed in wild-type neurons indicated that overexpressed nicastrin remains endoH sensitive even after prolonged chase
periods, indicating that, as is the case for PS (Thinakaran et al., 1997), limiting factor(s) are responsible for the maturation of this protein.

The glycosylation of nicastrin is not required for γ-secretase activity

Given the close correlation between mature nicastrin and its association with PS, we investigated whether glycosylation of nicastrin is required for γ-secretase cleavage of APP and Notch. We prepared membranes from fibroblasts treated with MNJ, an inhibitor of mannosidase I, and used them in the cell-free γ-secretase assay using recombinant synthetic APP-C99 peptides (Li et al., 2000a). Similar amounts of Aβ were produced from solubilized membranes generated from control cells (–MNJ in Fig. 7A) and from treated cells (+MNJ in Fig. 7A). Western blot confirmed that almost all nicastrin in the treated cell membranes was of the ‘high-mannose’ type (Fig. 7B). In a second experiment, we treated HEK cells stably transfected with APP695/Sw with the mannosidase I inhibitor kifunensine and measured the effect on Aβ production. Kifunensine treatment effectively inhibited nicastrin maturation beyond the level of the high-mannose type of glycosylation (Fig. 7C). This treatment did not inhibit Aβ production (Fig. 7C). In a similar experiment, we transfected these cells transiently with mNotchΔE and analyzed NICD generation (De Strooper et al., 1999). No differences between untreated or kifunensine-treated cells on NICD production could be observed (Fig. 7C). These data demonstrate that the maturation of the glycosyl residues on nicastrin is not required for γ-secretase activity.

Treatment with kifunensine also did not affect the transport of nicastrin to the cell surface as measured by cell-surface biotinylation (Fig. 7D). This indicates that the maturation of the glycosyl residues of nicastrin reflects in the first place its trafficking through the compartments of the biosynthetic pathway, and has little or no importance for its incorporation into the γ-secretase complex or its cell-surface localization.

**Discussion**

The major post-translational modification of nicastrin is glycosylation and we investigated here the functional relevance of this important modification. This question was particularly important given the dramatic effects of PS deficiency on nicastrin maturation that parallels the effects on γ-secretase activity, as was also observed by others (Edbauer et al., 2002; Leem et al., 2002). Deglycosylation of the 110 kDa immature nicastrin precursor form using endoH reduces its apparent Mr by about 40 kDa, indicating that several high-mannose oligosaccharide chains are added co-translationally to the core protein in the ER. These chains are modified in the Golgi apparatus by mannosidase I and II, and several glycosyltransferases to generate endoH-resistant complex oligosaccharide chains. The resulting mature nicastrin has an apparent Mr of 130 kDa. The glycosyl residues in this mature form can be removed with N-glycosidase F, yielding a protein core of about 70 kDa. Mature nicastrin is also sensitive to neuraminidase treatment and reacts with the lectin *Maackia amurensis* agglutinin (MAA) (Fig. 1B), indicating the presence of sialic acid residues. This implies that nicastrin must reach the trans-Golgi network, where sialylation occurs (Roth et al., 1985). We noticed that fully mature nicastrin remains partially sensitive to endoH treatment (Fig. 1A, lane 3), indicating that at least one of the oligosaccharide side-chains remains of the high-mannose type, which was confirmed with *Galanthus nivalis* agglutinin (GNA) (Fig. 1B). It is possible that nicastrin leaves the ER in a conformation that prevents access of mannosidase I to at least one of the oligosaccharide side chains. This assumption is further supported by the fact that mature nicastrin is partially resistant to N-glycosidase F and to endoH treatment at 37°C (A.H. and B.D.S., unpublished). Complete deglycosylation is only obtained after partially denaturation of nicastrin by heating the samples at 70°C in the presence of 0.1% SDS. Thus, at least one of the oligosaccharide chains in mature nicastrin is normally not accessible to enzymatic digestion. These results, first obtained in fibroblast cell lines,


**Fig. 7.γ-Secretase activity is not influenced by the glycosylation status of nicastrin.** (A) Cell-free γ-secretase assay using membranes from wild-type (WT) MEFs treated with MNJ. C−, negative control (substrate only); C+, positive control (solubilized γ-secretase from Hela cells); −s, solubilized γ-secretase from WT MEFs without substrate added. +s, substrate added. (B) Western blot analysis of the membranes used in panel A showing the absence of glycosylation maturation of nicastrin after MNJ treatment. (C) Cell-based γ-secretase assay. HEK cells stably expressing APP/Sw were transiently transfected with mNotchΔE or NICD and treated with kifunensine (1 μg/ml), a mannosidase I inhibitor resulting in inhibition of mature glycosylation of nicastrin. Aβ and NICD generation was analyzed as described. Note that inhibition of glycosylation of nicastrin does not inhibit the production of Aβ nor NICD. (D) Inhibition of glycosylation has no effect on surface expression of nicastrin. HEK293 cells were treated with kifunensine (1 μg/ml) and surface biotinylated after 72 hours kifunensine treatment. Biotinylated proteins were precipitated using streptavidin beads and immunoblotted with B59.2 (α-nicastrin). Western blot analysis of total cell extracts is shown as a control. Nicastrin is labeled in both treated and untreated cells, demonstrating that immature glycosylated nicastrin can reach the cell surface under these conditions.

were entirely confirmed with nicastrin analyzed in primary cultures of neurons. Interestingly, we detected almost no immature nicastrin in the wild-type neurons and, furthermore, immature and mature nicastrin have similar apparent Mᵣ in these cells, explaining why one band is observed in western blots of neurons, while two bands are seen with extracts from fibroblasts. We noticed that, upon overexpression, nicastrin becomes only glycosylated with high-mannose sugar chains (not shown) and suggests therefore that a rate-limiting step is involved in the maturation process of nicastrin. A similar rate-limiting step has been invoked for the stabilization and incorporation of PS into the γ-secretase complex (Thinakaran et al., 1996; Yu et al., 1998).

Since, under normal conditions, mainly mature nicastrin co-precipitates with PS1 (Fig. 2A), we investigated the possibility that glycosylation of nicastrin has direct functional implications for the activity of γ-secretase. However, we found normal γ-secretase activity towards Aβ and NICD generation when glycosylation of nicastrin was inhibited (Fig. 7). Therefore, the complex glycosylation of nicastrin is not directly needed for the γ-secretase processing of APP or Notch substrates. In previous work, we noticed that brefeldin A treatment of neurons, which fuses several components of the Golgi apparatus with the ER, is able to activate γ-secretase activity documented in the current work reinforces the hypothesis that passage of nicastrin and probably other components of the γ-secretase complex through the Golgi apparatus is a prerequisite for the maturation and activation of the protease. Thus, whereas the glycosylation of nicastrin is not a direct requisite for activity, it reflects faithfully the acquisition of one or more additional components/factors associated with the trafficking through the Golgi apparatus that makes the protease fully operational.

Previous work (Chung and Struhl, 2001; Goutte et al., 2002; Hu et al., 2002) showed that PS becomes destabilized in the absence of nicastrin. In the current work, we find an inverse relationship: PSs are needed for the trafficking and maturation of nicastrin. The effects caused by PS1 or PS2 deficiency on nicastrin maturation reflects very closely the effects on γ-secretase inhibition by the different PS-deficient genotypes. Thus, in PS1- and 2-deficient cells, which do not display γ-secretase activity, no glycosyl maturation of nicastrin was observed at all. In PS1-deficient cells that display a quantitatively important but not complete γ-secretase deficiency, a tiny fraction of nicastrin became complex glycosylated. Finally, in PS2-deficient cells that display an almost normal γ-secretase activity (Herreman et al., 1999; Herreman et al., 2000; Zhang et al., 2000), no changes in the glycosylation pattern of nicastrin compared with wild-type cells were observed. These observations, together with the profound effects of PS deficiency on nicastrin subcellular localization, underscore the possibility that nicastrin and PS travel, maturate and become stabilized together to constitute a functional unit displaying γ-secretase activity. It should be noted that a fraction of PS and nicastrin do not co-distribute in the cell (Fig. 5), perhaps reflecting dynamic changes in the stoichiometry of the proteins or indicating that both proteins could be involved in functions where they are not operating in one functional unit. Pulse-chase experiments and immunocytochemical data demonstrate that, in the absence of
PS, nicastrin remains largely caught in the ER. This suggests that nicastrin needs to associate with PS to leave the ER, or at least that PSs are involved in the transport of nicastrin out of the ER. Since only little or no immature nicastrin is associated with PS in wild-type cells, we suggest that nicastrin and PS rapidly leave the ER once they become associated and travel together towards the Golgi apparatus and perhaps to the cell surface. In the Golgi compartments, nicastrin undergoes post-translational modifications, but this maturation is not required for γ-secretase function nor for further trafficking to the cell surface, as convincingly demonstrated using the mannosidase I inhibitors kifunensine or MNJ (Fig. 7). Our biotinylation data unequivocally demonstrate the presence of mature nicastrin at the cell surface in wild-type cells. This could also indicate the presence of PSs at the cell surface (Ray et al., 1999a; Kaether et al., 2002). By contrast, several groups have demonstrated that the bulk of PS immunoreactivity is distributed in the ER, intermediate compartment and to a certain extent into the early Golgi (Annaert et al., 1999; Culvenor et al., 1997; Lah et al., 1997). Since γ-secretase activity is believed to occur mainly in endosomes, Golgi and at the cell surface, some discrepancy still exists between the subcellular distribution of PS, which is the putative catalytic subunit, and the γ-secretase activity, agreeing with the ‘spatial paradox’ (Annaert and De Strooper, 1999). The current data indicate that this spatial paradox is not absolute since small amounts of PS are apparently leaving the ER/intermediate compartment/cis-Golgi, as deduced from the appearance of nicastrin at the cell surface. Nevertheless, it should be stressed that this does not answer several questions. For example, what is the function of the bulk of PS in the ER or why are the PSs, containing the putative catalytic site of the complex, not functionally active in the ER (Cupers et al., 2001a; Maltese et al., 2001)?

Our data suggest that the PSs have, in addition to their role in γ-secretase activity, an important function in the trafficking and maturation of nicastrin. One could argue that the disturbances in nicastrin trafficking and maturation observed in PS-deficient cells does not necessarily imply such an intrinsic function of PS since it is not unusual that an oligomeric complex needs to be fully assembled before all subunits can travel through the biosynthetic pathway. By contrast, deficiency of PSs also affects the subcellular distribution of other proteins, such as APP and the Trk receptor (Naruse et al., 1998) and telencephalin (Annaert et al., 2001). Therefore, it becomes likely that PS has, in addition to its role as a γ-secretase catalytic subunit, an important function in subcellular trafficking of a selected group of proteins. This dual function could probably explain the large amounts of PSs in the early biosynthetic compartments having no role in proteolysis (Cupers et al., 2001a).

In conclusion, the current manuscript provides basic information regarding the cellular distribution of nicastrin, its post-translational modifications and their role in the generation of the PS/nicastrin complex, and the effect of PS deficiency on nicastrin biosynthesis and trafficking.

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