

SorLA Signaling by Regulated Intramembrane Proteolysis*

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The single-transmembrane receptor SorLA/LR11 contains binding domains typical for lipoprotein receptors and a VPS10 domain, which binds the neuropeptide head-activator. This undecapeptide enhances proliferation of neuronal precursor cells in a SorLA-dependent manner. Using specific inhibitors we found previously that head activator activates shedding of SorLA by the metalloprotease TACE close to the transmembrane domain releasing the large extracellular part of the receptor. Here we show that the remaining COOH-terminal membrane fragment of SorLA is processed by γ -secretase. Inhibition of γ -secretase by specific inhibitors or overexpression of dominant negative presenilin mutants and knock out of the presenilin genes led to accumulation of the SorLA membrane fragment and also of full-length SorLA in the membrane. In an *in vitro* assay we observed the γ -secretase-dependent release of the two soluble cleavage products, the SorLA cytoplasmic domain and the SorLA β -peptide. These processing steps are reminiscent of a novel signaling pathway that has been described for the notch receptor. Here, the notch cytoplasmic domain is released into the cytoplasm by the γ -secretase and migrates to the nucleus where it acts as a transcriptional regulator. In parallel we found that a fusion protein of the released cytoplasmic tail of SorLA with EGFP located to the nucleus only if the nuclear localization signal of SorLA was intact. In a reporter gene assay the cytoplasmic domain of SorLA acted as a transcriptional activator indicating that SorLA might directly regulate transcription after activation by γ -secretase.

The γ -secretase complex was first described to process the amyloid precursor protein (APP),² thereby releasing the A β -peptide, which accumulates in the brain of patients with Alzheimer disease (1). γ -Secretase consists of presenilin and at least three other integral membrane proteins and catalyzes the hydrolysis of a peptide bond in the transmembrane region of substrate proteins (2). In recent years, γ -secretase substrates in addition to APP were discovered (3). All of them are single spanning type I transmembrane proteins. Usually, γ -secretase does not proteolyse the full-length proteins, but so-called COOH-terminal fragments (CTFs). These CTFs are produced from the full-length proteins in a process called "ectodomain shedding," in which

another protease, often a metalloprotease, cleaves the precursor proteins close to the outer face of the transmembrane region.

In the case of the notch protein, this "regulated intramembrane proteolysis (RIP, for comparison see Fig. 8)" is part of a signal transduction cascade (4). After a preceding activation by the Golgi-localized protease furin, the notch receptor can bind its ligand. The ligand-bound receptor is subsequently a substrate for a metalloprotease, which sheds the notch ectodomain. The remaining CTF is cleaved by γ -secretase into a β -peptide at the outer face of the membrane and the notch intracellular domain. The notch intracellular domain migrates into the nucleus and, after binding to transcription factors, it regulates the transcription of target genes (5). Such a signaling cascade was proposed for other γ -secretase substrates, such as APP, ErbB-4, CD44, LRP1, and the β 2 sodium channel subunit (6–9). For CD44 a signaling cascade similar to that of notch indeed seems to exist (10). Nevertheless, in the other cases either the ligand is unknown (APP, β 2), the ICD could not be shown to enter the nucleus (LRP1), or transcriptional activation has not been observed (ErbB-4). Therefore it was proposed that the function of the γ -secretase complex for most of its substrates lies in the degradation of membrane spanning segments and only in some instances in signal transduction (3).

SorLA, also called LR11, is a type I transmembrane receptor with a 56-amino acid-long cytoplasmic COOH-terminal tail strongly expressed in neurons (11–13). Its large extracellular part is composed of a VPS10 domain followed by repeats as found in the family of lipoprotein receptors and fibronectin type III domains. The best characterized human protein with a VPS10 domain is sortilin (14), which binds the neuropeptide neurotensin (15). Sortilin can form heterodimers with the G-protein-coupled neurotensin receptors (16). In addition it also initiates a neurotensin signaling cascade in cells lacking other neurotensin receptors by activation of Erk/Akt kinases (17).

The first neuropeptide ligand identified for SorLA was the head activator. Head activator is involved in head regeneration of coelenterates (18) and binds to the hydra orthologue of SorLA with nanomolar affinity (19–21). In mammals, head activator mediates entry into mitosis and proliferation of neuronal and neuroendocrine precursor cells after binding to the VPS10 domain of SorLA (22). Other ligands of SorLA include lipoproteins (12), PDGF-BB (23), and GDNF (24). In accordance with the multiple ligands, SorLA also seems to be involved in several functions including protein sorting (25), lipoprotein uptake (26), smooth muscle cell migration, and the development of atherosclerosis (27), but so far no signaling cascade has been identified. Recently, SorLA was identified to be involved in trafficking APP in neuronal cells. The presence of SorLA seems to inhibit the production of the amyloid A β -peptide. Therefore, SorLA might play an important role in the development of Alzheimer disease (28–31).

SorLA is proteolytically processed in several steps. After removal of the signal peptide, a propeptide is cleaved off in the Golgi apparatus by furin (32). In a second step the large ectodomain of SorLA can be

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² The abbreviations used are: APP, amyloid precursor protein; CTF, COOH-terminal fragment; DAPT, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-5-phenylglycine t-butyl ester; LRP, low density lipoprotein receptor-related protein; SorLCD, SorLA intracellular domain; wt, wild-type; TACE, tumor necrosis factor- α -converting enzyme; EGFP, enhanced green fluorescent protein; RT, real-time.

released by the metalloprotease TACE (32, 33). This shedding seems to be constitutive at a low level, but is stimulated by phorbol esters and by the neuropeptide head activator (32). Since the released ectodomain is of the same apparent molecular mass as the transmembrane precursor, the metalloprotease cleavage site has to be close to the transmembrane domain.

These processing steps are reminiscent of the above mentioned notch signaling cascade. Therefore we analyzed whether SorLA is, like notch, a substrate for the γ -secretase. Here we provide evidence that the SorLA-CTF is cleaved in a presenilin-dependent manner into a β -peptide and the SorLA intracellular domain (SorICD). Dependent on a nuclear localization signal the SorICD can migrate into the nucleus where it might regulate the transcription of target genes.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Human embryonic kidney 293 (HEK-293) cells stably transfected with APP₆₉₅ containing the Swedish mutation (swAPP) (34) and either presenilin 1 wild-type (wt) or presenilin 1 D358N (35) were cultured in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 200 μ g/ml zeocin to select for presenilin 1 expression, and 200 μ g/ml G418 to select for swAPP expression. In addition, these cell lines were stably transfected with a construct expressing full-length SorLA or SorLA-CTF*-myc (Fig. 1). After hygromycin B selection (200 μ g/ml) SorLA expression was analyzed by Western blotting. For most experiments cell lines generated from single cells were used. Immortalized mouse embryonic fibroblast cells derived from presenilin 1^{+/+}/presenilin 2^{+/+} or presenilin 1^{-/-}/presenilin 2^{-/-} mice (36) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin/streptomycin. The human teratocarcinoma cell line NT2 (Ntera2/D1) (37) was cultured in optimized modified Eagle's medium (Opti-MEM) supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. African green monkey kidney fibroblasts (COS-7) were cultured and transfected by electroporation as described (38).

Transfections were carried out using FuGENE 6 (Roche Applied Science) for HEK-293 cells or Lipofectamine 2000 (Invitrogen) for mouse embryonic fibroblasts according to the supplier's instructions.

For γ -secretase inhibition cells were treated for 15 h with DAPT (*N*-[*N*-(3,5 difluorophenylacetyl-L-alanyl)]-S-phenylglycine *t*-butyl ester) or L685,458 (both from Calbiochem) dissolved in dimethyl sulfoxide or, as control, with dimethyl sulfoxide alone.

cDNA Constructs and Protein Expression—The full-length SorLA cDNA (22) was cloned into pcDNA3.1/Hygro(+) (Invitrogen, Fig. 1). The SorLA-CTF* construct was generated by polymerase chain reaction (PCR) using the primers L5-StubSorLA (5'-GGAAGCTTAACAGATCTGTGGGGAGCCT-3') and L3-SorLAStop(-) (5'-GGCTCGAGGGCTATCACCATGGGGACGTC-3') and the full-length SorLA cDNA as template. The PCR product was cloned into pSecTag B (Invitrogen). The resulting construct SorLA-CTF*-myc (Fig. 1) encoding an NH₂-terminal signal peptide and a COOH-terminal myc/His-tag was transferred into pcDNA3.1/Hygro(+). FLAG-SorLA-CTF*-myc was generated by inserting the annealed primers 5FLAG (5'-TGGATTACAAGGATGACGACGATAAGCAGC-3') and 3FLAG (5'-GCTTATCGTCGTCATCCTTGTAATCCAGCT-3') into the SfiI site of SorLA-CTF*-myc.

For the EGFP constructs SorICD was amplified by PCR with the primers 5SICD (5'-GGAAGCTTCTACACGAAGCACCAGGAGGCTG-3') and 3SICD (5'-GGGGATCCTCAGGCTATCACCATGGGGAC-3') from pcDNA3.1/Hygro(+)-SorLA and cloned into pEGFP-C2 (Clontech).

pEGFP-MutSorICD was generated by PCR-based site-directed mutagenesis of pEGFP-SorICD with the primers fNLS-Mut (5'-CTTCTACACGAAGCAGCCGCGCTGCAGAGCAGCTTTCAC-3') and rNLS-Mut (5'-GTGAAGCTGCTCTGCAGCGCGGCGTGCTTCGTGTAGAAG-3'). The pBind-SorICD construct expressing SorICD fused to the NH₂-terminal DNA-binding domain of Gal4 was synthesized by cloning the SorICD fragment from pEGFP-SorICD into the pBind vector (Promega). All cDNA constructs have been verified by sequencing.

Cell Lysis and Immunoblotting—Cells were lysed using STEN-lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% (v/v) Nonidet P-40). Insoluble particles were removed by centrifugation at 14,000 \times *g* for 15 min.

Immunoblotting was performed as described (21) using a polyclonal antiserum (anti-SorICD, 1:3000) directed against a COOH-terminal peptide of the intracellular domain of SorLA (32) or monoclonal antibodies against the myc- and the FLAG-M2-epitope (Sigma). Detection was performed by peroxidase-based chemiluminescence (Super Signal West Dura Extended, Pierce).

In Vitro Generation of SorICD—HEK-293 cells stably transfected with SorLA-CTF*-myc were resuspended (0.5 ml/10-cm dish) in homogenization buffer (10 mM HEPES, pH 7.0, 10 mM KCl, protease inhibitor mix (Complete mini, Roche Applied Science)) and homogenized by passing them 10 times through a syringe (23-gauge). The post-nuclear supernatant was prepared by centrifugation at 1000 \times *g* for 15 min. Membranes were pelleted from the supernatant by centrifugation at 14,000 \times *g* for 40 min and resuspended (50 μ l/10 cm dish) in assay buffer (150 mM sodium citrate, pH 6.4, protease inhibitor mix). For the generation of SorICD, 25- μ l membrane aliquots were incubated for 2 h at 37 $^{\circ}$ C. Soluble proteins were separated from the membranes by ultracentrifugation at 100,000 \times *g* for 1 h and analyzed by Western blotting.

Confocal Microscopy—24 h after transient transfection with EGFP-SorICD, EGFP-MutSorICD, or EGFP, COS-7 cells were fixed with 4% paraformaldehyde for 10 min and washed with phosphate-buffered saline. Microscopy was performed using a LSM 510 Meta instrument (Zeiss), which allows the quantification of fluorescence intensity in selected areas. To account for the different absolute fluorescence of the cells, the ratio of nuclear over cytoplasmic fluorescence intensity was calculated. The ratio of 40 cells for each construct was determined and the constructs compared by a paired two-sided Student's *t* test.

Luciferase Assay—COS-7 cells were transfected with equal DNA amounts of the pG5Luc luciferase reporter (Promega), pBIND (Promega), or pBIND-SorICD. As positive control cells were transfected with pG5Luc, pACT-MyoD (Promega), and pBind-Id (Promega). 48 h later cells were tested for luciferase expression with BrightGlo (Promega) according to the supplier's instructions. All values were calculated over pBIND activation. Data represent the average of duplicate wells in five independent experiments.

Immunoprecipitation—SorLA-CTF* was precipitated from solubilized cell membranes using anti-SorICD as described (32). Sor β was precipitated from the conditioned medium of HEK-293 cells 24 h after transient transfection with the FLAG-SorLA-CTF*-myc construct using anti-FLAG-M2-agarose for 3 h at 4 $^{\circ}$ C. Immunoprecipitates were washed four times with washing buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 0.2% Nonidet P-40) and analyzed for Sor β by immunoblotting with the FLAG-M2 antibody.

Quantitative RT-PCR—Total RNA was prepared using the Nucleospin RNA II kit (Macherey-Nagel). cDNA was synthesized using oligo(dT) as primer and the polymerase Moloney murine leukemia virus (Invitrogen) according to the supplier's instructions. 2 μ g of cDNA were used for the amplification of SorLA-specific cDNA in a quantitative

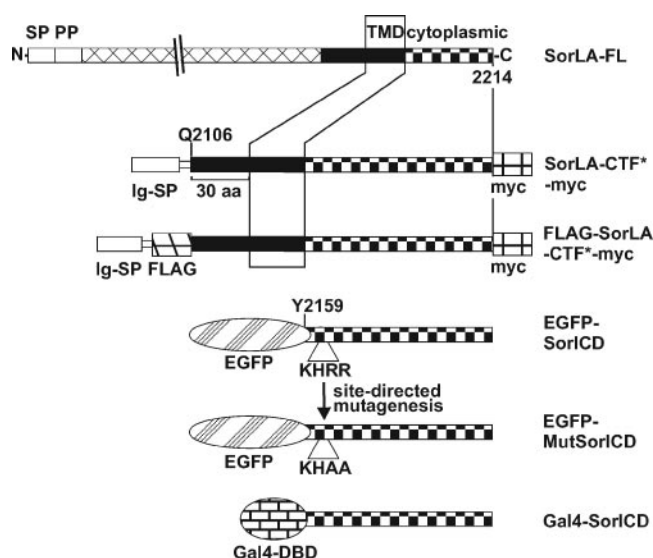


FIGURE 1. Expression constructs. Full-length SorLA (SorLA-FL) includes the signal peptide (SP), the propeptide (PP), and the large ectodomain. In the other constructs the COOH-terminal part around the transmembrane domain (TMD) is enlarged. SorLA-CTF*-myc and FLAG-SorLA-CTF*-myc contain the signal peptide of the immunoglobulin κ chain (Ig-SP). In the three lower constructs, the SorICD is preceded by either the EGFP or by the DNA-binding domain of Gal4 (Gal4-DBD).

real-time PCR in a LightCycler system (Roche Applied Science) with the primers L5-StubSorLA and L3-SorLAStop(-) using the LC-DNA Master-SYBR-Green Kit (Roche Applied Science). In parallel all samples were subjected to a quantitative PCR to amplify glyceraldehyde-3-phosphate dehydrogenase with the primers AS03 (GAGTCAACGGATTTGGTCGT) and AS04 (TTGATTTGGAGGGATCTCG). The integrity of the amplified DNA was analyzed by agarose gel electrophoresis. Samples were run in triplicates; all runs included a negative control. PCR efficiency of each value was taken into account using the freeware program LinRegPCR (39). Target gene values were normalized to their corresponding glyceraldehyde-3-phosphate dehydrogenase values.

Degradation of SorICD—SorICD was generated in the *in vitro* assay as described above in the absence of protease inhibitors in a 2-h incubation at 37 °C. Cytosol was prepared from NT2 cells by ultracentrifugation of the post-nuclear supernatant for 1 h at 100,000 \times g at 4 °C. To test the stability of the SorICD, 5 μ l of NT2 cytosol were added to 20 μ l of the SorICD sample and incubated for 2 h at 37 °C in the absence or presence of insulin, EDTA, or bovine serum albumin in the indicated amounts. The undegraded SorICD was analyzed by Western blotting with anti-SorICD.

RESULTS

Presenilin-dependent Release of SorICD—To find out whether SorLA is a substrate for presenilin-dependent proteolytic processing, we expressed a short membrane-anchored form of SorLA (SorLA-CTF*), which resembles the putative γ -secretase substrate after ectodomain shedding (SorLA-CTF). To investigate the influence of the γ -secretase, we stably expressed SorLA-CTF*-myc (Fig. 1) in HEK-293 cells producing presenilin 1 wt or the dominant negative presenilin 1 mutant D385N (40). Using Western blots with antibodies directed against SorICD or the attached myc-tag we identified the SorLA-CTF*-myc as an ~20-kDa protein in HEK-293 cells stably expressing wt presenilin 1 (Fig. 2A). Incubation with the specific γ -secretase inhibitor DAPT (41) led to accumulation of SorLA-CTF*-myc (Fig. 2A). This accumulation is even more pronounced in cells stably expressing the dominant negative presenilin 1 mutant D385N (Fig. 2B). To exclude side effects of the γ -se-

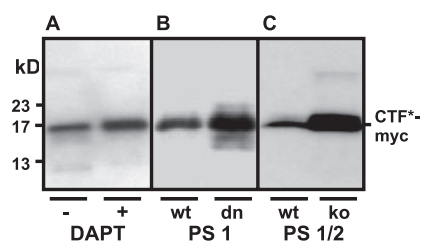


FIGURE 2. Accumulation of SorLA-CTF*-myc after reduction of γ -secretase activity. Cells were analyzed for the amount of SorLA-CTF*-myc by Western blotting using an anti-myc antiserum. In each lane equal amounts of protein from cell lysates were applied. **A**, HEK-293 cells stably expressing SorLA-CTF*-myc and presenilin 1 wild type were treated with the γ -secretase inhibitor DAPT (1 μ M) or the equivalent volume of the solvent Me₂SO for 15 h. **B**, HEK-293 cells stably expressing presenilin 1 wild-type (wt) or the dominant negative presenilin 1 mutant D385N (dn) were transiently transfected with SorLA-CTF*-myc and analyzed after 48 h. **C**, mouse embryonic fibroblasts from wild-type (wt) or presenilin 1^{-/-}/presenilin 2^{-/-} double knock-out (ko) mice were transiently transfected with SorLA-CTF*-myc. After 48 h an immunoprecipitation with anti-SorICD was performed to enrich SorLA-CTF*-myc for the Western analysis.

tase inhibitor or the presenilin 1 mutant D385N on the expression of SorLA-CTF*-myc we verified the results with immortalized mouse embryonic fibroblasts from wt and double knock-out mice for presenilin 1 and 2. As in cells expressing the dominant negative presenilin 1 mutant, SorLA-CTF*-myc was enriched in the fibroblasts from the knock-out mice (Fig. 2C).

One product of the γ -secretase intramembrane proteolysis should be the released intracellular domain of SorLA (SorICD). The cytoplasmic domain of SorLA consists of 56 amino acid residues. Together with some residues of the transmembrane domain and the attached tags (Fig. 1) we estimated the molecular mass of SorICD to be about 9 kDa. In Western blots of cell lysates the SorICD was usually missing. Nevertheless, in some experiments an immunoreactive fragment of ~13 kDa was detected in the cell lysate of HEK-293 cells overexpressing the SorLA-CTF*-myc. Upon incubation with DAPT this band vanished (Fig. 2A). For a more reliable analysis we adapted an *in vitro* assay developed for the detection of the intracellular domain of APP (42). The idea of the assay is to remove cellular proteases by a membrane preparation prior to the *in vitro* generation of the otherwise unstable SorICD. Incubation of membranes prepared from HEK-293 cells stably expressing SorLA-CTF*-myc led to a soluble SorICD fragment of ~13 kDa (Fig. 3). This fragment has the same apparent molecular mass as the SorICD detected in cell lysates (Fig. 2A) and could be visualized with antisera directed against the SorICD itself or against the attached myc-tag. Upon reduction of the temperature to inhibit proteases the fragment was absent. A similar inhibition was observed in the presence of the specific γ -secretase inhibitors DAPT and L685,458 (Fig. 3). Metalloprotease inhibitors like EDTA and proteasome inhibitors like lactacystin did not interfere with the SorICD production (data not shown).

The molecular mass of 9 kDa calculated for the myc/His-tagged SorICD is smaller than the observed apparent molecular weight of 13 kDa. This might be due to a reduced electrophoretic movement as observed for other proteins with highly charged tags or to phosphorylation of the intracellular domain. Taken together, these data indicate that SorLA is a novel γ -secretase substrate.

Secretion of the Sor β -peptide—Processing of β APP through γ -secretase results in the production of the cytoplasmic APP intracellular domain and the secreted A β -peptide. To analyze the production of the equivalent Sor β -peptide, we transiently transfected HEK-293 cells expressing wt or dominant negative presenilin with an NH₂-terminally FLAG-tagged and a COOH-terminally myc/His-tagged SorLA-CTF* (FLAG-SorLA-CTF*-myc, Fig. 1). Similar to SorLA-CTF*-myc, the FLAG-tagged CTF* accumulated in cells expressing the dominant neg-

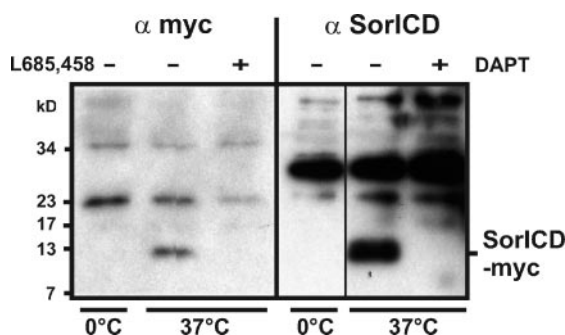


FIGURE 3. *In vitro* production of SorICD. Membranes from HEK-293 cells stably expressing SorLA-CTF*-myc and presenilin 1 wild type were incubated at the indicated temperature in the absence or presence of the γ -secretase inhibitors L685,458 or DAPT (1 μ M). The amount of SorICD*-myc was determined by Western blotting using the given antisera.

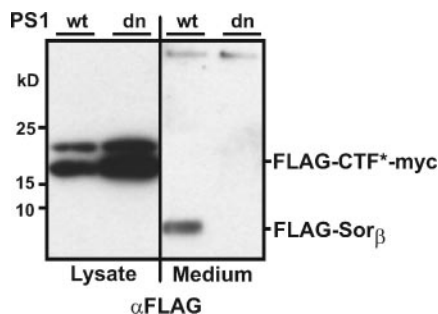


FIGURE 4. Release of Sor β . HEK-293 cells stably expressing presenilin 1 wild type (wt) or the dominant negative presenilin 1 mutant D385N (dn) were transiently transfected with FLAG-SorLA-CTF*-myc. After 72 h FLAG-Sor β was precipitated from the culture medium using anti-FLAG-agarose and the remaining cells were lysed. Sor β and the SorLA-CTF* were visualized by Western blotting with anti-FLAG. The two bands in the lysates might represent the overexpressed proteins with or without either the uncleaved signal peptide or a proteolyzed myc-tag. In each lane equal amounts of protein from cell lysates were applied.

active presenilin 1 mutant demonstrating that also FLAG-SorLA-CTF*-myc is a substrate for the γ -secretase (Fig. 4). We then precipitated soluble secreted FLAG-tagged proteins from conditioned medium of transfected HEK-293 cells with anti-FLAG-agarose. In a Western blot using an anti-FLAG antibody we identified the FLAG-tagged Sor β with a molecular mass of \sim 7 kDa in the precipitate (Fig. 4). Cells expressing the dominant negative presenilin 1 mutant did not produce Sor β .

Accumulation of Full-length SorLA after γ -Secretase Inhibition—To investigate the influence of presenilin on full-length SorLA (SorLA-FL), we stably expressed SorLA-FL (Fig. 1) in HEK-293 cells. In addition to full-length SorLA (Fig. 5A), the native SorLA-CTF was barely detectable with a molecular mass of 13 kDa. Incubation of the cells with DAPT led to an accumulation of SorLA-CTF as it was seen even more pronounced in cells expressing the dominant negative presenilin mutant. Interestingly, full-length SorLA also seems to accumulate depending on the level of presenilin inhibition. This effect is unusual and has not been observed in this magnitude for other γ -secretase substrates.

To rule out that the accumulation of full-length SorLA and the SorLA-CTF are artifacts of SorLA overexpression, we treated the human neurogenic embryonal carcinoma cell line NT2, which expresses SorLA endogenously, with the γ -secretase inhibitor DAPT (Fig. 5B). As in the HEK-293 cells, inhibition of the endogenous γ -secretase activity resulted in accumulation of the SorLA-CTF, confirming that SorLA is a natural γ -secretase substrate. The apparent molecular mass of the endogenous SorLA-CTF (about 13 kDa) was comparable with the mass of the CTF released from overexpressed full-length SorLA in HEK-293 cells (13.6 kDa). In contrast, the artificial SorLA-CTF* without tags, which bears 30 amino

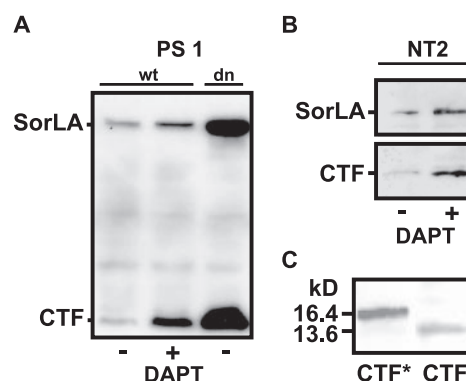


FIGURE 5. Accumulation of full-length SorLA after γ -secretase inhibition. A, heterologously expressed SorLA. HEK-293 cells stably expressing full-length SorLA and either presenilin 1 wild-type (wt) or the dominant negative presenilin 1 mutant D385N (dn) were treated with 5 μ M DAPT or the equivalent volume of the solvent Me₂SO for 15 h. Cell lysates were analyzed by Western blotting with anti-SorICD. B, endogenous SorLA. NT2 cells were treated as described for A. Due to the low amount of endogenous SorLA, total membranes instead of whole cell lysates had to be used for Western analysis. C, size determination of the CTF. Lysates from HEK-293 cells transiently overexpressing D385N presenilin and either the SorLA-CTF* (without tags) or full-length SorLA were analyzed by Western blotting. The CTF derived by metalloprotease cleavage of full-length SorLA was smaller than CTF*. In each lane equal amounts of protein were applied.

acids NH₂-terminal of the transmembrane domain (Fig. 1), showed a molecular mass of 16.4 kDa after overexpression in HEK-293 cells (Fig. 5C). Therefore the metalloprotease cleavage site in full-length SorLA seems to be less than 30 amino acids away from the membrane region. The mass difference of nearly 3 kDa indicates that less than 10 extracellular/luminal amino acids are enclosed in the endogenous SorLA-CTF.

In addition, again comparable with the experiments with heterologously expressed SorLA, also the endogenous full-length SorLA accumulated after γ -secretase inhibition.

The elevated levels of full-length SorLA could be explained either by a reduced activity of the metalloprotease or by an elevated production of SorLA mRNA and/or protein. To investigate whether the inhibition of the γ -secretase has an effect on the mRNA expression, we measured the SorLA mRNA levels in cells in the absence or presence of the γ -secretase inhibitor DAPT by quantitative RT-PCR. In HEK-293 cells stably expressing either full-length SorLA or SorLA-CTF*-myc under the control of a viral promoter, we did not observe increased levels of SorLA mRNA after treatment with DAPT. In contrast, the endogenous SorLA mRNA in NT2 cells was 3.2 times more abundant after γ -secretase inhibitor treatment.

Localization and Transactivation Activity of SorICD—Analysis of the SorICD by the program PSORT II resulted in the recognition of a putative nuclear localization sequence with the sequence KHRR (Fig. 6A). These basic amino acids might also be important in determining the topology of SorLA according to the “positive inside rule” (43). Nevertheless, the equivalent basic signal of human APP is not recognized as a nuclear localization sequence. To test the activity of the SorLA nuclear localization sequence, we constructed vectors encoding NH₂-terminally EGFP-tagged SorICDs (Fig. 1) with an intact putative nuclear localization sequence (EGFP-SorICD) or with a mutated motif (EGFP-MutSorICD). Confocal analysis revealed that EGFP-SorICD was localized in the nucleus of transfected COS-7 cells (Fig. 6B). In contrast, EGFP-MutSorICD is like EGFP alone distributed uniformly in the cytoplasm and nucleus. In a blind study we quantified the fluorescence intensity in defined areas of nucleus and cytoplasm of the transfected COS-7 cells with the confocal microscope. The ratio of the nuclear over cytoplasmic intensity was significantly higher in cells expressing EGFP-SorICD (Fig. 6C) than in cells with the mutated SorICD ($p = 0.0003$) or EGFP alone ($p = 0.000002$), thus indicating that SorICD relies on

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2132 RSTDVA~~AAVVVPIFLILL~~SLGVGF~~ALL~~YTKHRRIQSSFTAF~~A~~

2174 NSHYSSRLGSAIFSSGDDLGEDDEAPMITGFSDDVPMVIA*

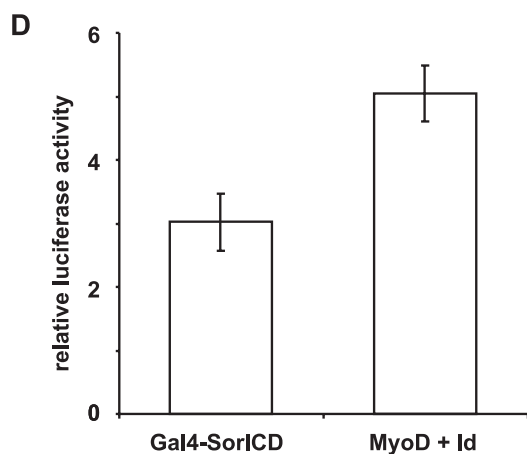
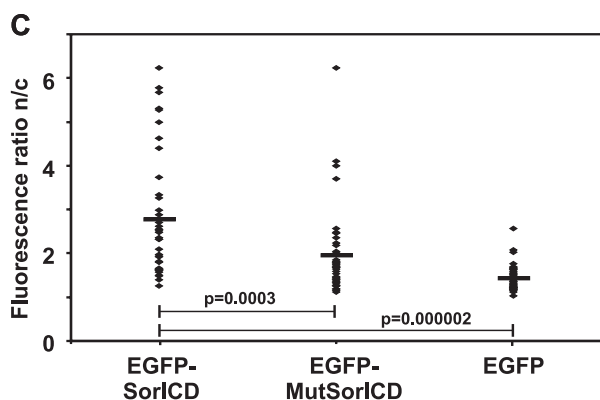
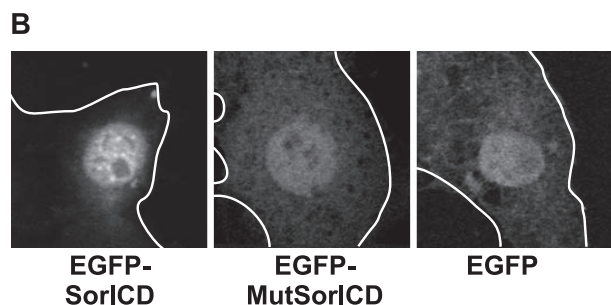


FIGURE 6. Nuclear localization and transcriptional activity of SorICD. *A*, sequence of the final 83 amino acids of human SorLA. The transmembrane domain is underlined, the nuclear localization signal KHRR is marked by a broad bar. Numbers indicate the position in the full-length protein. *B*, COS-7 cells expressing EGFP or a fusion protein composed of EGFP and SorICD with either the intact (KHRR) or a mutated nuclear localization signal (KHAA) were fixed 48 h after transfection and analyzed by confocal microscopy. The cell surface is indicated. *C*, ratio of the fluorescence intensity in nucleus and cytoplasm calculated for 40 cells of each construct. Mean values are indicated as black bars. Statistical significance as calculated with the paired two-sided Student's *t* test is given. *D*, transcriptional activation of a luciferase reporter. On the ordinate the induction of the luciferase activity over the Gal4 negative control is indicated. As positive control the well known transcriptional activator MyoD was used together with its binding partner Id.

its own nuclear localization sequence to migrate to the nucleus. Since EGFP alone shows some tendency to enter the nucleus, we tried to construct a larger fluorescent reporter protein containing two consecutive EGFP molecules preceding the SorLA tail. We verified the integrity of the larger protein by gel electrophoresis with subsequent visualization of the proteins on a fluorescence reader (Typhoon 9410, Amersham Biosciences). Nevertheless, the tend-

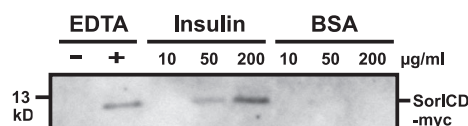


FIGURE 7. Degradation of SorICD. *In vitro* generated SorICD-myc was incubated at room temperature for 2 h with cytosol from NT2 cells in the absence or presence of EDTA (5 mM), insulin, or bovine serum albumin (BSA) in the indicated concentrations. The amount of remaining SorICD-myc was analyzed by Western blotting using anti-SorICD.

ency of the double-EGFP protein to enter the nucleus was similar to that of EGFP alone (data not shown).

Localization of the SorICD in the nucleus suggests a regulation of transcriptional processes. We therefore analyzed the capability of the SorICD to stimulate the transcription of a Gal4-dependent-luciferase reporter (Fig. 6*D*). Cells transfected with the construct expressing SorICD fused to the DNA-binding domain of yeast transcription factor Gal4 (pBIND-SorICD) showed a 3-fold higher stimulation of transcription compared with the DNA-binding domain of Gal4 alone. The positive control with Gal4-MyoD and VP16-Id expressing constructs exhibited a 5-fold higher activation than the Gal4 construct.

Degradation of SorICD by an Insulin-degrading Enzyme-like Activity—In general the intracellular domains of γ -secretase substrates are highly unstable and substrates for cytoplasmic proteases. Since lactacystin did not prevent SorICD degradation (data not shown), the proteasome is not responsible for the degradation. We then addressed the question whether the SorICD is like the APP intracellular domain (44) a substrate for the insulin degrading enzyme. For this purpose we generated SorICD in an *in vitro* assay and incubated it with cytoplasm derived from NT2 cells to provide the necessary proteolytic activity. In the presence of insulin as a competitive substrate for the insulin degrading enzyme, the degradation of the SorICD was prevented although equal amounts of bovine serum albumin had no influence (Fig. 7). As a metalloprotease the insulin-degrading enzyme is also inhibited by chelators of divalent cations. Inhibition of the breakdown of SorICD by EDTA is therefore another hint that the SorICD is degraded by the insulin-degrading enzyme similar to the APP intracellular domain.

DISCUSSION

SorLA binds multiple ligands and is involved in the regulation of head activator-induced mitosis (22), migration of smooth muscle cells (23), atherosclerosis (27, 45), and in the pathogenesis of Alzheimer disease (28–31). So far the signal transduction pathways are not well understood. SorLA is a mosaic receptor containing repeats as found in the family of lipoprotein receptors and a VPS10 domain. In addition to their role in ligand uptake, lipoprotein receptors also mediate signaling cascades involving protein phosphorylation, e.g. as LRP in the transmission of the PDGF signal (46, 47). Nevertheless, LRP1 and the VPS10 receptor sortilin are both substrates for sheddases, which remove their extracellular domains leaving behind membrane-bound CTFs (8, 48). Therefore, an alternative signaling pathway depending on regulated intramembrane proteolysis similar to notch signaling is possible.

Here we provide evidence that SorLA might also signal by regulated intramembrane proteolysis (Fig. 8). As in the case of notch, SorLA is first activated by a furin-like activity, then it can undergo ligand-induced ectodomain shedding via a metalloprotease. In a third step a constitutive intramembrane cleavage mediated by the presenilin- γ -secretase complex releases the SorICD into the cytosol and the Sor β -peptide into the extracellular space. Reduction of γ -secretase activity by specific inhibitors, by overexpression of dominant negative presenilin, or by knockout of the presenilin genes prevents proteolysis.

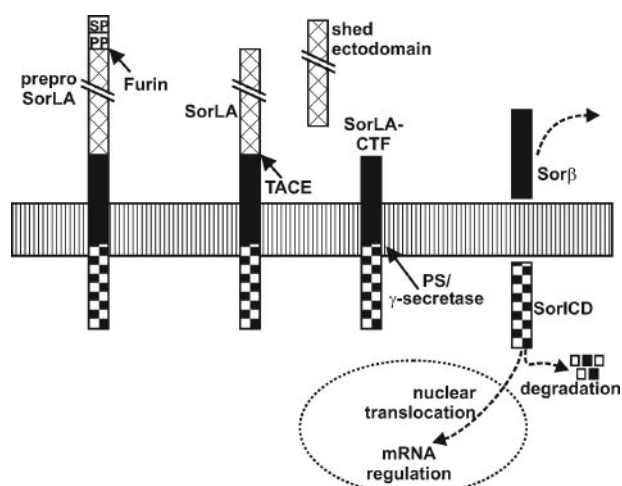


FIGURE 8. **Model for regulated intramembrane proteolysis of SorLA.** Mature SorLA with removed signal peptide (SP) and propeptide (PP) is produced in the Golgi compartment by furin cleavage. The metalloprotease TACE can shed the large soluble ectodomain in a ligand-induced manner. The remaining SorLA CTF is cleaved by the γ -secretase complex. The SorICD can subsequently enter the nucleus and might regulate the transcription or the mRNA stability of target genes.

It is difficult to detect the SorICD *in vivo*, since it is rapidly degraded as is the case for other ICDs. Even after overexpression we could not detect a myc-tagged SorICD by immunocytochemistry (data not shown). In contrast to the notch intracellular domain (5), the SorICD is not degraded in the proteasome. The APP intracellular domain is metabolized by an insulin-degrading enzyme-like activity (44). In our experiments we could prevent degradation of the SorICD with insulin and metal chelators, both known inhibitors of the metalloprotease insulin-degrading enzyme, similar to the experiments performed with the APP intracellular domain. The instability of the SorICD might be important for the inactivation of the signaling cascade.

For localization studies we expressed fusion proteins composed of EGFP and SorICD. These proteins migrate with high efficiency into the nucleus only if they contain the nuclear localization signal of SorICD. EGFP has the characteristic to enter to some extent the nucleus even in the absence of an additional nuclear localization signal. To reduce this background signal, we first tried to express a myc-tagged SorICD, but failed to detect it by immunocytochemistry. Next we analyzed fusion proteins consisting of two consecutive EGFP molecules, which, due to their extended size, were expected to be excluded from the nucleus. We confirmed the integrity of these fusion proteins by electrophoresis. Nevertheless, they entered the nucleus like EGFP alone. Our assumption is therefore based on our single-EGFP fusion proteins, which, after overexpression, depend on the SorICD to effectively enter the nucleus. This is similar to the notch intracellular domain, which also relies on its own nuclear localization sequence (5, 50), although other factors might be involved. In contrast the APP intracellular domain either enters the nucleus in a complex with Fe65 (51) or exhibits its signaling function outside the nucleus (52).

In the nucleus the notch intracellular domain participates in transcriptional activation through binding transcription factors (3). Since target genes of SorLA signaling are unknown, we performed a Gal4-based transactivation assay with the SorICD. We found a significant, but weak, stimulation of luciferase activity by the SorICD. A similar observation was made for the APP intracellular domain, where only in the presence of coactivators was a stronger stimulation achieved (53). We therefore assume that the SorICD might activate transcription but needs additional factors to be efficient.

Surprisingly, inhibition of γ -secretase led not only to the accumulation of the direct substrate, SorLA-CTF, but also of full-length SorLA. The accumulation was observed in HEK-293 cells stably overexpressing SorLA and presenilin 1 after incubation with the γ -secretase inhibitor DAPT. These results were confirmed in HEK-293 cells stably overexpressing a dominant negative mutant of presenilin and in NT2 cells, which endogenously express SorLA. Therefore, the accumulation cannot be an artifact of SorLA overexpression. For other γ -secretase substrates, if at all, only a less pronounced rise was observed. One explanation would be that full-length SorLA is, like the CTF, a substrate for the secretase as is the case for the cell-cell adhesion molecule E-cadherin (54). Nevertheless, we can exclude this, since metalloprotease inhibitors totally block the release of the SorLA ectodomain (32). Therefore, the accumulation suggests some kind of feedback mechanism in which the released SorICD reduces the amount of full-length SorLA. To test an influence of γ -secretase inhibition on the amount of the SorLA mRNA, we performed quantitative RT-PCR. Indeed, we found elevated mRNA levels after DAPT treatment of NT2 cells that endogenously express SorLA. These data hint at either a stimulation of SorLA transcription or a stabilization of the SorLA mRNA by the SorICD. In HEK-293 cells stably expressing either full-length SorLA or SorLA-CTF^{*}-myc under the control of a viral promoter, we did not observe increased levels of SorLA mRNA after treatment with DAPT. Since on the protein level heterologously expressed full-length SorLA accumulates after γ -secretase inhibition even more pronounced (Fig. 5A) than in NT2 cells (Fig. 5B), we conclude that at least in the case of the overexpressed SorLA a mechanism different from mRNA production or stabilization must be involved. This could include either a more efficient translation of the SorLA mRNA or, more probable, a decrease in the degradation of full-length SorLA.

Several links connect SorLA to Alzheimer disease: SorLA binds and internalizes apolipoprotein E-containing lipoproteins, which are genetically linked to the disease. SorLA is most highly expressed in brain but is down-regulated in the brains of Alzheimer patients (31). Recently it was shown that SorLA interacts directly with APP and inhibits A_{β} production by regulating the trafficking of APP (28–30). Finally we could show here that SorLA is, like the amyloidogenic protein APP, a substrate for the presenilin-containing γ -secretase complex, which is necessary for the production of the A_{β} -peptide. Therefore, SorLA might be a competitive substrate of APP for γ -secretase similar to notch (55) and LRP (49). This could explain why reduced SorLA levels in brain would lead to an increased production of A_{β} and finally to the development of Alzheimer disease.

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