Mature Glycosylation and Trafficking of Nicastrin Modulate Its Binding to Presenilins*Ⓢ

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Nicastrin is an integral component of the high molecular weight presenilin complexes that control proteolytic processing of the amyloid precursor protein and Notch. We report here that nicastrin is most probably a type 1 transmembrane glycoprotein that is expressed at moderate levels in the brain and in cultured neurons. Immunofluorescence studies demonstrate that nicastrin is localized in the endoplasmic reticulum, Golgi, and a discrete population of vesicles. Glycosidase analyses reveal that endogenous nicastrin undergoes a conventional, trafficking-dependent maturation process. However, when highly expressed in transfected cells, there is a disproportionate accumulation of the endo-β-N-acetylglucosaminidase H-sensitive, immature form, with no significant increase in the levels of the fully mature species. Immunoprecipitation revealed that presenilin-1 interacts preferentially with mature nicastrin, suggesting that correct trafficking and co-localization of the presenilin complex components are essential for activity. These findings demonstrate that trafficking and post-translational modifications of nicastrin are tightly regulated processes that accompany the assembly of the active presenilin complexes that execute γ-secretase cleavage. These results also underscore the caveat that simple overexpression of nicastrin in transfected cells may result in the accumulation of large amounts of the immature protein, which is apparently unable to assemble into the active complexes capable of processing amyloid precursor protein and Notch.

Nicastrin is an integral membrane glycoprotein, a major binding partner for the presenilins, and a participant in the presenilin-dependent proteolytic processing of Notch and the amyloid precursor protein (APP)[1] (1, 2). Mutations in the presenilin-1 (PS1) and presenilin-2 (PS2) genes, which encode multispanning transmembrane proteins, cause early-onset familial Alzheimer disease (3, 4).

Because of the roles that the presenilins play in Notch processing during embryonic dorsal axis development and in the genesis of Alzheimer disease, their biology has been examined intensively. PS1 and PS2 are localized primarily within the endoplasmic reticulum and Golgi (5–8). The presenilin holoproteins undergo highly regulated endoproteolytic processing within their large cytoplasmic loop domains to yield N- and C-terminal fragments (NTFs and CTFs, respectively) (9–11). The presenilin NTFs and CTFs remain tightly associated as components of multimeric, high molecular weight complexes (12–15). Examination of these complexes has revealed that they contain additional protein elements, such as β/δ-catenins (16–18), which may be related to the developmental aspects of presenilin function (19). Nicastrin is also a major component of this complex and participates in the APP and Notch processing pathways (1, 2). This role has recently been confirmed using affinity resins specific for APP γ-secretase (20). Comparable activities were also observed both for the nicastrin homologue, *aph-2* (21, 22), in *Caenorhabditis elegans*, as well as for the *Drosophila* nicastrin (23–25). These results suggest that nicastrin plays an integral role in the regulated, intramembranous, and proteolytic events involved in processing APP, Notch, and possibly other transmembrane proteins.

The presenilin-nicastrin complexes are functionally necessary for an unusual form of proteolytic processing in which several membrane-bound proteins (including Notch and APP) are cleaved within their transmembrane domains (26–29). PS1 and PS2 facilitate the S3-site intramembranous cleavage of Notch that generates the Notch intracellular domain, which then acts as a transcriptional activator. PS1 and PS2 play similar roles in the γ-site cleavage of the membrane-bound, APP C-terminal fragments (APP-CTFs) produced by α- or β-secretase cleavage of the APP holoprotein. This intramembranous cleavage generates the amyloid-β (Aβ) peptide (30) and a labile C-terminal derivative (γ-stub), which may trans-

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[1] The abbreviations used are: APP, amyloid precursor protein; PS1, presenilin-1; Endo H, endo-β-N-acetylglucosaminidase H; PNGase, peptide N-glycosidase; NTFs, N-terminal fragments; CTFs, C-terminal fragments; Aβ, amyloid-β; MDCK, Madin-Darby canine kidney; ER, endoplasmic reticulum; Pipes, 1,4-piperazinediethanesulfonic acid; BiP, immunoglobulin heavy chain binding protein.

Nicastrin Distribution and Expression

locate to the nucleus (31–33). Loss of functional PS1 expression, either alone or in combination with PS2, results in severe Notch-like defects in embryonic dorsal axis development in both invertebrates and vertebrates (34–38). Similarly, absence of PS1 reduces Aβ secretion and causes the accumulation of APP-CTFs derived from α- and β-secretase-mediated cleavage (39). Currently, it is not clear how the presenilins mediate their effects on Notch and APP processing. Some experimental results have suggested that the presenilins may belong to a novel class of di-aspartyl proteases (15, 40–43). Other roles that have been postulated for presenilins include serving either as an adaptor/regulator of γ-secretase and S3 cleavages (39) or as a regulator of protein trafficking (7, 44). Indeed, *spe-4*, a distant member of the presenilin family in *C. elegans*, regulates shuttling of a Golgi-like organelle (45).

As would be predicted for a functional component of the presenilin complexes, nicastrin plays a similar role in these activities. RNA interference suppression of nicastrin expression in *C. elegans* causes an embryonic lethal *glp-1*-like phenotype similar to that induced by loss of both presenilin homologues (*sel-12* and *hop-1*) (1). This is consistent with the recent evidence of *aph-2* links to the *lin-12*/Notch pathway (22). Loss-of-function nicastrin mutations also suppress γ-secretase cleavage of APP and reduce Aβ secretion *in vitro* (1). Although nicastrin does display some sequence similarities to dipeptidases, no intrinsic enzymatic activity can be demonstrated, suggesting that nicastrin is not itself a protease (46).

Although much is known about the processing and intracellular localization of the presenilins, relatively little is known about the processing and intracellular localization of nicastrin. We have explored the subcellular localization and post-translational modification of nicastrin in several cells types, including primary neurons. We report here that nicastrin is expressed at higher levels in the brain than in other tissues. We also found that nicastrin undergoes a complex pattern of glycosylation and that it is the fully mature, glycosylated form that preferentially interacts with PS1. Only a small proportion of PS1 is bound to immature species, even when nicastrin is expressed at high levels in transfected cells. A loss-of-function deletion mutation in nicastrin, which inhibits its trafficking and glycosylation, reduces the PS1-nicastrin interactions. This suggests that proper trafficking/compartmentalizations of nicastrin and presenilins and/or specific patterns of glycosylation may be essential for the formation of functional γ-secretase complexes.

EXPERIMENTAL PROCEDURES

Nicastrin Expression Vectors—Wild-type and mutant (missense DYIGS-AAIGS and deletion) nicastrins, with and without V5 epitope tags, were prepared as described previously (1, 2). To examine compartment-specific oligosaccharide processing, wild-type and D336A/Y337A mutant nicastrin cDNAs were engineered by site-directed mutagenesis to express a C-terminal KKAA ER retention tag and ligated into pcDNA6/V5-HisA vector, using *Kpn*I and *Pme*I sites. The original expression vector was engineered to retain the V5 epitope at the C terminus of nicastrin, to which the di-lysine motif was attached. Retention of the V5 tag permitted the transfected protein to be immunoprecipitated and distinguished from its endogenous counterpart.

Cell Cultures and Immunofluorescence—Madin-Darby canine kidney (MDCK) and SHSY-5Y cells were cultured on collagen-coated glass coverslips in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (Invitrogen). Where required, cells were then transiently transfected with nicastrin using LipofectAMINE. SHSY-5Y cells were plated on uncoated glass coverslips and maintained in Dulbecco's modified Eagle's medium plus 10% FCS.

Primary cultures of cerebellar granule neurons were obtained from 6-day-old mice. Cells were plated on poly-D-lysine-coated glass coverslips and maintained in minimal essential medium supplemented with 10% fetal bovine serum, 0.5% glucose, and 25 mM KCl. Cytosine arabinoside (4 μM) was added at 18 h post-plating. Cultures were ~95%

neuronal (as indicated by immunostaining with neuron-specific enolase), and differentiated cells were examined at 7 days post-plating.

Immunofluorescence was performed as described (5, 47). Briefly, cells were washed with phosphate-buffered saline, fixed with 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.02% Triton X-100 (v/v) in Tris-buffered saline. Nicastrin immunoreactivity was detected using polyclonal antibodies raised either to the C-terminal domain at residues 691–709 or to a GST fusion protein containing residues 320–420 of human nicastrin. Double labeling for nicastrin and organelle marker proteins was achieved using mouse monoclonal antibodies directed to the following: BiP (an endoplasmic reticulum marker) (StressGen, Victoria, British Columbia, Canada); p58 (a Golgi marker) (Sigma); the PS1 N-terminal fragment (NT1) (48); or the APP C-terminal fragment (antibody C1/6.1) (49). To examine the orientation of nicastrin within the membrane, the plasma membrane was selectively permeabilized by incubation for 15 min at 4 °C in 10 mM Pipes buffer (pH 6.8) containing 0.3 M sucrose, 0.1 M KCl, 2.5 mM MgCl$_2$, 1 mM EDTA, and 5 μg/ml digitonin (5). Cells were further processed as described above and co-stained for ER-lumenal BiP to ensure that the intracellular membranes remained intact. Immunofluorescence was visualized with Cy3-conjugated goat anti-rabbit or fluorescein isothiocyanate-conjugated horse anti-mouse antisera (Vector, Burlingame, CA). Cells were mounted with Dako Fluorescent Mounting Medium, and images were collected using the Radiance2000 (Bio-Rad) confocal microscope.

Tissue Extraction and Protein Analysis—Total protein extracts from cultured cells and brain tissue were prepared by Triton X-100 solubilization, as described previously (13). Proteins were separated by SDS-PAGE on Tris glycine or Tricine gradient gels (NOVEX) and transferred to polyvinylidene difluoride membranes (13). Nicastrin-containing total protein extracts were examined for glycosylation patterns using Endo H and PNGase (New England Biolabs), as per the manufacturer's protocols.

Interactions between PS1 and the various forms of nicastrin were examined by immunoprecipitation and immunoblotting. Membrane preparations from cells expressing endogenous protein or transiently transfected V5-tagged nicastrin constructs were isolated and extracted with digitonin, as described (1). Fractionation of the endogenous PS1 complex from SHSY-5Y digitonin extracts was performed using glycerol gradients, as described (13). PS-containing complexes were precipitated using an antiserum directed toward PS1 residues 1–25 and subjected to SDS-PAGE. The presence of mature and immature nicastrin was examined using antisera directed to the C terminus of nicastrin.

Fractionation of Cellular Membranes and Organelles on Iodixanol Gradients—To examine the distribution of intracellular nicastrin, ER and Golgi-enriched fractions were obtained from HEK293 cells expressing endogenous and transfected nicastrin as indicated. These membrane fractions were homogenized in buffer containing 130 mM KCl, 25 mM NaCl, 1 mM EGTA, 25 mM Tris (pH 7.4), plus protease inhibitors. Extracts were centrifuged for 10 min at 1,000 × *g* to remove nuclear debris, and the supernatants were collected and re-centrifuged for 10 min at 3,000 × *g*. These extracts were then layered on a step gradient consisting of 30, 25, 20, 15, 12.5, 10, 7.5, 5, and 2.5% iodixanol (v/v) (Accurate Chemicals) in homogenization buffer and centrifuged at 126,000 × *g* for 30 min. Eleven fractions were collected from the top of the gradient, and the distribution of nicastrin was examined by Western blotting. The fractions were also examined for the presence of the following intracellular markers of the ER (BiP and calnexin; StressGen) and the Golgi (p59 and GM130; Sigma) to confirm the localization of the nicastrin constructs.

RESULTS AND DISCUSSION

Nicastrin Topology and Subcellular Localization—To assess the topology of nicastrin within cellular membranes, antibodies raised to different domains were used for immunofluorescence studies in several different cell types expressing either endogenous nicastrin or transiently overexpressed exogenous nicastrin. These cells were permeabilized with digitonin to selectively disrupt the plasma membrane and selectively expose cytoplasmic epitopes. Comparable cells were treated with Triton X-100 to disrupt all membranes, thereby exposing epitopes sequestered within the lumina of vesicles and organelles in addition to cytoplasmic epitopes.

Strong perinuclear staining was detected when antibodies to the C-terminal tail (residues 691–709) of nicastrin were used to
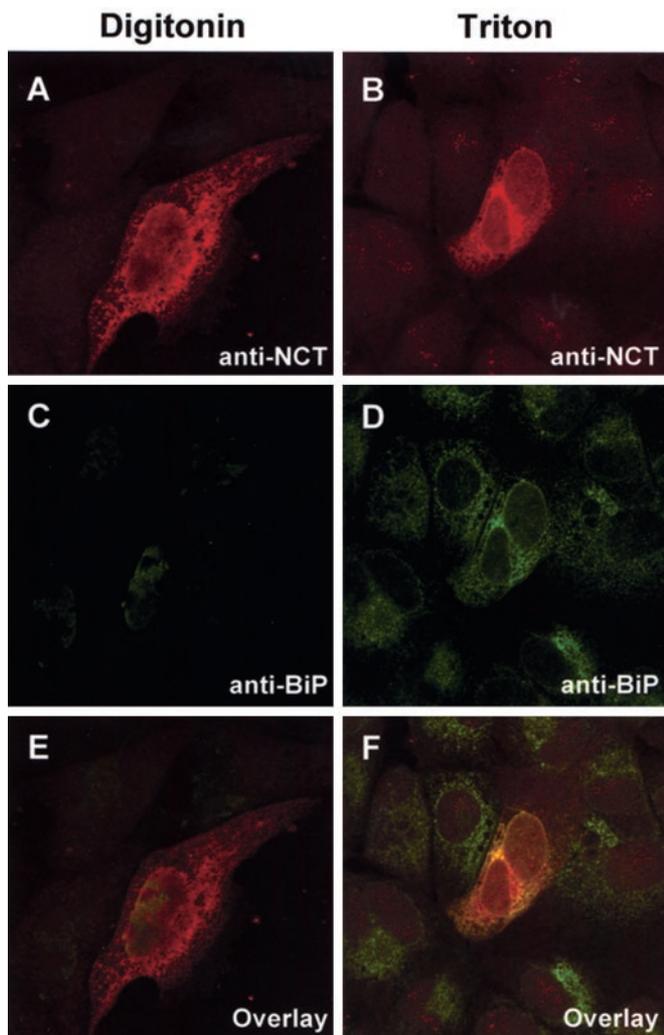
Digitonin

Triton

A anti-NCT

B anti-NCT

C anti-BiP

D anti-BiP

E Overlay

F Overlay

FIG. 1. **Topology of nicastrin.** Orientation of nicastrin was determined by immunofluorescence of semi-permeabilized cells. *A*, MDCK cells were treated with digitonin and stained with antibodies directed toward the C terminus of nicastrin. Similar staining was observed following complete permeabilization with Triton X-100 (*B*), suggesting that the nicastrin is a type 1 transmembrane protein with the C terminus in the cytoplasm. Staining for the lumenal ER marker, BiP, used a monoclonal antibody on the same cells treated with either digitonin (*C*) or Triton X-100 (*D*). *Overlays* show the nicastrin staining (*red*) and the BiP (*green*) immunoreactivity for digitonin (*E*) and Triton-treated cells (*F*).

investigate MDCK cells transfected with wild-type human nicastrin and permeabilized with digitonin (Fig. 1*A*). In regions more remote from the nucleus, the nicastrin-immunoreactive structures had a punctate appearance that may reflect either localization within a discrete vesicular compartment or the presence of clusters of membrane-bound nicastrin. In some cells, a small amount of nicastrin immunoreactivity localized to the nuclear envelope (PS1 immunoreactivity has a similar distribution) (17). All immunoreactivity was abolished when the antisera were preabsorbed with the appropriate antigen, and no immunoreactivity was detected by preimmune serum (not shown). To establish whether digitonin had selectively exposed the C terminus of nicastrin in the cytoplasm by permeabilizing only the plasmalemma, the cells were co-immunostained with an antibody to BiP, a protein resident in the ER lumen. No BiP immunoreactivity was detectable, confirming that digitonin had selectively exposed only epitopes oriented toward the cytoplasm (Fig. 1*C*; *overlay* image Fig. 1*E*). In contrast, upon permeabilization of all cellular membranes with Triton X-100,

intense immunoreactivity was observed for both nicastrin and BiP (Fig. 1, *B* and *D*; *overlay* image Fig. 1*F*). A cytoplasmic orientation for the C terminus was confirmed by independent experiments, in which identical results were obtained using anti-V5 antibodies to detect a C-terminal V5-tagged nicastrin in transfected cells. When an antibody to nicastrin residues 320–420 in the hydrophilic N terminus was used, no staining was detected following digitonin treatment, implying that the N terminus is located within the lumen of an intracellular compartment (data not shown). This result was supported by subsequent biochemical studies, which revealed that nicastrin undergoes *N*-linked glycosylation (see below).

These immunofluorescence results are consistent with the predicted type 1 transmembrane topology of nicastrin with a large ectodomain ($\sim$670 residues) and relatively short cytoplasmic tail of $\sim$20 residues (1). However, it should be noted that Kyte-Doolittle hydrophobicity plots reveal an additional hydrophobic domain that, in the mammalian nicastrins, encompassed residues 16–36. Despite the sequence divergence among the various species, this hydrophobic structure is maintained in all of the nicastrin sequences but does not conform to a typical signal peptide. In fact, in murine and *C. elegans* nicastrin, Kyte-Doolittle hydropathy plots predict this domain to be a second transmembrane helix. Furthermore, the presence of two charged residues (arginine 19 and 32) in the human sequence are atypical for a signal sequence and are more consistent with a membrane-associated domain than with a membrane-spanning helix. Initial investigations into the possible orientation of the nicastrin N terminus have run into difficulties. Antibodies raised to residues 1–25 of human nicastrin failed to recognize the endogenous protein but were cross-reactive with the peptide antigen (data not shown). Possible explanations for this finding are that the epitope may be blocked or may undergo some form of proteolytic processing. In addition, topological investigations using N-terminal Myc-tagged nicastrin indicated that the transfected protein was not expressed at high levels, which is consistent with its being an inherently unstable protein (data not shown). The conservation of this hydrophobic domain in nicastrin suggests that it may have some functional significance, possibly playing a role in membrane binding and/or insertion. However, the contributions that this domain may make to nicastrin function will require further investigation.

In our initial investigations, transfected nicastrin was shown to be localized primarily within perinuclear structures, including the ER and Golgi (1, 2). This distribution overlapped to a large extent with PS1, which has been shown in several cell types, including primary hippocampal neurons, to be concentrated in these compartments (50). To investigate further the subcellular localization of endogenous nicastrin and its distribution to various subcellular compartments, we examined mouse primary neuronal cultures. Nicastrin immunoreactivity in granule neurons was located primarily in the perinuclear region but extended into processes, where it often had a punctate appearance. In contrast to other cells, but in agreement with reports that nicastrin is constitutively expressed in neurons (51), staining was considerably higher in neurons than in any of the other cell types that were examined (Fig. 2*A*). Double immunostaining with antibodies to nicastrin and to the ER resident BiP protein displayed the expected perinuclear localization for BiP, but there was only modest overlap with nicastrin (Fig. 2*B*). More extensive co-localization was observed when cells were double-labeled for nicastrin and the Golgi marker, p58 (Fig. 2*C*; higher magnification of Golgi staining for endogenous and additional cells types are provided in the Supplemental Material). However, there remained a significant
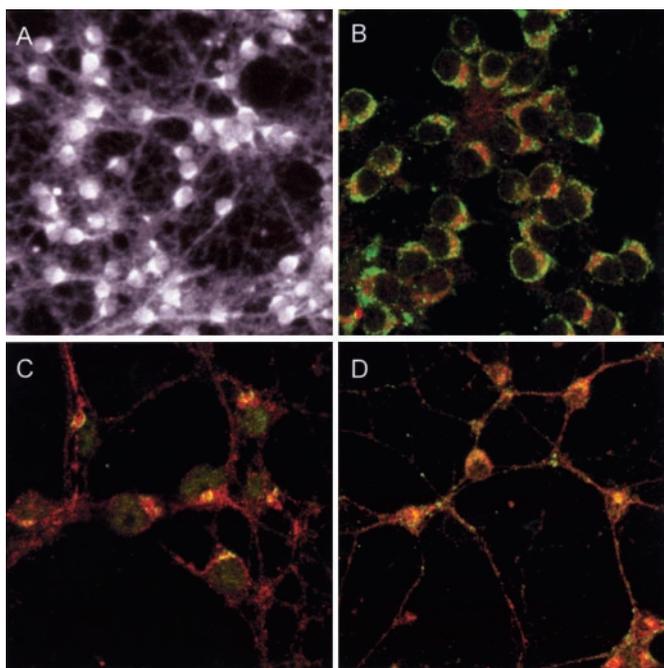
FIG. 2. **Expression and localization of nicastrin in neurons.** *A*, immunofluorescence staining for nicastrin in primary cultures of mouse granule neurons showing intense perinuclear staining and weaker immunoreactivity within neurites. Nicastrin (*red*) and the ER marker, BiP (*green*), show only modest overlap (*B*). *C*, nicastrin (*red*) and the Golgi marker p58 (*green*) show extensive overlap. Some nonspecific nuclear staining was observed with the p58 antibody. *D*, double labeling of similar neurons for nicastrin (*red*) and for APP, using the monoclonal antibody C1/6.1 directed toward the APP C-terminal tail (*green*).

FIG. 3. **Examining the maturation process of nicastrin.** Western blotting and glycosidase treatments of nicastrin in cellular and brain extracts are shown. Endogenous nicastrin expression in HEK293 cells of mature and immature forms was detected by affinity-purified polyclonal antibodies. Endo H and PNGase treatments show the sensitivity of the various bands to deglycosylation. Native, fully deglycosylated nicastrin migrates at ~80 kDa, which corresponds to the predicted molecular weight for the native protein (*left panel*). Similar patterns were observed in stably transfected cells except that a greater abundance of the immature form was recorded from transfected cells (*center panel*). Mature and immature forms observed in mouse brain extracts show that the fully mature form migrated at a slightly higher apparent molecular weight. Brain nicastrin also displayed a different sensitivity to Endo H treatment. However, treatment with PNGase produced a de-glycosylated form similar to that observed in cultured cells (*right panel*).

portion of nicastrin immunoreactivity within the cell body and in the processes that did not fully overlap with either ER or Golgi markers. These may correspond to compartments late in the secretory pathway, such as the trans-Golgi network or even endosomes. Attempts to identify these nicastrin-only structures with other subcellular protein markers (*e.g.* EEA1) were not successful (data not shown), and additional investigations as to the exact nature of these compartments will be necessary. Some of the nicastrin-positive membrane structures within the perinuclear region, proximal neurites, and more distal processes also stained for APP holoprotein and APP C-terminal fragments (using a monoclonal antibody to the APP C-terminal tail) (Fig. 2D). It has been established that APP co-localizes with trans-Golgi network markers (52). Other structures stained nicastrin. These results lead us to conclude that nicastrin is located in the Golgi and, to a lesser extent, in the ER.

A previous immunofluorescence study of the nicastrin *C. elegans* homologue, *aph-2*, indicated a cell surface localization that was enhanced at cell-cell junctions (21). This result raises the possibility that the invertebrate protein may be directed to the cell surface and may be involved in membrane adhesion and/or signaling mechanisms. However, no neuronal plasma membrane staining was detected in the present study of mammalian nicastrin, and preliminary cell surface labeling experiments have failed to detect nicastrin at the cell surface (data not shown). Although it is possible that, under certain circumstances, small amounts of nicastrin may be located near the cell surface, our data suggest that mammalian nicastrin is primarily confined to intracellular membranes.

Glycosylation of Nicastrin—The primary amino acid sequence of nicastrin contains 16 *N*-linked glycosylation consensus sequences, all within the hydrophilic, N-terminal domain. We have reported previously (1) that in cells overexpressing the wild-type protein nicastrin was detectable as an Endo H-sen-
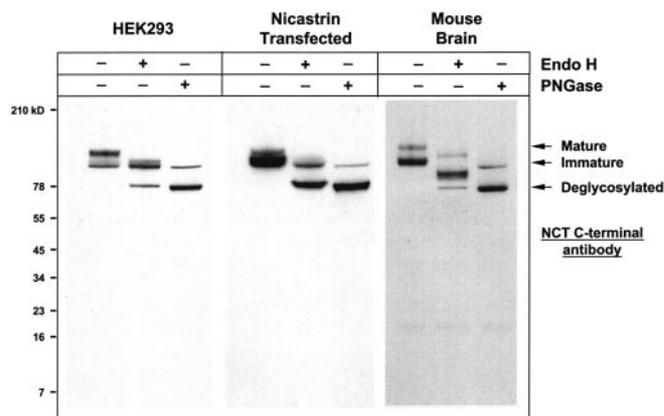
sitive doublet with estimated molecular masses of ~110–120 kDa. The slightly smaller species in this doublet was consistently present as the major band, whereas the higher molecular weight band was diffuse, less intense, and less consistently present. To resolve the origin and significance of these modified forms, we undertook a detailed analysis of the glycosylation of nicastrin using affinity-purified antibodies directed to the C-terminal cytoplasmic tail.

Western blotting of lysates from untransfected HEK293 and MDCK cells revealed two bands of approximately equal intensities migrating at ~120 and ~110 kDa (Fig. 3). Treatment with Endo-H resulted in a mobility shift of the fully mature (~120 kDa) glycosylated species, the loss of the ~120-kDa band, and the formation of a major band at ~110 kDa. This shows that the high molecular weight mature nicastrin has only limited sensitivity to Endo-H. In contrast, the lower molecular weight immature species seemed to be completely Endo-H-sensitive, as the appearance of a new minor band at ~80 kDa corresponds to the predicted weight of unglycosylated nicastrin (Fig. 3). The partial Endo-H sensitivity of the mature species suggests that nicastrin contains a mixture of both ER-derived high mannose residues and also Golgi-derived complex oligosaccharides. This is consistent with the immunofluorescence data that indicate that nicastrin is present in Golgi compartments. It would be expected that most or all of the glycosylation would be Endo-H-resistant. However, as described below, there are several precedents for oligosaccharide heterogeneity, particularly in heavily glycosylated proteins.

Previous investigations have demonstrated that the position of the *N*-linked glycosylation site can determine whether complex sugars are added, even for proteins that are translocated beyond the Golgi to the cell surface. For example, studies on the erythrocyte anion exchanger have revealed that repositioning of an *N*-linked glycosylation site determined whether glycosylation was Endo-H-sensitive or -resistant (53). This occurred irrespective of the fact that the mutant protein was correctly trafficked through the Golgi to the plasma membrane, as
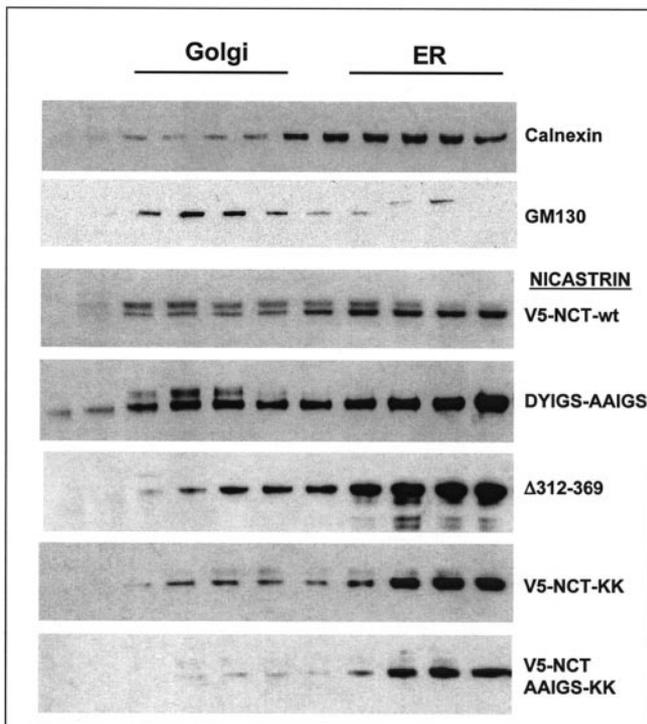
FIG. 4. **Subcellular fractionation of nicastrin and the effects of mutations.** Gradient fractionation of extracts from HEK293 cells that were transfected with wild-type and mutant nicastrins are shown. Maturation of wild-type nicastrin (untagged and C-terminal V5-tagged) from the endoplasmic reticulum to the Golgi was observed, as judged by co-fractionation of organelle markers, calnexin and GM130. Fractionation of the missense mutant nicastrin, DYIGS to AAIGS, revealed a distribution similar to that of the wild-type protein and a normal conversion to the higher molecular weight mature form. The deletion mutant, Δ312–369, displayed the largest change in trafficking with the majority of the protein remaining confined to the ER fractions. Only a small proportion of the deletion mutant was trafficked to the Golgi, and although the deleted sequence does not contain any glycosylation sites, no detectable mature form was observed. Similar ER retention was observed for wild-type and the AAIGS mutant containing C-terminal di-lysine (KK) motifs although some leakage was observed with the wild-type proteins.

FIG. 5. **Comparing the subcellular localization of wild-type and deletion mutants.** Immunofluorescence of MDCK cells transfected with wild-type nicastrin (*red*) show the ER marker BiP (*green*) and wide cytoplasmic distribution (*A*). Subcellular localization of the nicastrin Δ312–369 deletion mutant (*red*) and its almost exclusive localization to the ER are shown by complete overlap with BiP (*green*) staining (*B*).

shown by surface biotinylation. A similar event may occur with nicastrin. Although the majority of the glycosylation in the mature species is Endo-H-resistant, there are still some sites that do not contain complex oligosaccharides. Complex glycosylation of nicastrin was confirmed by PNGase treatment, which removes all carbohydrate chains. PNGase treatment generated a major ~80-kDa band (corresponding to deglycosylated native nicastrin) and a minor band at ~110 kDa. This minor band may reflect either incomplete deglycosylation or the presence of other post-translational modifications, such as phosphorylation, sulfation, and/or lipidation. However, preliminary experiments to investigate these possibilities have failed to detect incorporation of $^{32}PO_4$ or $^{35}SO_4$ transfected into nicastrin in HEK293 cells (data not shown).

In contrast to endogenous nicastrin in untransfected cells, HEK293 cells overexpressing transfected wild-type human nicastrin displayed a variable glycosylation pattern, with the predominant species being the completely Endo H-sensitive, immature ~110-kDa species (Fig. 3, *center panel*). PNGase treatment of these extracts gave a pattern similar to that of the endogenous proteins, except that the completely deglycosylated band was considerably more intense. These results suggest that much of the overexpressed exogenous nicastrin does not undergo complete maturation, and therefore may not be incorporated into functional presenilin-nicastrin complexes. This
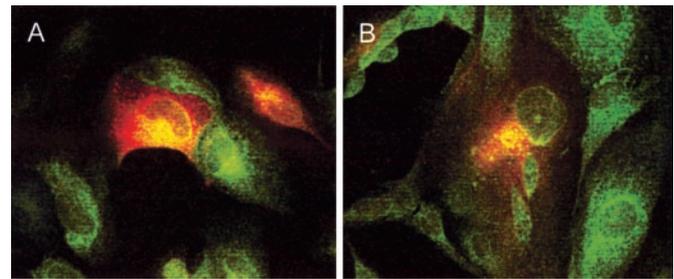
observation is significant because it suggests that exogenous nicastrin may be processed inefficiently and rather variably. As a result, the glycosylation state of transfected nicastrin will need to be considered for a correct interpretation of future studies using transfected exogenous nicastrin to investigate nicastrin function. The absence of any observable nicastrin-dependent effect may simply reflect incomplete glycosylation of the transfected nicastrin so that only the biologically inactive immature species accumulates.

Western blot analysis of homogenates from mouse brain or primary neuronal cultures revealed the presence of both mature and immature forms of nicastrin (Fig. 3, *right panel*). However, overall levels of endogenous brain and neuronal nicastrin are considerably higher than in HEK293 or MDCK cells when the relative intensities of immunoblot bonds from samples containing similar amounts of total protein are compared. Furthermore, when compared with nicastrin processing in other cell types, the brain-derived, mature, and glycosylated form of nicastrin had both a higher apparent molecular weight (~125 kDa compared with ~120 kDa) and a slightly different pattern of sensitivity to glycosidases. Endo-H digestion of brain homogenates generated a major band at ~90 kDa (which was not observed in nicastrin derived from cultured cells) and a minor band with a molecular mass of ~115 kDa (as compared with ~110 kDa in HEK293 cell lines). PNGase treatment of brain lysates resulted in a nicastrin band pattern similar to that observed in HEK293 cells, with a predominant ~80-kDa, fully deglycosylated species and a minor ~110-kDa band. These subtle differences in Endo-H sensitivity raise the possibility that nicastrin may undergo cell type-specific processing glycosylation.

To investigate the intracellular distribution of the different glycosylated forms of nicastrin, cells were transfected with either wild-type nicastrin or one of several mutant proteins. Lysates of these cells were examined by gradient fractionation and indirect immunofluorescence (Fig. 4). The subcellular compartments within these fractions were determined by staining with antibodies to marker proteins for the ER (BiP and calnexin) and Golgi-derived GM130 (1, 2). Wild-type nicastrin (native and V5-tagged proteins) underwent a typical pattern of ER-to-Golgi maturation with the majority of the mature species localized to the Golgi (Fig. 4, *third panel from the top*). Investigation of a double missense mutation (DYIGS to AAIGS), which has been shown to enhance APP processing, indicated that this mutant nicastrin undergoes a trafficking and glycosylation in a pattern comparable with that of the wild-type protein (Fig. 4). Localization of the missense mutant was confirmed by immunofluorescence in MDCK cells transfected with an untagged AAIGS construct (Fig. 5*A*). Probing with antibodies directed to the C terminus of nicastrin or while
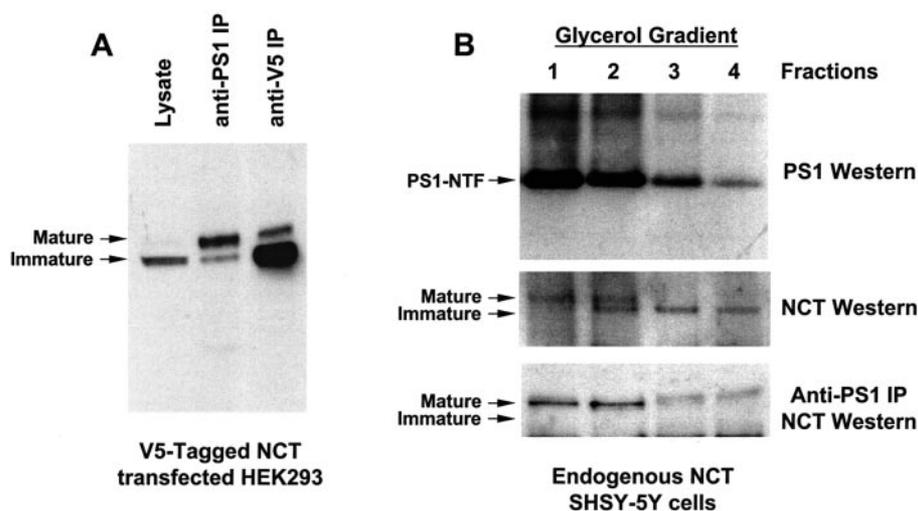
FIG. 6. **Specific interaction of PS1 with mature form of nicastrin.** Specificity of presenilin-1 interactions with nicastrin. HEK293 cells stably expressing a V5-tagged wild-type human nicastrin (*NCT*). Western blotting with an anti-V5 antibody indicated that the immature form was the major species, and very little mature nicastrin was observed on standard exposures (*A*, *lysate*). Immunoprecipitation with an anti-PS1 antibody followed by Western blotting for V5-tagged nicastrin revealed that a greater proportion of the mature form was recovered suggesting a preferential association of PS1 with the fully mature nicastrin (*A*, *anti-PS1 IP*). This is contrasted by immunoprecipitation with anti-V5 antibodies that resulted in recovery of an overwhelming amount of immature nicastrin (*A*, *anti-V5 IP*). Similar examination of the endogenous proteins was performed using SH-SY5Y neuroblastomas that express both immature and mature nicastrin (*B*). Digitonin extracts were fractionated on a glycerol gradient to semi-purify the PS1 complex which displayed a significant overlap with nicastrin (*B*, *top/middle panels*). Immunoprecipitation of all fractions with an anti-PS1 antibody followed by Western blotting for nicastrin revealed that only the fully mature form of nicastrin was associated with PS1 (*B*, *bottom panel*).
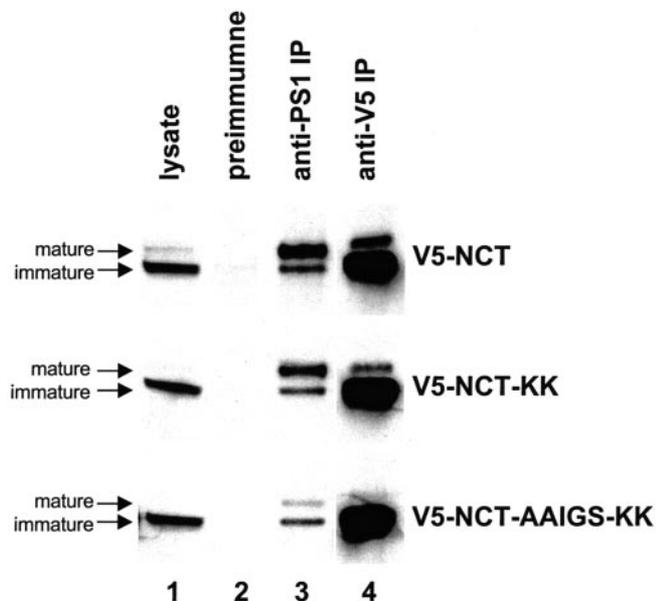

FIG. 7. **Effects of ER retention mutants on PS1-nicastrin interactions.** Co-immunoprecipitation of V5-tagged nicastrin containing ER retention di-lysine (KK) motifs. Levels of mature and immature nicastrin in total lysates of HEK293 cells stably expressing wild-type (*V5-NCT*), di-lysine nicastrin (*V5-NCT-KK*), and missense mutation (*V5-NCT-AAIGS-KK*) contained a C-terminal KK sequence (*lane 1*). No precipitated proteins were observed with preimmune serum (*lane 2*). Immunoprecipitation with an anti-PS1 antibody and probing for nicastrin revealed the specificity of the interaction for wild-type and a reduction of co-precipitating PS1 upon retention of nicastrin in the ER for both di-lysine mutants (*lane 3*). Immunoprecipitation with anti-V5 antibodies and probing for nicastrin indicated the predominance of the immature form for all cells examined (*lane 4*).

counterstaining with a BiP monoclonal revealed a wide distribution (*i.e.* Golgi and ER), consistent with the fractionation data.

In contrast to wild-type and the missense mutant protein, iodixanol fractionation of a nicastrin deletion mutant (Δ312–369) indicated that this species was localized almost exclu-

sively to the ER and that little of the higher molecular weight mature species was observed (Fig. 4, *bottom two panels*). Similar KK mutants of APP are concentrated within the ER and have been effective tools for investigating the role of trafficking (54–55) in APP maturation. ER retention of the nicastrin Δ312–369 mutant was confirmed by immunofluorescence, which indicated a complete overlap with BiP staining and no appreciable staining beyond the ER compartment (Fig. 5*B*). This deletion mutant has been shown previously to decrease the production of Aβ (1). This effect may be due to the fact that the Δ312–369 nicastrin mutant protein is not properly glycosylated and trafficked to the Golgi, where mature nicastrin interacts with PS1 and formation of a functional complex (see below).

To examine the maturation process further, ER retention mutants containing C-terminal di-lysine (KK) motifs were transfected into HEK293 cells. Gradient fractionation of a V5-tagged, wild-type nicastrin containing a KK motif indicated that it was retained primarily in the ER (Fig. 4). However, a very weak high molecular weight band, corresponding to the predicted mature form, was observed. This suggests some leakage of the system. Transfection of a comparable AAIGS-KK mutant revealed that it was largely confined to the ER fractions and was poorly trafficked to the Golgi (Fig. 4, *bottom panel*). These findings were confirmed by immunofluorescence with V5-specific monoclonal antibodies (data not shown). These observations suggest that normal shuttling of nicastrin to the Golgi compartment is required for formation of the mature glycosylated species.

Specificity of the Nicastrin Interaction with Presenilins—To determine whether the presenilin-nicastrin complex might be influenced by glycosylation of nicastrin, we examined the interaction of PS1 with mature and immature nicastrin in transfected cells expressing high levels of V5-tagged or endogenous wild-type nicastrin. PS1 was immunoprecipitated with an N-terminal antibody, and the level of co-precipitated nicastrin (mature and immature) was determined by Western blotting with polyclonal anti-nicastrin antibodies. Western blotting of HEK293 cells stably expressing a C-terminal, V5-tagged nicas-

trin revealed the immature form primarily and barely detectable levels of the mature protein (Fig. 6). An anti-V5 immunoprecipitation of an identical cell extract further illustrated the disproportionate amount of the immature form of nicastrin that is present in these cells. Although immature nicastrin is the major species expressed in transfected cells (>10-fold), only a very small proportion of the immature form co-precipitated with PS1 (Fig. 6*A*). In contrast, a much higher fraction of mature, glycosylated nicastrin co-immunoprecipitated with PS1 even though the mature glycosylated forms of nicastrin were less abundant within the cells.

An identical result was observed when the interaction between nicastrin and PS1 was assessed in native SHSY-5Y neuroblastoma cells expressing endogenous nicastrin and PS1. Digitonin extracts were fractionated on a glycerol gradient to examine the extent to which nicastrin distribution overlaps with that of the high molecular weight PS1 complex. From a total of 10 fractions, the PS1 NTF and CTF were contained within four major fractions that coincided with nicastrin (Fig. 5*B*). A tendency toward preferential distribution of the mature nicastrin to the higher molecular weight fractions was observed (fractions 1 and 2). PS1 was immunoprecipitated from all samples, and the amount and type of nicastrin were assessed by Western blotting. Although levels of mature and immature protein appeared similar, as determined by Western blotting, virtually no immature nicastrin co-precipitated with PS1 (Fig. 6*B*, *bottom panel*). Even in fractions wherein the immature form predominated (*i.e. fractions 3* and *4*), no detectable endogenous immature form could be co-precipitated with PS1.

The specificity of the interaction between nicastrin and PS1 was investigated further with the ER-retained di-lysine (KK) mutants. Cells transfected with V5-tagged wild-type nicastrin contained predominantly immature protein in Western blots of total lysates (Fig. 7). This was confirmed by immunoprecipitation with V5-specific antibodies and probing for nicastrin. According to this protocol, the higher molecular weight mature nicastrin was preferentially associated with PS1. Examination of wild-type nicastrin containing the C-terminal KK motif revealed the expected reduction of the higher molecular weight species (Fig. 7). However, what high molecular weight KK-tagged nicastrin was present displayed a specificity of interaction with PS1 similar to that observed for wild-type nicastrin. Examination of an AAIGS-KK mutant by gradient fractionation revealed a high level of expression but almost no detectable mature species. Despite the high level of expression, only a minor amount of nicastrin could be co-immunoprecipitated with PS1 (Fig. 7). These observations further support the contention that normal trafficking of nicastrin to the Golgi, as shown by its glycosylation pattern, is essential for the formation of a stable complex with PS1.

There are two potential interpretations for the preferential interaction of PS1 with the mature, glycosylated form of nicastrin. One possibility is that the interaction is directly due to the glycosylation state of the mature form, which alters nicastrin structure and facilitates binding to PS1. However, it is more likely that the maturation process is simply a marker of trafficking and is, therefore, an indicator of movement of nicastrin past the ER and to compartments where it can assemble with PS1 to produce a functional complex. Evidence to support the hypothesis that the trafficking of nicastrin is functionally important is provided by deletion mutants within the conserved N-terminal hydrophilic domain (*e.g.* residues 312–369). These deletions remove a conserved domain containing the amino acid sequence motif DYIGS, significantly altering the ability of the protein to interact with PS1 (1) without affecting any of the glycosylation sites within nicastrin. Although these nicastrin

deletion mutations do not directly modify or delete glycosylation sites, they do significantly reduce both the amount of nicastrin that is trafficked to the Golgi and the amount of nicastrin that undergoes mature glycosylation (Fig. 4). In contrast, cells transfected with wild-type nicastrin or with the DYIGS to AAIGS missense mutant underwent significant trafficking and maturation of the exogenous nicastrin within the Golgi compartments (Fig. 4). These observations indicate that nicastrin must be shuttled beyond the ER into the later stages of the secretory pathway before it can associate with PS1, and possibly other proteins, to form a functional complex.

These observations have several implications for understanding the role of nicastrin in APP and Notch processing. One primary conclusion is that PS1 displays preferential binding to the mature form of nicastrin that is produced during ER to Golgi trafficking. Post-translational events are likely to be functionally important because nicastrin deletion mutants, which fail to undergo mature glycosylation and association with PS1, cause loss of Aβ production. A secondary conclusion is that important, cell type-specific differences in the type and pattern of glycosylation are likely, because brain-derived nicastrin shows different molecular weights and different sensitivity to glycosidases. Finally, cellular systems that are used to examine nicastrin function must be monitored carefully both for the maturation and proper trafficking of any transfected nicastrin constructs as well as for displacement of endogenous forms by the transfected proteins. Unless the exogenous nicastrin has a dominant negative effect (like the $\Delta312-369$ mutation), in the absence of normal trafficking and glycosylation of the transfected proteins, no functional effect of the protein may be discerned.

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