Glu<sup>332</sup> in the Nicastrin Ectodomain Is Essential for γ-Secretase Complex Maturation but Not for Its Activity<br>Received for publication, April 21, 2008. Published, JBC Papers in Press, May 23, 2008, DOI 10.1074/jbc.M803040200

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The γ-secretase complex is responsible for the proteolysis of integral membrane proteins. Nicastrin has been proposed to operate as the substrate receptor of the complex with the glutamate 332 (Glu<sup>332</sup> in human) serving as the anionic binding site for the α-amino-terminal group of substrates. The putative binding site is located within the aminopeptidase-like domain of Nicastrin. The Glu<sup>332</sup> is proposed to function as the counterpart of the exopeptidase Glu located in the active site of these peptidases. Although Glu<sup>332</sup> could bind the α-amino-terminal group of substrates, we hypothesized, in analogy with M28-aminopeptidases, that other residues in the putative binding site of Nicastrin should participate in the interaction as well. Surprisingly, mutagenesis of these residues affected the in vivo processing of APP and Notch substrates only weakly. In addition, the E332Q mutation, which completely abolishes the anionic α-amino-terminal binding function, remained fully active. When we introduced the previously characterized E332A mutation, we found strongly decreased γ-secretase complex levels, but the remaining complex appeared as active as the wild-type complex. We confirmed in two independent in vitro assays that the specific enzymatic activity of the E332A mutant was comparable with that of the wild-type complex. Thus, Glu<sup>332</sup> crucially affects complex maturation rather than substrate recognition. Moreover, other Nicastrin mutants, designed to either impede or alter substantially the putative binding pocket, affected only marginally γ-secretase activity. Consequently, these studies indicate that the main role of the Glu<sup>332</sup> is in the maturation and assembly of γ-secretase rather than in the recognition of the substrates.

The γ-secretase is a multimeric membrane complex with aspartyl proteolytic activity. In contrast to other intramembrane proteases, the γ-secretase cleaves type I transmembrane proteins with broad specificity (1, 2). The size of the ectodomain determines whether a type I membrane protein is a γ-secretase substrate (3). In addition to other substrates, this protease complex cleaves the COOH-terminal 99-amino acid fragment of the amyloid precursor protein (APP) within the membrane at different positions, to release the COOH-terminal intracellular domain (AICD) and COOH-terminal heterogeneous amyloid-β peptides (Aβ) (4, 5). Aβ peptides are the major component of amyloid deposits in the brains of patients with Alzheimer disease, and the longer Aβ42 peptide generation appears to be crucial in the Alzheimer amyloid cascade (6, 7). Therefore, reduction of Aβ42 by specific inhibition of one of the key enzymes involved in its generation, the β- or the γ-secretase, is highly desirable.

The γ-secretase complex is formed by presenilin (Ps), Nicastrin (Nct), anterior pharynx defective (Aph1), and presenilin enhancer-2 (Pen-2) (4, 8–11). Ps is the catalytic subunit (reviewed in Ref. 12) and Nct, a type I transmembrane protein with a large and highly glycosylated ectodomain (Nct-ECD), has been implicated in the initial recognition of substrates (13). Aph1 and Pen-2 are multipass transmembrane proteins that have been proposed to play a structural role in the assembly and maturation of the complex (14, 15). Although the sequence of events preceding full assembly and activation of the protease complex is controversial, the current hypothesis suggests that formation of the Nct-Aph1 subcomplex is the first step in γ-secretase assembly, followed by sequential incorporation of the Ps and Pen-2 subunits, respectively, or alternatively, incorporation of the Ps-Pen-2 subcomplex (16–19).

Nct homology with the M28 aminopeptidase superfamily initially led to speculation that the proposed Nct aminopeptidase (AP)-like domain might possess catalytic activity or could serve as a binding domain for substrates (20). More recently, demonstration that Nct acts as a substrate receptor (13) and is rate-limiting for access to the protease active site, suggests that this function could be exploited as a therapeutic target for Alzheimer disease.

According to the prevailing Nct receptor model, the initial binding of substrates to the γ-secretase complex crucially depends on a salt interaction between human Nct-E333 (Glu<sup>332</sup> in mouse) and the α-amino-terminal group of the substrate.

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The abbreviations used are: APP, amyloid precursor protein; Aβ, amyloid β peptide; AICD, APP intracellular domain; AP-like domain, aminopeptidase-like domain; CTF, COOH-terminal fragment; Nct<sup>−/−</sup> cells, Nicastrin knockout cells; MEF, mouse embryonic fibroblasts; NCT, Nicastrin; NICD, Notch intracellular domain; Ps, presenilin; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxyethyl)propane-1,3-diol; MOPS, 4-morpholinepropanesulfonic acid; CHAPS, 3-[[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; WT, wild type.
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which leads to translocation and docking of the substrate in the catalytic core (13). This model proposes that Nct-Glu\(^{333}\) is the counterpart of the Glu residue present within the exopeptidase motif of APs (21, 22), which is essential for the binding of the \(\alpha\)-amino-terminal group of substrates and inhibitors. Furthermore, the model suggests that apart from Glu\(^{333}\) no other key structural determinants are involved in the Nct recognition mechanism, explaining the broad specificity of the \(\gamma\)-secretase. However, no further quantitative data are available to support this assumption.

Indeed, functional and structural analysis of aminopeptidases, such as acidic and arginyl aminopeptidase LTA4H, has demonstrated that other residues in addition to the exopeptidase Glu are involved in substrate recognition (23, 24). In particular, this has been demonstrated for the Streptomyces griseus aminopeptidase, one of the most similar counterparts of Nct (25). The sequence similarity between Nct and the M28 enzymes and the conserved functional role of Nct-Glu\(^{332}\) (exopeptidase Glu in aminopeptidases) suggests a common substrate binding mode for aminopeptidases and this \(\gamma\)-secretase subunit. Thus, we hypothesized that additional residues in the Nct AP-like domain, as in the aminopeptidases, participate in the interaction with the substrates. To further characterize the contribution of Nct to \(\gamma\)-secretase function, we constructed a structural model of the AP-like domain of Nct, and then used this model to identify critical residues within the putative substrate binding site that may play a role in substrate recognition.

Our results demonstrate that the mouse Nct-E332A and E332Q mutants strongly impair complex assembly but not complex activity as assessed by their specific activities, indicating that the substrate recognition/binding mechanism is mainly unaffected by these substitutions. Furthermore, additional mutations in the putative binding pocket altered \(\gamma\)-secretase complex levels but not its specific activity, supporting the crucial involvement of this domain in the maturation and proper assembly of the \(\gamma\)-secretase complex.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Polyclonal antibodies against mouse Ps1 NTF (B19.3), APP11a (B80.3), PEN-2 (B126.1), and APP COOH terminus (B63.3) and monoclonal 9C3 against the COOH terminus of Nicastrin have been described (26, 27). Other antibodies purchased were: anti-NICD (cleaved Notch1 Val\(^{1744}\)) from Cell Signaling, anti-Myc (A-14, Santa Cruz Biotechnology), and MAB5232 against Ps1 CTF (Chemicon).

**Generation of Stable Nct Cell Lines**—MEF derived from Nct\(^{-/-}\) mice (28) and immortalized by large T antigen transfection were maintained in Dulbecco’s modified Eagle’s medium/F-12 containing 10% fetal bovine serum. Nct\(^{-/-}\) MEFs were transduced using a replication-defective recombinant retroviral expression system (Clontech) with either wild-type or mutant mouse Nct. Stable Nct cell lines were selected with puromycin (5 \(\mu\)g/ml).

Transduction with APP Adenovirus and Enzyme-linked Immunosorbent Assay—MEF cell lines were transduced with the recombinant adenovirus Ad5/CVM-APP bearing human APP-695 (29) as previously described (30). Briefly, medium was refreshed after transduction and cultures were kept for 24 h.

The conditioned medium was collected, cleared by centrifugation, and assayed for the production of A\(\beta\)40 and A\(\beta\)42 by specific ELISA kits (The Genetics Company) according to the manufacturer’s protocol. Samples were also used for determination of the amount of secreted APP fragments (APPs) by SDS-PAGE and Western blotting with the polyclonal antibody 22C11 (Chemicon).

**Analysis of Notch Processing**—Stable MEF cell lines were infected with the Ad5/dE1E2a/CMV Myc-tagged Notch \(\Delta E\) adenovirus (31) for 24 h. Cell cultures were treated with the proteasomal inhibitor lactacystin (Calbiochem) for 4 h and cell extracts were prepared. 12 \(\mu\)g of total protein were loaded onto a 4–12% BisTris gel (Invitrogen), electrophoresed in MOPS Running Buffer (Invitrogen), and transferred to nitrocellulose membranes. NICD levels were determined with a cleavage specific antibody (cleaved Notch1 Val\(^{1744}\)) and quantified relative to the levels of Notch \(\Delta E\) using an anti-Myc antibody.

**Blue Native Gel Electrophoresis**—This method was performed as described previously (32), with the exception that the samples (5 \(\mu\)g) were separated on a 5–16% polyacrylamide gradient at a constant voltage of 200 V for 4 h.

**Cell Surface Biotinylation**—This method was performed as described previously (33).

**Expression and Purification of APP C99-3xFLAG—**COS1 cells were transiently transfected with pSG5-C99-3xFLAG vector (34). Before collection, cells were treated overnight with 10 \(\mu\)M GM6001 inhibitor (Sigma) to avoid C83-3xFLAG generation. Cells were harvested and resuspended in 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, complete protease inhibitor mixture (Roche) and incubated on ice for 1 h. Membrane-solubilized protein fractions were obtained by ultracentrifugation at 245,000 \(\times\) g for 20 min. Immunoaffinity purification was carried out with the anti-FLAG M2-agarose beads (Sigma), according to the manufacturer’s protocols. APP C99-3xFLAG was eluted in 100 mM glycine HCl, pH 2.7, 0.25% N-dodecyl \(\beta\)-D-maltoside (Sigma) and immediately neutralized to pH 7 by the addition of Tris-HCl, pH 8.0.

**In Vitro Activity Assay Using Solubilized \(\gamma\)-Secretase—**In vitro assay was done as previously described (35) with minor modifications. Briefly, MEF microsomal fractions (10 mg/ml) were solubilized in 1% CHAPSO buffer (50 mM PIPES, pH 7.0, 0.25 mM sucrose, 1 mM EGTA, 1X Complete protease inhibitor mixture (Roche)) and incubated on ice for 1 h. Afterward, membrane-solubilized protein fractions were obtained by ultracentrifugation at 100,000 \(\times\) g for 1 h. 3–4 \(\mu\)g were used in the in vitro assay or 10 \(\mu\)g were analyzed by Western immunoblot with Ps1 CTF and Pen-2-specific antibodies. In vitro reactions with 0.8 \(\mu\)M APP C99-3xFLAG (in 20 \(\mu\)l final volume) were carried out in 50 mM PIPES, pH 7.0, 0.25 mM sucrose, 1 mM EGTA, 1X EDTA-free Complete protease inhibitors (Roche), 2.5% DMSO, 0.1% phosphatidylcholine, and 0.0125% phosphatidylethanolamine (N\(\left[\mathrm{N}^\text{-}ight]-(3,5	ext{-difluorophenacetyl-L-alanyl})\text{-}l\text{-alanyl})\text{-}(s\text{-)phenylglycine t-butyl ester at 37 °C}.

**In Vitro Activity Assays Using Microsomal Preparations—**MEF cell lines were transiently transfected with an expression vector for C99-3xFLAG using Lipofectamine Plus reagent (Invitrogen) and collected 48 h post-transfection. Before collection, cultures were treated overnight with 5 \(\mu\)M inhibitor IX

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A STRUCTURE-SEQUENCE ALIGNMENT

![Structure-Sequence Alignment Diagram]

B NCT sequences alignment

| Species       | Sequence                        |
|---------------|---------------------------------|
| Mouse         | AATRLDSRSF...AESAVA...FQGETFDIGSS...SFVELQVA...QALPPSSLQRF5LAR...RYQ |  |
| Human         | AATRLDSRSF...AESAVA...FQGETFDIGSS...SFVELQVA...QALPPSSLQRF5LAR...RYQ |  |
| Rat           | AATRLDSRSF...AESAVA...FQGETFDIGSS...SFVELQVA...QALPPSSLQRF5LAR...RYQ |  |
| Chicken       | VTRIDEHSPF...ABSSVS...FQGETFDIGSS...SFVELQVA...QALPPSSLQRF5LAR...RYQ |  |
| Fruit Fly     | VTRIDEHSPF...ABSSVS...FQGETFDIGSS...SFVELQVA...QALPPSSLQRF5LAR...RYQ |  |
| Marathon Bug  | VTRIDEHSPF...ABSSVS...FQGETFDIGSS...SFVELQVA...QALPPSSLQRF5LAR...RYQ |  |
| Zebra F.      | VTRIDEHSPF...ABSSVS...FQGETFDIGSS...SFVELQVA...QALPPSSLQRF5LAR...RYQ |  |
| Pugu          | VTRIDEHSPF...ABSSVS...FQGETFDIGSS...SFVELQVA...QALPPSSLQRF5LAR...RYQ |  |
| C. elegans    | VTRIDEHSPF...ABSSVS...FQGETFDIGSS...SFVELQVA...QALPPSSLQRF5LAR...RYQ |  |
| ARATH         | VTRIDEHSPF...ABSSVS...FQGETFDIGSS...SFVELQVA...QALPPSSLQRF5LAR...RYQ |  |

(N-[N-(3,5-difluorophenacetyl-l-alanyl)-(L)-phénylglycine t-butyl ester). Membranes corresponding to one-fourth of a 75-cm² flask were resuspended in 25 µl of 50 mM PIPES, pH 7, 0.25 mM sucrose, 1 mM EGTA and incubated on ice or at 37 °C, followed by ultracentrifugation. AICD amounts were estimated from supernatants.

**AICD Quantification and Normalization**—Unless indicated otherwise, AICD product was analyzed after 4 h incubation at 37 °C. Lipids and substrate were extracted with chloroform/methanol (2:1, v/v) and AICD determined by semi-quantitative Western blot using the anti-FLAG M2 antibody from Sigma and IR detection at 800 nm using the Odyssey Infrared Imaging System. For specific activities AICD was normalized against Ps1 CTF and PEN-2 present in the reactions.

**Trypsin-proteolytic Digestion Assays**—0.5% n-dodecyl β-D-maltoside-extracted membrane proteins MEFs (see “Microsomal Preparations”) were incubated with increasing concentrations of trypsin (0–100 µg/ml) in 25 mM BisTris/HCl, pH 7.0, 150 mM NaCl, 5 mM EDTA, for 30 min at 30 °C. Proteolysis was stopped with addition of SDS-PAGE sample buffer and samples were analyzed by Western blot with a NCT-specific antibody.

All the experiments in this paper were performed at least three times in duplicates or triplicates. The GraphPad Prism 4 software was used for statistical analysis and graphing.

**RESULTS AND DISCUSSION**

**Identification of Critical Amino Acid Residues in the Aminopeptidase Fold of Nicastrin**—To identify critical residues that contribute to the proposed substrate binding by Nct, and in the absence of structural information, we utilized the similarity between Nct and M28 enzymes, with a known crystal structure, to construct a three-dimensional model of the peptidase-like domain of Nct. In accordance with the literature (20), tertiary structure prediction software like Phyre (36), GenTHREADER (37, 38), and 3D-PSSM (39) identified members of the M28 superfamily as proteins that share structural homology with Nct. The aminopeptidase of S. griseus (SGAP) (40) and the human glutaminyl-peptide cyclotransferase (41), two homologous structures, were selected as
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TABLE 1
Selection of residues for site-directed mutagenesis

| S. griseus leucyl aminopeptidase | Human glutaminyl cyclase | Analogous positions in mNCT | Residues conservation | Mutants |
|---------------------------------|-------------------------|-----------------------------|-----------------------|--------|
| His285                          | Asp284                  | Asp280                      | Ser in plants         | Ala    |
| Asp287                          | Asp286                  | Asp282                      | Ala in insects        | Gly    |
| Glu318                          | Glu320                  | Glu316                      | Ass in chinchin, Glu in C. elegans | Asn    |
| Thr332                          | Thr335                  | Thr331                      | Ala in fish/plants, Ser in insects | Ala    |
| Asp363                          | Asp365                  | Asp361                      | Ala                   | Ala    |
| Arg431                          | Arg432                  | Arg431                      | NOT in the pocket     | Ala    |

Table showing the selection of residues for site-directed mutagenesis.

Structural templates and Modeler (42) were used to construct a Nct peptidase-like domain model (residues 253 to 502) (Fig. 1A). Despite the low sequence identity between Nct and M28 peptidases (<20%), all algorithms utilized predicted, for the Nct domain, a hydrolase-like fold with very high levels of confidence (>99.9%).

In agreement with Fagan et al. (20), three residues involved in the catalytic mechanism of aminopeptidases are conserved in the putative substrate binding pocket of the Nct model: Asp282, Glu332 (substrate binding site), and Tyr452 in mouse Nct, which are in analogous positions to residues Asp287, Glu318, and Tyr446 in SGAP. Asp287 is a second-shell residue that polarizes the His285 metal ligand; Glu318 participates as a general base during catalysis, involved in hydrogen bonding with the N terminus of the substrate, and Tyr452 stabilizes the transition state intermediate in this particular aminopeptidase. The other catalytic residues (metal ligands) in SGAP are, however, not conserved in Nct. These positions are occupied by residues Arg280, Ser296, Thr333, Gly365, and Glu453 in mouse Nct. Interestingly, the Nct model showed that Arg280 is located close to Glu332 and Glu365 residues, suggesting the existence of a salt bridge between these charged residues (Fig. 1B). Furthermore, the negatively charged residue conserved at position 363 in all Nct sequences suggests that it could be of functional significance. Accordingly, we selected Arg280, Asp282, Ser296, Thr333, Glu363, Gly365, and Tyr452, and Glu453 residues for further mutagenesis analysis (Table 1).

In addition, we used the x-ray crystal structures of SGAP and human glutaminyl-peptide cyclotransferase in complex with inhibitors (25, 41) to identify residues that could be involved in the recognition of substrates by Nct. In SGAP, the inhibitory amino acids bind to the two zinc ions, present in the active site of the peptidase, via their free carboxylate group. Further stabilization is achieved by their interaction with catalytic residues Tyr246 and Glu201, the metal ligand Asp142, and two other residues Arg204 and Phe199 (25). Interestingly, the Arg202 residue is presented by a loop that approaches the SGAP catalytic site. Similarly, the corresponding loop in the human glutaminyl-peptide cyclotransferase structure (Ile303–Asp305) approaches the active site and interacts with the backbone of the inhibitors (41). In our Nct model, the equivalent loop contains the 423PSS425 residues. Interestingly, multiple sequence alignments show that the 422PPSS425 motif is highly conserved, in particular Pro422, Pro423, and Ser425 are completely conserved in all reported Nct sequences. Our model also suggests that the 421LPPSS425 loop delineates the binding pocket and that Ser425 may interact by a hydrogen bond with Glu332 (Fig. 1A). Based on this comparison, the Ser425 residue was included in our mutagenesis analysis. Finally, the alanine substitution of the previously identified Glu332 critical residue (Glu333 in human NCT) was included as a positive control and the R431A substitution, which is not located in the pocket, was used as a negative control. For a summary, see Table 1.

Site-directed Mutagenesis and Expression in Cell Culture—To investigate the contribution of the selected residues to the putative substrate binding site of Nct, we introduced single amino acid substitutions that minimally disturb the overall folding of the domain but should substantially change the binding properties of the pocket. As indicated in Table 1, in most cases we replaced the selected residues with alanine. We also used additional substitutions taking into account sequence information from the NCT orthologues (see Table 1). Finally, we investigated mutations that should impede substrate binding, either by eliminating the anionic binding site (E332Q), or by introducing bulky residues at Ser296, a position supposed to sterically block the pocket or neutralize the Glu332 side chain: S296W, S296K, respectively.

In our analysis, we used Nct knock-out mouse fibroblasts (Nct−/− MEFs) to avoid interference of endogenously expressed wild-type Nct, which also allowed us to investigate to what extent the different mutants were able to restore γ-secretase assembly. We generated at least two different cell lines stably expressing each mutant. We confirmed that Nct expression levels were similar to endogenous expression levels in Nct wild-type cells, minimizing overexpression artifacts (Fig. 2). Interestingly, most mutants became glycosylated, indicating transport throughout the secretory pathway, with the exception of the E332A. An alanine substitution at position Glu332 dramatically decreased Nct complex glycosylation and its incorporation into the γ-secretase complex (see below). We have previously shown that γ-secretase activity does not depend on glycosylation of Nct (33) and therefore the functional significance of decreased or increased glycosylation of Nct mutants is difficult to assess.

Residues Located in the Putative Binding Pocket of Nct Affect Notch and APP Cleavage to a Limited Extent—We next investigated whether any of the Nct substitutions influenced γ-secretase processing of APP or Notch, by transducing the different mutant stable cell lines with full-length APPswe or Myc-tagged NotchΔE. AP40/42 peptides released to the extracellular medium or the production of NICD were quantified by...
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Although supposed to affect or modulate substrate binding, most of the introduced mutations unexpectedly displayed only a small or no effect on the processing of the substrates. In fact, APP processing was only (and admittedly mildly) affected by the S296W substitution, which was designed to sterically hinder substrate binding. However, on the other hand, NotchΔE processing was not affected, indicating that the prediction that this bulky substitution should prevent substrate binding to Nicastrin did not hold true. Only a few other mutants, i.e. T333A, G365N, and S425A, slightly or moderately reduced NICD production, without affecting the release of Aβ peptides. Overall, the changes in γ-secretase processing caused by the different mutations are mild (if for instance compared with the effect of Presenilin clinical mutations (34)), and in fact, only the E332A substitution dramatically affected the activity of the complex. However, and in contrast to Shah et al. (13), we found that even this mutant did not completely inhibit cleavage of either substrate. More intriguingly, the removal of the putative negative charge (E332Q) did not affect γ-secretase activity at all, indicating that the anionic function of Nicastrin is not needed for substrate interaction.

E332A Mutant Impairs γ-Secretase Complex Maturation—The Glu332 is the key residue in the current hypothesis for the function of Nct, therefore we focused in detail on this amino acid. As demonstrated by blue native gel electrophoresis, the E332A substitution has a deep impact on the maturation of the γ-secretase complex (Fig. 4, A and B). Although formation of the high molecular weight complex was reduced, the E332A Nct-Aph1 mutant subcomplexes were still present (Fig. 4A). The impairment of γ-secretase complex assembly was further corroborated by SDS-PAGE and Western blot analysis. Indeed, the E332A mutant showed reduced stabilization of Ps1 fragments and Pen-2, whereas Aph1 expression was mainly unaffected (Fig. 4B). The “neutralizing” E332Q mutant had an effect on γ-secretase complex formation but to a clearly lesser extent than the E332A mutant, which is in agreement with a previous study in HEK293 cells (14). It should be noticed that both the E332A and E332Q mutations did not interfere with the Nct-Aph1 subcomplex formation, suggesting that the disruption occurs mainly at the level of the incorporation of Presenilin into the complex. Finally, as a control, the conservative E332D mutation fully rescued the complex maturation (Fig. 4A).

In addition, cell surface biotinylations experiments showed less γ-secretase subunits at the plasma membrane in the E332A mutant cell lines, and indicated that the E332A mutant reached the cell surface mainly as a non-glycosylated form, although upon overexposure some mature E332A was also observed (Fig. 4C). Taken together, our results indicate that the negative charge at position 332 is essential for proper maturation of the γ-secretase complex.

E332A Mutant Impairs γ-Secretase Complex Maturation but Not Its Activity—Intriguingly, minimal γ-secretase activity was still detected in the E332A mutant cell lines, which led us to speculate that the loss of activity could be entirely explained by the defect in γ-secretase assembly and did not necessarily reflect reduced interaction between substrate and protease. Therefore, we determined the specific activity of the E332A mutant complex. We also included the mutants that appeared to affect processing of either APP or Notch in our previous experiments. We performed in vitro activity assays measuring the total amount of γ-secretase complex in our mixture and relating that to the amount of product generated after 4 h. The γ-secretase complex was solubilized in 0.25% CHAPSO from total membranes and AICD production was measured, using C99-3xFLAG as substrate. Specific activities were defined as AICD normalized against the γ-secretase Ps1 CTF and Pen2 subunits (Fig. 5A). Importantly, addition of 10 μM inhibitor X to the activity reactions demonstrated that AICD production was dependent on γ-secretase activity (Fig. 5B).

Strikingly, these in vitro assays showed that specific activities of the wild-type γ-secretase and the E332A mutant did not differ (Fig. 5A, black bars, AICD normalized to Ps1 CTF and Pen2 levels), demonstrating that the effect of E332A substitution occurs solely at the level of complex maturation, and that, once a complex is generated, the mutant enzyme is as active as the wild-type. Moreover, we found a similar correlation between the levels of γ-secretase produced and activity levels of the T333A, G365N, and S425A mutants. The similar specific activities of these mutants relative to the wild-type complex were well correlated with the effect of Presenilin clinical mutations (34)). Stabilization of the γ-secretase high molecular complex (HMC) was assessed by blue native gel electrophoresis and Western blot with an Aph1 specific antibody as shown B. KNO, Nicastrin knockout cells.

FIGURE 2. Expression of the wild-type Nct and mutants on a Nct−/− background rescues the glycosylation of Nct and the maturation of the γ-secretase complex, with the exception of E332A. Nct−/− fibroblasts were stably transfected with the different indicated Nct constructs and expression levels of Nct, Ps1 CTF, and Pen-2 components were analyzed by Western blot using the appropriate antibodies (A). Stabilization of the γ-secretase high molecular complex (HMC) was assessed by blue native gel electrophoresis and Western blot with an Aph1 specific antibody as shown B. KNO, Nicastrin knockout cells.
required interaction between Nicastrin and the substrate prior to entering into the active site of the complex, making this interaction no longer rate-limiting. For that reason, we chose to determine /H9253-secretase activity under non-detergent solubilized conditions.

Therefore, we transiently transfected our stable Nct/H11002/, WT, E332A-, E332Q-, and E332D-Nct rescued cell lines with the C99-3xFLAG substrate and treated them with a /H9253-secretase inhibitor overnight to allow the substrate to accumulate to a similar extent in the different lines. We then prepared total membranes and incubated them at 37 °C for 4 h. We collected and quantified AICD levels generated de novo from the supernatants (Fig. 6, A and C, black bars) and γ-secretase levels present in the reaction mixture were estimated by Western blot (Fig. 6B). In contrast to Nct/H11021/, cells that did not generate any product, the Glu/H11052/ mutants rescued γ-secretase activity to different extents. Similar to what we had previously observed in the detergent-containing assays, the E332A substitution led to the generation of a minimal amount of AICD (Fig. 6C, black bars), which correlated with the amount of enzyme (Fig. 6C, white bars), demonstrating again that the lack of activity in the E332A mutant is only due to the defect in the complex formation. Furthermore, the neutralizing E332Q mutation, which showed absolutely no effect on the cleavage of either Notch or

FIGURE 3. Analysis of APP and Notch ΔE processing. APPswe or Myc-tagged Notch ΔE substrate were overexpressed using adenoviral vectors in Nct stably transduced Nct/H9004/ MEF cell lines. A, Aβ40 and Aβ42 secreted to the conditioned medium were measured by enzyme-linked immunosorbent assay 24 h after APPswe infection; or B, Notch ΔE transduced MEF cell lines were treated with the proteasomal inhibitor lactacystin and NICD levels were determined with a cleavage specific antibody and quantified relative to the levels of Notch ΔE infection, using an anti-Myc antibody. p values (*, p < 0.05; **, p < 0.01) were determined for the average of three independent experiments performed in triplicate (n = 9). KNO, Nicastrin knockout cells.
APP, displayed a similar specific activity as the wild-type. These observations are difficult to reconcile with the previous assumption that Nct functions as a substrate receptor, as this model entirely relies on the anionic binding role of Glu332, and was mainly demonstrated by the fact that E332A substitution abrogated Nct receptor function.

Interestingly, the effects observed in the current study with the E332A mutant correlate with earlier reports that demonstrate that deletions in the AP-like domain (H9004312–369 or H9004312–340) significantly reduce the Nct-Ps1 interaction and proportionately affect the activity of the H9253-secretase (9, 43, 44).

Furthermore, immunoprecipitation experiments in digitonin-solubilized extracts of HEK293 overexpressing wild-type NCT or deletions H9004312–369 or H9004312–340 show that all, WT and mutants, bind equally to the C99/C83 and Notch ΔE substrates, indicating that the interaction between NCT and the substrate remains unaltered under conditions that maintain the integrity of the complex, even when the presumed binding Glu residue is not present. On the other hand, NCT pull-down experiments from 1% Triton X-100 lysates of HEK293 cells stably expressing C99 substrate and overexpressing wild-type NCT, NCT-TMS (NCT with the transmembrane region of E-selectin), or the Δ312–369 and Δ312–340 NCT deletions show that WT NCT and NCT-TMS (but none of the deletion mutants) co-precipitate C99 (13). These observations suggest that the ectodomain of NCT (312–369 region) may indeed interact with the substrate under stringent conditions, but under biologically relevant conditions the interaction is eventually overridden by the contribution of the NCT transmembrane region or other sequences in the complex to substrate binding. Taken together, our results demonstrate that the conserved negative charge at position 332 is required for the proper assembly of the enzyme but not for its activity, indicating that the recognition mechanism of the γ-secretase complex does not rely on the Nct-Glu332 residue.

E332A Mutation Does Not Affect Nicastrin Conformation—Finally, we sought to address whether impairment in assembly of the γ-secretase was due to a folding defect in the E332A Nct mutant by using partial proteolytic digestion. It has been shown that immature Nct is sensitive to trypsin, whereas mature Nct is resistant, indicating that Nct undergoes a conformational change during complex assembly (44). Thus, we took advantage
of mild trypsinization experiments to compare WT Nct and the E332A mutant. Total membranes from WT and E332A rescued knock-out MEF cells solubilized in n-dodecyl β-D-maltoside were incubated with increasing concentrations of trypsin. In agreement with previous findings, the WT and E332A mature forms were trypsin resistant, whereas the immature forms were digested to different extents but displayed a common band pattern (Fig. 7), suggesting no significant conformational difference between wild-type Nct and mutant E332A. Supporting our observation, structural data from the Nct homologous M28-proteins show that the equivalent glutamate is not essential for the protease-like domain fold; for instance, this residue is not conserved in the transferrin receptor structure (45), and in the case of the human glutaminyl-peptide cyclotransferase (41), the E201Q mutant (equivalent to the E332Q in Nct) affects the activity but not the structure of the enzyme (41).
Site-directed Mutagenesis of the Nicastrin Ectodomain

Thus, the role of the protease-like domain in Nct remains somewhat elusive. However, structure-functional studies of the transferrin receptor have involved its protease-like domain in protein-protein interactions. More specifically, the regions at the base or at the N-terminal part of this domain, which are both distant from the remnants of the “active site,” contribute to the binding of the iron-transferrin or the HFE (hereditary hemochromatosis protein) ligands, respectively (46, 47). We suggest therefore that the Nct AP-like domain may have evolved in the context of the γ-secretase complex, as a binding scaffold for the presenilin subunit, presenilin-Pen-2 subcomplex, or another unknown auxiliary subunit, in which the region containing the Glu\textsuperscript{332} residue contributes significantly to the assembly of the multimeric protease. Alternatively or additionally, the AP-like domain of NCT could participate, alone or in concert with other domains in Nct, in the conformational change needed during the assembly of the γ-secretase complex.

CONCLUSIONS

Our mutagenesis data of residues present in the putative substrate binding pocket of Nct, although supposed to affect or modulate substrate binding, unexpectedly displayed only small or no effects in the processing of the substrates, with the exception of the previously identified Glu\textsuperscript{332} residue (human Glu\textsuperscript{333}). Furthermore, our results showed that E332A critically affects assembly and maturation of the γ-secretase complex. Although our experiments do not rule out that the ectodomain of Nct also participates in the enzymatic function of the γ-secretase complex, our results clearly demonstrate that the previously proposed mechanism cannot be maintained as an explanation for the available data and reopen a discussion about nicastrin function in the γ-secretase-mediated regulated intramembrane proteolysis.

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