

The Nicastrin ectodomain adopts a highly thermostable structure

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Abstract

Nicastrin is a type I transmembrane glycoprotein, which is part of the high molecular weight γ -secretase complex. γ -Secretase is one of the key players associated with the generation of Alzheimer's disease pathology, since it liberates the neurotoxic amyloid β -peptide. Four proteins Nicastrin, anterior pharynx-defective-1 (Aph-1), presenilin enhancer-2 (Pen-2) and Presenilin are essential to form the active γ -secretase complex. Recently it has been shown, that Nicastrin has a key function in stabilizing the mature γ -secretase complex and may also be involved in substrate recognition. So far no structural data for the Nicastrin ectodomain or any other γ -secretase component are available. We therefore used Circular Dichroism (CD) spectroscopy to demonstrate that Nicastrin, similar to its homologues, the *Streptomyces griseus* aminopeptidase (SGAP) and the transferrin receptor (TfR), adopts a thermostable secondary structure. Furthermore, the Nicastrin ectodomain has an exceptionally high propensity to refold after thermal denaturation. These findings provide evidence to further support the hypothesis that Nicastrin may share evolutionary conserved properties with the aminopeptidase and the transferrin receptor family.

Keywords: A β -peptide; Alzheimer's disease; γ -secretase; Presenilin; regulated intramembrane proteolysis (RIP); *Streptomyces griseus* aminopeptidase.

Introduction

Nicastrin is a 709 amino acid type I transmembrane glycoprotein, which is known to be an essential component of the γ -secretase complex (Yu et al., 2000; Edbauer et al., 2002; Leem et al., 2002; Edbauer et al., 2003). γ -Secretase cleaves a variety of substrates within the hydrophobic bilayer of cellular membranes and is a key player in the generation of

the neurotoxic Amyloid β -peptide (A β) found in neuritic plaques of Alzheimer's disease (AD) patients (Haass and Selkoe, 2007). A β is generated via regulated intramembrane proteolysis (RIP) from the β -Amyloid Precursor Protein (APP). During amyloidogenic processing APP first undergoes shedding by the β -site APP cleaving enzyme (BACE), releasing a large part of the APP ectodomain and generating a membrane bound C-terminal stub (APP CTF). In a second proteolytic step the APP CTF is a substrate for intramembrane cleavage by γ -secretase releasing the APP intracellular domain (AICD) into the cytosol and A β into the extracellular space (Steiner et al., 2008). The active center of the γ -secretase complex is composed of either Presenilin (PS) 1 or PS2 (De Strooper et al., 1998; Steiner et al., 1999; Wolfe et al., 1999; Herreman et al., 2000; Zhang et al., 2000). PSs are nine-transmembrane proteins and belong to the GxGD-type intramembrane aspartyl proteases (Steiner et al., 2008), as one of their critical active site aspartate residues is located within a conserved GxGD-motif in transmembrane domain (TMD) 7 (Steiner et al., 2000). In addition to Nicastrin and PS two other integral membrane proteins, Aph-1 (Aph-1a or Aph-1b) and Pen-2 (Goutte et al., 2000; Francis et al., 2002), are essential to form a stable and catalytically active γ -secretase complex (Lee et al., 2002; Steiner et al., 2002; Edbauer et al., 2003; Kimberly et al., 2003; Takasugi et al., 2003). The mature γ -secretase is a stable complex with a half-life time of more than 24 h in mammalian cells (Ratovitski et al., 1997). During the assembly of the γ -secretase complex in the endoplasmic reticulum (ER) Nicastrin and Aph-1 form a premature subcomplex, which then recruits and stabilizes PS (LaVoie et al., 2003; Takasugi et al., 2003; Shirotni et al., 2004). Upon incorporation of Pen-2, PS is autoproteolytically cleaved within its large cytosolic loop between TMD 6 and 7 resulting in an N- and a C-terminal fragment, which remain tightly associated within the mature γ -secretase complex (Thinakaran et al., 1996; Capell et al., 1998; Yu et al., 2001; Takasugi et al., 2003; Prokop et al., 2004; Fukumori et al., 2010). After its complete assembly the γ -secretase complex is exported to later secretory compartments where Nicastrin undergoes complex glycosylation (Kaether et al., 2002). Nicastrin maturation in mammalian cells is dependent on the correct binding of Nicastrin to the other complex components (Edbauer et al., 2002; Kimberly et al., 2002; Leem et al., 2002; Tomita et al., 2002; Yang et al., 2002). Interestingly, complex glycosylation of Nicastrin in later secretory compartments is not necessarily required for γ -secretase activity or cell surface transport of the complex (Herreman et al., 2003; Shirotni et al., 2003). On the other hand, core glycosylation and folding of Nicas-

trin in the ER seems to be the critical step determining stability and activity of the γ -secretase complex (Hayashi et al., 2009). Using multiple sequence alignment analysis, it has been shown that the Nicastrin ectodomain shares some homology with the aminopeptidase and transferrin receptor family. In particular, *Streptomyces griseus* aminopeptidase (SGAP) and the human transferrin receptor (TfR) ectodomain exhibit substantial homology with large parts of the Nicastrin ectodomain (Fagan et al., 2001). SGAP is a zinc-metalloprotease, which is characterized by a high thermal stability of up to 70°C–80°C (Vosbeck et al., 1973). The human TfR is a type II transmembrane glycoprotein, which mediates the cellular uptake of iron by binding to and endocytosis of the serum iron transport protein transferrin (Richardson and Ponka, 1997). Like SGAP, the secondary structure of TfR exhibits an enhanced stability against thermal denaturation (Orberger et al., 2001). Due to its homology to the aminopeptidase superfamily, it was suggested that Nicastrin may serve as a substrate receptor. It was shown that Glu333 of the Nicastrin ectodomain binds to the N-termini of type I transmembrane proteins, which were truncated by the initial shedding step and serve as the immediate substrates for γ -secretase (Shah et al., 2005; Dries et al., 2009). However, these findings have been recently challenged (Chavez-Gutierrez et al., 2008; Martin et al., 2009) and it was suggested that the Nicastrin protein instead plays a critical role in stabilizing the γ -secretase complex (Zhang et al., 2005, 2010).

To provide further evidence that Nicastrin may share evolutionary conserved properties with the aminopeptidase/transferrin receptor superfamily we investigated whether the Nicastrin ectodomain is able to adopt a characteristic and stable secondary structure with similar biophysical properties like its proposed homologues.

Results

The Nicastrin ectodomain adopts a defined secondary structure

In order to determine the structural properties and the stability of the Nicastrin ectodomain (ectoNicastrin) we expressed a Nicastrin IgG fusion protein (Figure 1A), containing the entire ectoNicastrin (amino acids 1–669) including the native N-terminal ER targeting signal sequence in HEK293 cells. Cell lysates of HEK293 cells expressing the Nicastrin IgG fusion protein predominantly contain the immature variant of the fusion protein, while the mature form is secreted efficiently into the culture medium (Figure 1B). To ensure that Nicastrin IgG undergoes complex glycosylation similar to that of native Nicastrin, lysates and conditioned media from cells expressing endogenous Nicastrin or additionally Nicastrin IgG were treated with Endoglycosidase H (EndoH) to selectively remove non-complex sugars. The immature Nicastrin IgG found in the cell lysates was sensitive to treatment with EndoH whereas the secreted version of the fusion protein was partially resistant like the mature form of native Nicastrin [(Edbauer et al., 2002; Shi-

rotani et al., 2003) and Figure 1B]. This confirms that the Nicastrin IgG fusion protein is correctly translated into the ER and undergoes complex N-glycosylation similar to endogenous Nicastrin. After demonstrating physiological maturation of Nicastrin IgG we purified the fusion protein via affinity binding to a protein A column and selectively cleaved off ectoNicastrin using Faktor Xa (Figure 1C). Faktor Xa was subsequently removed using benzamidine and ectoNicastrin was further purified by size exclusion chromatography (Figure 1C). Silver staining of the respective fractions demonstrates the purity of the individual fractions (Figure 1C). To investigate whether ectoNicastrin adopts a defined secondary structure we performed Circular Dichroism (CD) spectroscopy. As indicated by the minima of the ellipticity at 210 nm and 222 nm and the maximum at 195 nm ectoNicastrin is predominantly in an α -helical conformation at 20°C (Figure 1D). This result demonstrates that recombinant Nicastrin adopts a highly structured secondary conformation.

The secondary structure of the Nicastrin ectodomain is highly thermostable

To elucidate whether ectoNicastrin exhibits a characteristic thermostability like its potential relatives SGAP (Vosbeck et al., 1973) and TfR (Orberger et al., 2001) CD spectra were collected after stepwise heating (Figure 2). EctoNicastrin was indeed particularly stable. Upon heating only little unfolding was observed whereas the majority of ectoNicastrin remained in its predominantly α -helical conformation up to 85°C (Figure 2A). Similar findings were made for the unfolding process of SGAP. SGAP lost only little of its α -helical conformation up to 75°C (Figure 2B), which is consistent with previously published data (Vosbeck et al., 1973). A structural switch occurred only at temperatures above 85°C or 75°C for ectoNicastrin or SGAP, respectively, as indicated by a shift of the CD minimum towards 200 nm and the increase in CD at 222 nm (Figure 2A and B). At temperatures >90°C SGAP started to aggregate and precipitated (data not shown). In the temperature range from 20°C up to 75°C the CD spectra of ectoNicastrin show an isodichroic point at 204 nm suggesting that there is a two state equilibrium between unfolded and folded states of the protein. In contrast, SGAP seems to undergo a gradual transition into an irreversibly denatured state, although the bulk of the protein remains folded up to 75°C. Taken together these data suggest that the Nicastrin ectodomain acquires a highly thermostable secondary structure similar to its homologues of the aminopeptidase superfamily.

The Nicastrin ectodomain refolds efficiently after thermal denaturation

Since ectoNicastrin turned out to be extremely stable during heating, we next investigated its refolding properties. After heating to 100°C the sample containing ectoNicastrin was cooled to 20°C. CD spectra were collected prior to heating, at 100°C and after cooling (Figure 3A). The two spectra acquired prior to heating and after cooling were highly similar and no shift of the characteristic minima was observed,

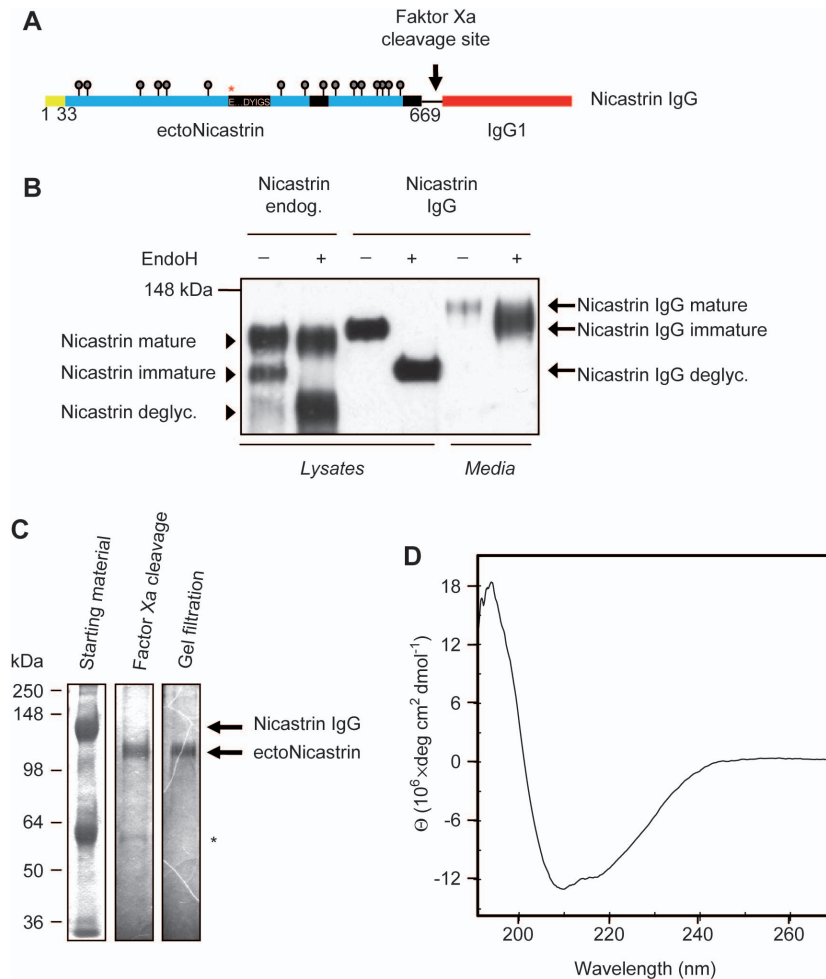


Figure 1 The Nicastrin ectodomain adopts a defined secondary structure.

(A) Model depicting the Nicastrin IgG fusion protein. The Nicastrin ectodomain is shown in blue, the IgG fusion in red and the Nicastrin signal sequence is highlighted in yellow. Black boxes indicate the conserved regions including the DYIGS (amino acid 336–340) motif and potential glycosylation sites are marked by black circles. Glu 333, which was reported to be involved in γ -secretase substrate recognition (Shah et al., 2005) is marked by an asterisk. (B) Endo H resistance of Nicastrin IgG. Immunoprecipitated endogenous Nicastrin and Nicastrin IgG were incubated in the presence or absence of EndoH. Nicastrin variants were detected with a polyclonal antibody directed against the N-terminus of Nicastrin. (C) Purification of ectoNicastrin. Starting material: Conditioned media from HEK293 cells, expressing the Nicastrin IgG fusion protein were separated using SDS polyacrylamid gel electrophoresis and stained with coomassie blue. Factor Xa cleavage: The starting material was applied to a Protein A column. Upon factor Xa cleavage ectoNicastrin was eluted and factor Xa was removed using a benzamidine. The eluate was separated on SDS polyacrylamid gel electrophoresis and silver-stained. Gel filtration: The concentrated benzamidine eluate was applied to a Superdex 200 HR 30/10 gel filtration column and a silver stain of the fraction used for CD spectroscopy was performed. The asterisk indicates bovine serum albumin (BSA) from the conditioned media. (D) CD spectroscopy of ectoNicastrin: The CD spectrum of ectoNicastrin was recorded at 20°C. Note that ectoNicastrin adopts a predominantly α -helical structure as indicated by the minima of the ellipticity (Θ) at 210 nm and 222 nm and the maximum at 195 nm.

while the spectrum collected at 100°C showed a significant change in secondary structure (Figure 3A). In contrast SGAP did not regain its original structure after thermal denaturation, but rather remained in an unfolded conformation (Figure 3B). This suggests that ectoNicastrin, in contrast to SGAP, has the ability to almost completely refold to its original conformation after thermal unfolding. Taken together these data indicate that the Nicastrin ectodomain adopts a highly stable structure, which remains refoldable even upon denaturation.

A N-terminal deletion of Nicastrin reduces thermostability and the capacity of refolding

We previously demonstrated, that deletions in the Nicastrin ectodomain abolish the incorporation of Nicastrin into the γ -secretase complex (Shirotani et al., 2003). To investigate whether such a deletion also influences the thermostability and the refolding capacity of the Nicastrin ectodomain, we expressed a Nicastrin IgG fusion protein, which lacks amino acid 40–164 of the Nicastrin ectodomain (Nicastrin IgG

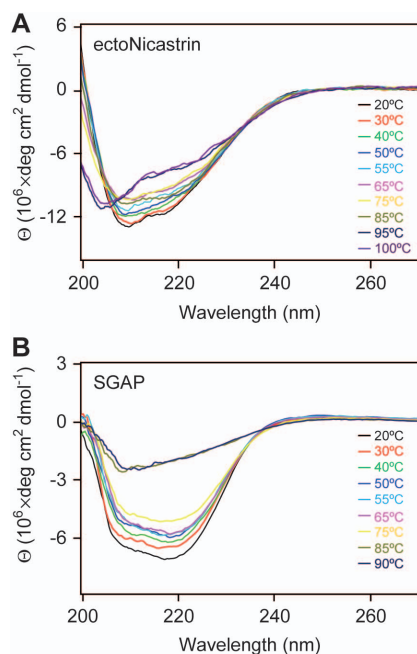


Figure 2 The ectoNicastrin secondary structure is stable up to 85°C.

CD spectra of ectoNicastrin (A) or SGAP (B) were recorded at increasing temperatures as indicated. Both ectoNicastrin and SGAP did not significantly change their secondary structures at temperatures up to 85°C or 75°C, respectively. In contrast to ectoNicastrin, which undergoes a significant structural change at temperatures >85°C, SGAP started to unfold at temperatures >75°C and aggregated at temperatures >90°C.

Δ40–164) (Figure 4A). The fusion protein was purified analogous to ectoNicastrin and the refolding capacity was determined. Compared to ectoNicastrin (Figure 3A) the refolding capacity of ectoNicastrin Δ40–164 was reduced, since the characteristic minima for an α-helical conformation were not fully regained after sample cooling (Figure 4B). These data suggest that deletions in the ectodomain of Nicastrin might affect the thermal properties of its secondary structure.

Discussion

Here we demonstrate a surprising thermostability and refolding capacity of the recombinant Nicastrin ectodomain. This is particularly interesting since other members of the aminopeptidase family such as SGAP, which share substantial homologies with the Nicastrin ectodomain (Fagan et al., 2001) show similar properties (Vosbeck et al., 1973; Orberger et al., 2001).

Remarkably the thermostability and the capability of refolding of the Nicastrin ectodomain is even more pronounced than that of SGAP. Since recently it has been proposed, that the thermodynamic stabilization of a protein might correlate with its degree of glycosylation (Shental-Bechor and Levy, 2008), the complex glycosylation pattern within the Nicastrin ectodomain may contribute to its extraordinary thermosta-

bility. The finding that a mutant variant of Nicastrin (Nicastrin Δ40–164) that lacks at least two glycosylation sites in the N-terminal domain shows a slightly reduced thermostability and refolding capacity, may further support this hypothesis. This idea may be additionally supported by the observation that interference with early glycosylation events in the ER causes inappropriate glycosylation of Nicastrin leading to a destabilization of the γ-secretase complex and a reduced intrinsic activity (Hayashi et al., 2009), while blocking later steps in the N-glycosylation pathway still retains γ-secretase activity (Herreman et al., 2003; Shirotani et al., 2003). However, deletions and mutations could also affect secondary structure, thermostability and function independent of the glycosylation pattern. To finally prove the hypothesis that the degree of glycosylation within the Nicastrin ectodomain, which is determined in the ER, is responsible for stability and integrity of the γ-secretase complex, rather than the size of the polysaccharide side chains, Nicastrin mutants, which lack defined N-glycosylation sites, should be investigated.

Although ectoNicastrin Δ40–164, according to the CD spectrum at 20°C, seems to adopt a similar structure like ectoNicastrin this mutant variant is non-functional in a cell culture system (Shirotani et al., 2003). This suggests that

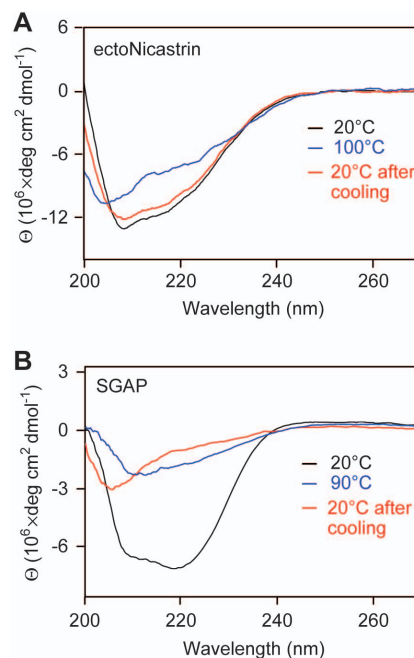


Figure 3 The ectoNicastrin refolds efficiently upon heat denaturation.

(A) The CD spectra of ectoNicastrin or (B) SGAP prior to heating were recorded at 20°C as described in Figure 1D (black line). The proteins were subsequently heated to 100°C (ectoNicastrin) or 90°C (SGAP; blue line) and cooled again followed by the recording of a third CD spectrum at 20°C (red line). For ectoNicastrin only a minor difference between the two spectra prior to heating and after cooling was observed, indicating that ectoNicastrin almost completely refolded after thermal unfolding. In contrast SGAP was not capable of refolding after thermal denaturation.

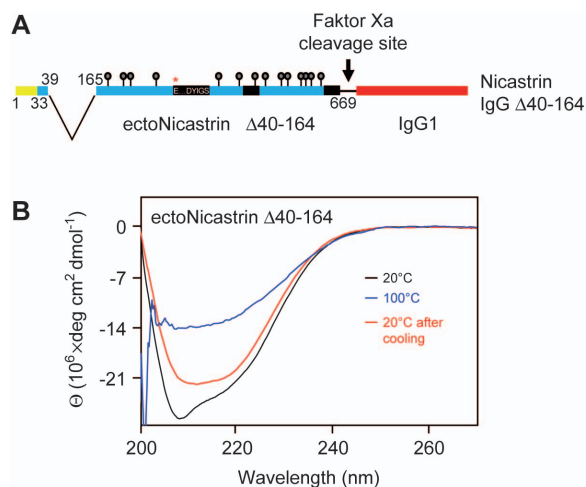


Figure 4 Reduced refolding propensities of ectoNicastrin $\Delta 40-164$.

(A) Model depicting the Nicastrin IgG $\Delta 40-164$ fusion protein, which lacks amino acid 40–164 of the Nicastrin ectodomain. The mutated Nicastrin ectodomain is shown in blue, the IgG fusion in red and the Nicastrin signal sequence is highlighted in yellow. Black boxes indicate the conserved regions including the DYIGS (amino acid 336–340) motif and potential glycosylation sites are marked by black circles. Glu 333, which was reported to be involved in γ -secretase substrate recognition (Shah et al., 2005) is marked by an asterisk. (B) CD spectra of ectoNicastrin $\Delta 40-164$ prior to heating (black line), at 100°C (blue line) and after heating (red line) were recorded as described in Figure 3A. Note that in contrast to ectoNicastrin (Figure 3A) the refolding capacity of ectoNicastrin $\Delta 40-164$ is impaired, since the minima of the ellipticity (Θ) characteristic for an α -helical conformation after heating are less pronounced than before heat denaturation.

already small changes in the secondary structure of Nicastrin are sufficient to affect the proper function of the γ -secretase complex.

Taken together, although Nicastrin lacks an aminopeptidase activity, it shows a similar extraordinary and even further improved highly thermostable secondary structure like its homologs. Whether these findings prove the hypothesis, that Nicastrin evolved from the aminopeptidase and transferrin receptor family remains, however, to be shown.

Materials and methods

Cell culture, cDNAs and transfection

HEK293EBNA cells were cultured in DMEM with Glutamax (Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Invitrogen GmbH), and 1% Penicillin/Streptomycin (Invitrogen GmbH). Using PCR the Nicastrin ectodomain (amino acid 1–669; amino acid numbering according to Yu et al. (2000); GenBank: AAG11412.1) or the Nicastrin ectodomain carrying a deletion from amino acid 40–164 ($\Delta 40-164$; amino acid numbering according to Yu et al. (2000); GenBank: AAG11412.1) was fused to the human IgG1 hinge-CH3 heavy chain. Between the two genes a spacer of 27 basepairs containing a Factor Xa cleavage site was included. The fusion construct was subcloned into the NheI and

NotI sites of pcDNA 3.1. Zeo – (Invitrogen GmbH), sequence verified and stably transfected into HEK293EBNA cells. Transfection of cells was carried out using Lipofectamine 2000 (Invitrogen GmbH) according to the manufacturer's instructions and single cell clones were generated by selection in 200 $\mu\text{g/ml}$ zeocine (Invitrogen GmbH).

Purification of the Nicastrin ectodomain (ectoNicastrin)

Cells described above were cultured in TripleFlasks Nunclon™ Δ Surface (Thermo Fisher Scientific, Roskilde, Denmark), conditioned medium was collected and filtered using Bottle Top Filter 0.22 μm (Milipore GmbH, Schwalbach/Ts., Germany) to remove remaining cellular particles. To purify approximately 0.5 mg of the Nicastrin ectodomain 5 l conditioned medium were collected. The conditioned medium was directly applied to a Protein A column (GE Healthcare-Bio Science, Uppsala, Sweden) using an ÄKTA purifier (GE Healthcare-Bio Science) supplied with a separate sample pump (Direkt Load 960; DE Healthcare-Bio Science). After washing with 100 ml buffer A (50 mM Tris, pH 7.4), 6 ml Factor Xa solution [(20 mM Tris, 100 mM NaCl, 2 mM CaCl_2 , pH 8.0 supplemented with 20 $\mu\text{g/ml}$ Factor Xa (New England Biolabs, Ipswich MA, USA)] were added manually to the column and the column was incubated for roughly 16 h at 8°C. To remove factor Xa, the eluate from the Protein A column containing the cleaved Nicastrin ectodomain was directly applied to a benzamidine column (GE Healthcare-Bio Science) using 50 ml buffer B (50 mM Tris, 500 mM NaCl, pH 7.4). The eluate from the benzamidine column was fractionated and the fractions containing the ectoNicastrin were pooled and concentrated to 1/4 of the original volume using a Viva Spin column 50.000 MWCO (SartoriusStedim Biotech, Göttingen, Germany) according to the manufacturer's instructions. Finally the sample was applied to a Superdex 200 HR 30/10 gel filtration column (GE Healthcare-Bio Science) and separated using buffer C (20 mM sodium phosphate buffer, pH 7.2, supplemented with 100 mM KCl).

Deglycosylation experiments

For deglycosylation cells were lysed in STEN buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA) supplemented with 1% Nonidet P-40 (NP-40), 1% Triton X-100 and 2% bovine serum albumin (BSA) on ice for 20 min. Lysates were clarified by centrifugation for 45 min at 100 000 g and 4°C. Endogenous Nicastrin was immunoprecipitated using the polyclonal antibody N1660 (Sigma-Aldrich, St. Louis, MO, USA) against the C-terminus of Nicastrin. Nicastrin IgG was immunoprecipitated using Protein A coupled sepharose beads (Sigma-Aldrich). Precipitates were incubated in the presence of 50 mU/ml endoglycosidase H (endo H; Roche Diagnostics GmbH, Mannheim, Germany) for 14 h at 37°C in the appropriate buffer. Reactions were stopped by the addition of SDS sample buffer and reaction mixtures were separated by SDS-polyacrylamid gel electrophoresis. For Nicastrin detection the polyclonal antibody against the N-terminus of Nicastrin (NCT-NT, 1658) was used (Capell et al., 2003).

Circular Dichroism (CD) spectroscopy

Proteins were dissolved in buffer C (see above) at a concentration of approximately 1 $\mu\text{g}/\mu\text{l}$ and diluted to 0.1 $\mu\text{g}/\mu\text{l}$ with water. Aminopeptidase I from *Streptomyces griseus* (SGAP) was obtained from Sigma-Aldrich (Product Number A9934). CD spectra were measured with a Jasco J-810 Spectropolarimeter (Jasco, Inc., Easton MD, USA) using a cuvette with a path length of 0.2 cm. Spectra

of 10 scans were averaged. Temperature in the measuring cell was gradually increased as indicated in the respective figures, and at defined temperature intervals CD spectra were taken from 300–190 nm. Temperature control with an accuracy of 0.5°C in the cuvette was achieved with a heating/cooling accessory using a Peltier element. CD data are expressed as the mean residue ellipticity (Θ) in deg cm² dmol⁻¹ at the respective wave length in nm. The mean residue ellipticity was calculated from the measured ellipticity (θ , in mdeg) by dividing the molar ellipticity ($\theta \times 100 / ([\text{Protein}] \times d)$, d is the cuvette path length in cm) by the amount of residues (N) of the corresponding protein ($N=675$ for EctoNicastrin; $N=550$ for EctoNicastrin $\Delta 40-164$; $N=284$ for SGAP). The molecular weights of EctoNicastrin and EctoNicastrin $\Delta 40-164$ were 75 kDa and 61 kDa, respectively. The molecular weight of SGAP was 30 kDa.

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