

Ectodomain shedding, translocation and synthesis of SorLA are stimulated by its ligand head activator

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SUMMARY

The single transmembrane receptor SorLA is the mammalian orthologue of the head activator-binding protein, HAB, from hydra. The human neuronal precursor cell line NT2 and the neuroendocrine cell line BON produce head activator (HA) and respond to HA by entry into mitosis and cell proliferation. They express SorLA, and bind HA with nanomolar affinity. HA coupled to Sepharose is able to precipitate SorLA specifically proving that SorLA binds HA. Using antisera directed against extra- and intracellular epitopes we find SorLA as membrane receptor and as soluble protein released from cells into the culture medium. Cell lines differ strongly in processing of SorLA, with NT2 cells expressing SorLA mainly as membrane receptor, whereas release predominates in BON cells. Soluble SorLA lacks the intracellular domain and is shed from the transmembrane protein by a metalloprotease. Release from cells and brain slices is

stimulated by HA and by phorbol ester, and it is blocked by a metalloprotease inhibitor and by lowering the temperature to 20°C. Blockade of SorLA shedding and treatment of cells with SorLA antisense oligonucleotides lead to a decrease in the rate of cell proliferation. From this we conclude that SorLA is necessary to mediate the mitogenic effect of endogenous HA. HA enhances the translocation of SorLA from internal membranes to the cell surface and its internalization. In addition, HA stimulates SorLA synthesis hinting at an autocatalytic feedback loop in which the ligand activates production, processing, and translocation of its receptor.

Key words: Head-activator receptor, SorLA processing, Furin propeptide cleavage, Ectodomain shedding, Lipoprotein receptor homology, Metalloprotease, LR11

INTRODUCTION

Recently, we identified a receptor for the neuropeptide head activator (HA) from hydra by photoaffinity labeling, HA affinity purification, partial protein sequencing, and isolation of its cDNA and named it HAB for HA-binding protein (Franke et al., 1997; Hampe et al., 1996; Hampe et al., 1999b). HAB is a type I transmembrane receptor with a short carboxy-terminal cytoplasmic tail containing motifs involved in sorting, internalization, and G-protein coupling (Hampe et al., 1999b). In addition to repeats typical for members of the low density lipoprotein receptor (LDL-R) family, a VPS10 domain and fibronectin type III modules are found in the large extracellular part of HAB. Specific antibodies revealed the presence of HAB in hydra as a transmembrane receptor, but also as released protein, both capable of binding HA. Rapid release of soluble HAB from the membrane-bound protein during head regeneration hinted at a highly regulated process of ectodomain shedding necessary for HA action in hydra (Hampe et al., 1999a).

The undecapeptide HA mediates head-specific growth and differentiation processes in hydra, hence its name. HA is found in a gradient with maximal concentration in the head

region, and it plays an important role in hydra head regeneration. At the cellular level HA in hydra promotes proliferation of all cell types, at higher concentrations it triggers determination of stem cells to head-specific fates (Hampe et al., 1999a; Hobmayer et al., 1997). HA was isolated with identical sequence from mammals (Bodenmüller and Schaller, 1981). There it is found during early development of the nervous and neuroendocrine systems. In the adult the main locations are the hypothalamus, the upper part of the intestine, and various neuroendocrine organs. HA is secreted by neuroendocrine and neuronal cells and stimulates entry into mitosis and proliferation of precursor cells in an autocatalytic feedback loop. HA also stabilizes nerve-cell survival and enhances neurite outgrowth (Kajiwara and Sato, 1986; Kayser et al., 1998; Quach et al., 1992; Ulrich et al., 1996). The signaling cascade from HA to stimulation of mitosis includes activation of G proteins and of calcium and potassium channels in the plasma membrane. HA action can be blocked by respective inhibitors such as pertussis toxin, an inhibitor of the heterotrimeric inhibitory G protein, by SK&F96365 for the calcium channel, and by clotrimazole for the Gardos-type potassium channel (Hampe et al., 1999a; Kayser et al., 1998; Ulrich et al., 1996).

SorLA (also called LR11) was originally cloned from human, chicken, and rabbit cDNA libraries as a new member of the LDL-R family (Jacobsen et al., 1996; Mörwald et al., 1997; Yamazaki et al., 1996). Combination and alignment of domains are identical between hydra HAB and mammalian SorLA and not found in any other protein. Human SorLA binds the ER-resident LDL-R associated protein RAP (Bu and Schwartz, 1998; Jacobsen et al., 1996) and apolipoprotein E containing lipoproteins (Yamazaki et al., 1996). Highest concentrations of SorLA mRNA were found in the central nervous system. To gain insight into the function of this new type of protein we isolated the murine SorLA cDNA (Hermans-Borgmeyer et al., 1998). In situ RNA hybridization analysis revealed that SorLA expression is restricted to specific neuronal populations in the adult brain and to various locations in the peripheral nervous system and neuroendocrine glands. In the adult brain transcripts were most abundant in large neurons with long processes, such as pyramidal cells of the cerebral cortex and of the hippocampus and the Purkinje cells of the cerebellum. During embryonic development SorLA displayed a unique pattern of expression in specific regions of the brain (Hermans-Borgmeyer et al., 1998). The localization of HAB and SorLA on HA responsive cells and the expression pattern of SorLA in neurons of the developing and adult brain are compatible with a receptor function for the neuropeptide HA (Hampe et al., 1999a; Hermans-Borgmeyer et al., 1998). A link between SorLA and cell cycle control is suggested by the work of Kanaki et al. (1999) who found that SorLA is upregulated in proliferating smooth muscle cells and atherosclerotic lesions. In addition, Hirayama et al. (2000) recently described a higher SorLA expression in proliferating compared to differentiating neuroblastoma cells.

In this paper we show that SorLA binds HA and that it is present in cell lines which respond to HA by cell proliferation and entry into mitosis. SorLA exists as transmembrane protein, and it can be shed from cultured cells and from brain slices by a metalloprotease. Interference with SorLA shedding and synthesis acts antagonistically to the effect of HA on cell proliferation indicating a critical role of SorLA for HA function in mammals.

MATERIALS AND METHODS

Cell culture

NT2 cells (Pleasure and Lee, 1993) were cultured in Opti-MEM and BON cells (Evers et al., 1994) in Dulbecco's Nut-Mix F-12 (GibcoBRL), both supplemented with 5% fetal bovine serum. For the production of conditioned medium, the cells were grown in defined medium as described by Kayser et al. (1998). As indicated, brefeldin A (Sigma), the metalloprotease inhibitor BB-3103 (British Biotech), the phorbol ester PMA (Sigma), monomerized HA peptide (Bodenmüller et al., 1986), or HA homobipeptide (Neubauer et al., 1991) were added to the growth medium. Mitotic cells were counted after staining with Hoechst 33258, and cell proliferation was measured by coulter counting.

The antisense oligonucleotide 5'-TGCTGCTCCGTGTGCGCCAT-GTTCGGTCTCCGAA-3', directed against the translation start site of SorLA, was modified in the first three bases by phosphorothioate, and the last nine bases were added as loop. The first and last three bases of the sense control oligonucleotide 5'-CGAACATGGCGACA-CGGAGCAGCA-3' were also modified by phosphorothioate

(Eurogentec). The oligonucleotides were present in the culture medium of NT2 cells for 2 days and of BON cells for 4 days.

Binding studies

HA binding was determined by a filter binding assay (Hampe et al., 1996). For each data point 2×10^6 NT2 cells were solubilized with 100 μ l of a solution containing 1% Triton X-100, 50 mM MES, pH 6, 150 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 1 μ g/ml leupeptin, 1 mM Pefabloc SC, and 1 mM benzamidine for 1 hour at 4°C with gentle agitation. Non-solubilized material was removed by centrifugation at 100,000 *g*.

Quantitative competitive RT-PCR

Quantitative competitive reverse transcriptase polymerase chain reaction (RT-PCR) is based on the coamplification of a part of the mRNA of interest with an added RNA mimic of a different size with the same primers (Zhang et al., 1997). For better comparison of the SorLA-mRNA amount between different RNA sources, we also quantified the mRNA of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To amplify a 493 bp fragment from human and murine SorLA mRNA, the primers used extended from nucleotides 1592-1615 and 2084-2060 (numbers as in GenBank AF031816). The SorLA mimic of 367 bp was produced from murine SorLA cDNA using the same reverse primer and the forward primer extended at the 3' end by nucleotides 1741-1762. The amplified fragment was cloned into pGEM-T Easy (Promega) and the SorLA sense mRNA mimic was transcribed using T7 RNA polymerase (Ambion). Contaminating DNA was removed by DNase I digestion in the presence of MnCl₂ and by acidic extraction with phenol/chloroform. GAPDH was amplified using primers comprising nucleotides 4206-4225 and 4761-4742 (GenBank J04038). The forward primer extended by nucleotides 4350-4366 was used for mimic construction.

RNA was purified using Trizol (GibcoBRL) and QIAshredder (Qiagen) according to the suppliers' instructions. Contaminating DNA was removed by DNase I digestion. For quantification 0.1-5 μ g RNA were mixed with various concentrations of the SorLA or the GAPDH mimic, and an RT-PCR (Titan, Boehringer) with the respective primers was performed (30 cycles at an annealing temperature of 65°C). After gel electrophoresis and ethidium bromide staining the amount of RNA in the sample was expressed as the concentration of the mimic yielding the same amount of amplified product. The relative amount of SorLA mRNA was then calculated by dividing the concentrations of the SorLA and the GAPDH mRNAs.

Antibody production

A murine SorLA construct comprising amino acids 1384-1676 (numbers as in GenBank AAC16739), tagged amino-terminally with His₆, was produced in *E. coli* using the vector pQE31 (Qiagen). The protein was solubilized with 6 M guanidine hydrochloride and purified on Ni-NTA resin (Qiagen). The best antisera (F3) were obtained from rabbits immunized with an antigen which had been renatured by treating the resin with a gradient from 6-0 M guanidine hydrochloride. Preabsorption was carried out by incubation with the antigen blotted to Immobilon-P membranes (Millipore). As a control, the membrane was saturated with bovine serum albumin.

A polyclonal antiserum against the intracellular domain of murine SorLA (IC) was raised in rabbits against a peptide consisting of the last 18 carboxy-terminal amino acids coupled to keyhole limpet hemocyanin via an amino-terminally attached cysteine residue.

Expression of SorLA constructs

Fragments of the human SorLA cDNA (nucleotides 3053-6702 and 4720-6702 of GenBank Y08110) were cloned into pSecTag (Invitrogen) and electroporated into Cos7 cells. The smaller fragment was also introduced into a replication deficient adenovirus using the shuttle vector pAdTrack-CMV and homologous recombination in

bacteria (He et al., 1998). The recombinant virus was produced in 293 helper cells (Graham et al., 1977). BON cells were infected at a multiplicity of infection of 1-5 and harvested 2 days later.

Western blotting and sample preparation

Routinely, proteins from equivalent samples were separated on reducing 7% SDS-polyacrylamide gels and transferred to Immobilon-P membranes. SorLA was detected on the blots using the indicated primary antisera at a dilution of 1:3,000 and an alkaline phosphatase-conjugated secondary antibody. For analysis of the apparent molecular mass of SorLA we used 3.5% polyacrylamide gels and crosslinked phosphorylase B as a marker according to the Sigma technical bulletin MWS-877X.

Conditioned medium was centrifuged at 1000 *g* and 100,000 *g* to remove cell debris and concentrated 100-fold using ultrafiltration on Centricon 100 (Millipore) prior to western blotting. For membrane preparation cultured cells were disrupted with a Teflon homogenizer in 30 mM HEPES, pH 7.4, 0.1 mM ethylenediamine-tetraacetic acid (EDTA), 250 mM sucrose and protease inhibitors. Nuclei were removed by centrifugation at 1,000 *g*, and membranes were obtained by sedimentation at 100,000 *g*. Mouse brains were minced in liquid nitrogen by mechanical means and further processed on ice using a Teflon homogenizer (15 strokes at 150 rpm) in 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES pH 7.4, 1 µg/ml leupeptin, 1 mM Pefabloc SC, and 1 mM phenylmethylsulfonyl fluoride. The centrifugation steps were performed as given above.

Precipitation with HA Sepharose

HA was coupled to activated Sepharose 4B as described earlier (Franke et al., 1997). Conditioned medium from BON cells (4 ml) was incubated with 100 µl HA Sepharose or control Sepharose at 4°C overnight in the presence of 1 µg/ml leupeptin, 1 mM Pefabloc SC, and 1 mM phenylmethylsulfonyl fluoride. The Sepharoses were washed with 50 mM MES pH 6, 0.1% Triton X-100, supplemented at first with 75 mM, then with 2 M NaCl, and the amount of bound SorLA was assayed by western blotting.

Immunocytochemistry

Cells were permeabilized by fixation in 7% acetic acid, 7% glycerol, and 4% paraformaldehyde for 30 minutes at room temperature, followed by 3× 10 minutes washes with 0.1% Triton X-100 in PBS. Cells were preabsorbed using 1% bovine serum albumin in PBS/Triton and incubated with the primary antisera overnight at 4°C or for 1 hour at room temperature. To demonstrate immunoreactivity on the surface of cells, living cells were treated with the primary antisera diluted in defined medium for 20 minutes at 37°C. After PBS washing, cells were fixed and treated as above. In some experiments, as indicated, cells were fixed in 1% acetic acid in ethanol for 5 minutes at -20°C (Kanzaki et al., 1999). To visualize immune complexes Cy3-coupled secondary antibodies were used.

In vivo labeling and immunoprecipitation

NT2 cells were grown in labeling medium (Dulbecco's modified eagle medium without cysteine and methionine, GibcoBRL) supplemented as described by Kayser et al. (1998) and containing 0.14 mCi/ml of a mixture of [³⁵S]cysteine and [³⁵S]methionine (Promix, Amersham). For chase the cells were transferred to Opti-MEM with 5% fetal calf serum. Subsequently the cells were solubilized in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 50 mM Tris-HCl, pH 8) with protease inhibitors (complete, Roche Molecular Biochemicals) for 30 minutes at 4°C. After centrifugation at 100,000 *g* and preclearing with bovine serum albumin-treated Protein A Sepharose (Pharmacia, 20 µl), the supernatant (500 µl) was incubated with 1-5 µl antiserum for 2 hours on ice. Immune complexes were precipitated with Protein A Sepharose (20 µl), washed five times with RIPA buffer, and the pellets were subjected to reducing 7% SDS-page and autoradiography in a

phosphoimager (Fuji, Bas2000). As indicated, brefeldin A (Sigma) was added to the labeling medium at a concentration of 20 µg/ml. PNGaseF (Roche Molecular Biochemicals) digestion and furin (Alexis) cleavage of immunoprecipitated proteins were performed according to the manufacturers' instructions.

Removal of peripherally attached proteins from mouse brain membranes

Mouse brain membranes were incubated with 10 mM HEPES, pH 7.8, 0.14 M NaCl and 1% CHAPS, with 10 mM HEPES, pH 7.8, and 1 M NaCl, or with 100 mM Na₂CO₃ pH 11.5 as described (Hampe et al., 1999b). The amount of soluble SorLA was assayed by western blotting.

SorLA shedding from brain slices

Freshly prepared mouse brains were cut into 200 µm slices using a Vibroslice (Campden Instruments) while bathed in ice-cold artificial cerebrospinal fluid (Liss et al., 1999). After aspiration for 20 minutes at 4°C the slices were incubated in defined medium for 2 or 4 hours at 37°C. Conditioned medium was collected from these slices, cleared by 100,000 *g* centrifugation, and concentrated 50-100× by ultrafiltration (Centricon 100, Millipore). For membrane preparation the slices were ultrasonicated in 20 mM Tris-HCl, pH 7.5, 2 mM EDTA and protease inhibitors (complete, Roche Molecular Biochemicals) for 10 seconds at 4°C, followed by centrifugation at 100,000 *g*.

RESULTS

The HA-responsive cell lines NT2 and BON express SorLA

For the human neuroendocrine cell line BON we had shown earlier that HA stimulates entry into mitosis and cell proliferation (Kayser et al., 1998). Similarly proliferation and entry into mitosis of the human neurogenic embryonal carcinoma cell line NT2 were stimulated by the neuropeptide HA (Fig. 1A,B). These cells bound a radiolabeled HA analogue with nanomolar affinity (Fig. 1C), which we had also found for the head-activator binding protein HAB in hydra (Hampe et al., 1999b). The only mammalian homologue of hydra HAB is SorLA, which has the same unique combination of extracellular modules. No further mammalian homologues could be identified by PCR experiments using degenerate primers or by analysis of the EST-database. We therefore wanted to find out whether SorLA is present in the HA-responsive cell lines NT2 and BON. Using a quantitative competitive RT-PCR assay, which determines the amount of SorLA mRNA in relation to that of the house-keeping gene GAPDH, we detected high message levels in BON and NT2 cells in concentrations more than 100,000 times higher than found in murine 3T3 fibroblasts, which do not respond to HA (Fig. 2). Murine brain contained even higher levels of the SorLA mRNA in agreement with northern blotting and in-situ hybridization experiments (Hermans-Borgmeyer et al., 1998; Jacobsen et al., 1996).

SorLA is present as a transmembrane receptor in murine brain

For characterization of the protein we raised two antisera against different epitopes of murine SorLA. As antigens we used a recombinant protein comprising the first 4.5 extracellular fibronectin type III domains (F3), purified from

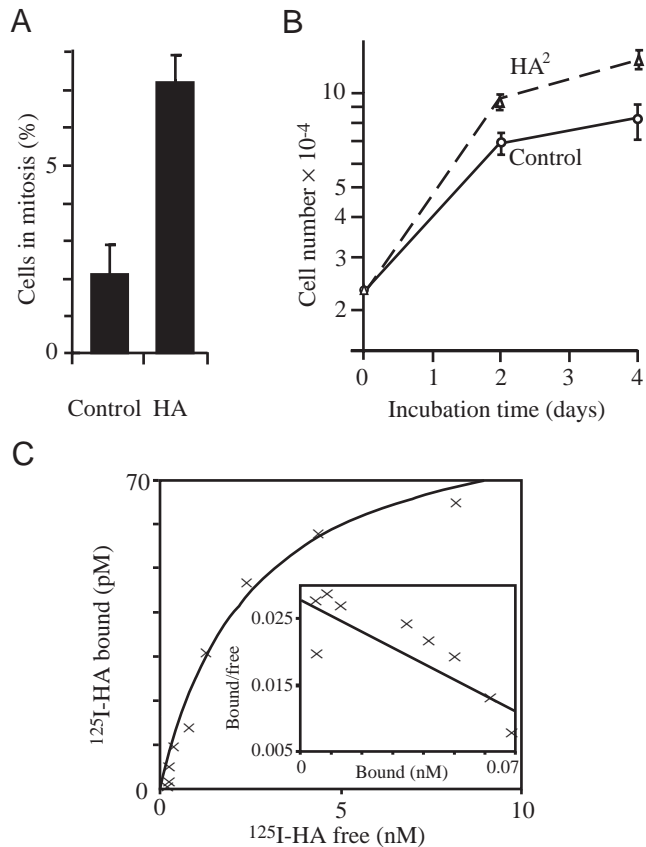


Fig. 1. NT2 cells respond to and bind HA. (A) HA stimulates mitosis. NT2 cells were incubated with or without 2 nM monomerized HA for 1.5 hours. After fixation cells were stained with Hoechst 33258. Mitotic nuclei were counted in 5×400 cells each and expressed as means ± s.e.m. (B) HA stimulates cell proliferation. NT2 cells were cultured in the absence or presence of 1 nM HA homopeptide (HA²). The total cell number was determined after 2 and 4 days by coulter counter. Three samples were counted each and shown as means ± s.e.m. (C) Saturation binding analysis of ¹²⁵I-HA bipptide to solubilized NT2 cells. Non specific binding measured in the presence of 0.5 μM [I-Tyr¹¹]HA was subtracted prior to analysis. The scatchard plot is given in the inset. A K_D of 2.5±0.4 nM was calculated by non-linear regression.

E. coli, and a synthetic peptide from the intracellular tail (IC) coupled to keyhole limpet hemocyanin. To test the specificity of the antisera, we heterologously expressed two carboxy-terminal SorLA fragments in Cos7 cells. Both contained the fibronectin type III module, the transmembrane domain, and the intracellular tail and were supplemented with a myc-tag. The antisera reacted with the SorLA fragments on western blots as did the α-myc antibody. No signal was seen on mock-transfected Cos7 cells (Fig. 3A). Both antisera recognized endogenous SorLA with its expected molecular mass of >250 kDa in a mouse brain membrane preparation (Fig. 3B). Serum preabsorbed with its SorLA antigen and the preimmune sera did not interact with SorLA. As second immunoreactivity a 100 kDa protein was detected in mouse brain by the antiserum directed against the extracellular domain, but not by the preabsorbed serum (Fig. 3B). It might be a fragment of full-length SorLA generated by alternative splicing or by

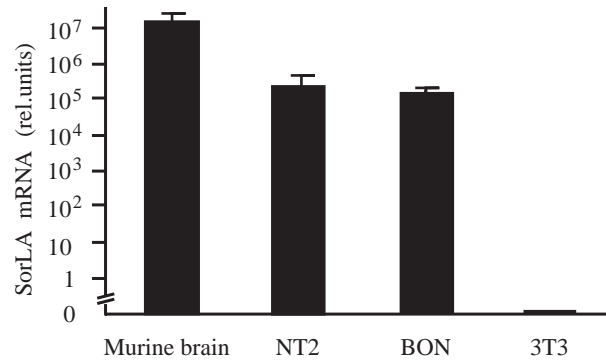


Fig. 2. Quantification of SorLA mRNA by quantitative competitive RT-PCR in murine brain, mouse 3T3 fibroblasts, and in human NT2 and BON cells. SorLA mRNA and GAPDH mRNA were independently quantified from the same sample of RNA, and the relative concentration of SorLA mRNA was calculated by division of the two values.

proteolytic cleavage. Using 3.5% polyacrylamide gels and crosslinked phosphorylase B as marker, we estimated the apparent molecular mass of SorLA to be 330 kDa, both in reducing and non-reducing SDS-page (Fig. 3C).

Soluble SorLA binds HA

We had previously found that the hydra homologue of SorLA, HAB, is present in hydra in a transmembrane and a slightly smaller soluble form, which lacks the transmembrane domain and the carboxy-terminal tail (Hampe et al., 1999b). Similarly, in addition to the membrane-bound form, the antiserum against the extracellular domain detected a soluble form of SorLA in conditioned medium of the cell lines BON and NT2 (Fig. 4A). The apparent molecular mass of soluble SorLA appeared to be slightly smaller than that of the membrane-bound form. Western blotting with the antiserum against the intracellular domain showed that soluble SorLA lacked the cytoplasmic tail (Fig. 4A). As estimated from western blots BON cells released about 95% of total SorLA into the medium during a 16 hours cultivation period. The membrane-bound form could often not be detected. In contrast, NT2 cells contained relatively high amounts of SorLA in the membrane fraction and released less into the medium. SorLA accumulated in the conditioned medium of BON cells starting with relatively low levels after one and much higher levels after 24 hours of cultivation (Fig. 4B). Released SorLA bound to HA immobilized on Sepharose but not to a control Sepharose (Fig. 4C), as had been found for soluble HAB from hydra.

To determine the localization of SorLA within cells, we performed immunocytochemistry on permeabilized NT2 cells and found SorLA predominantly in a perinuclear compartment (Fig. 5A,B). A punctate staining pattern was recognized by both types of antisera and hints at a localization of SorLA in intracellular vesicles.

SorLA is posttranslationally processed by furin

A propeptide of about 50 amino acids is cleaved from the N-terminus of the VPS10 domain of the neurotensin receptor sortilin by furin or a similar protease (Petersen et al., 1999). N-terminal sequencing of HAB and SorLA (Franke et al.,

1997; Jacobsen et al., 1996) also suggested cleavage of a similar propeptide at a putative furin processing site during maturation. Using NT2 cells we studied SorLA processing by metabolic ^{35}S -labeling and immunoprecipitation of solubilized SorLA with the antiserum directed against the extracellular domain. After 0.5 hours a proform of SorLA was detected (Fig. 6). Labeling for 4 hours resulted in a second immunoprecipitated band with a slightly larger molecular mass. Treatment with brefeldin A, which blocks protein translocation from the endoplasmic reticulum to the Golgi

apparatus, prevented the appearance of the larger form indicating that it was produced in a Golgi or post-Golgi compartment. After a chase of 2 hours with unlabeled medium the large molecular mass form was predominant in accordance with a production in a post-ER compartment. Incubation of the two SorLA forms with PNGaseF to remove N-linked sugars resulted in one broad band with a smaller molecular mass. Therefore, most of the difference between the two SorLA forms can be attributed to changes in N-glycosylation. To elucidate propeptide cleavage of the two SorLA forms we incubated them with furin (Fig. 6, lower panel). The size of the smaller ER form of SorLA was reduced by furin both in glycosylated and deglycosylated samples, whereas the larger form was unchanged. After deglycosylation and processing by furin the molecular masses of the two forms were indistinguishable. From these data we conclude that pro-SorLA is core-glycosylated in the ER. In a Golgi or post-Golgi compartment the glycosylation is modified, and the propeptide is cleaved off.

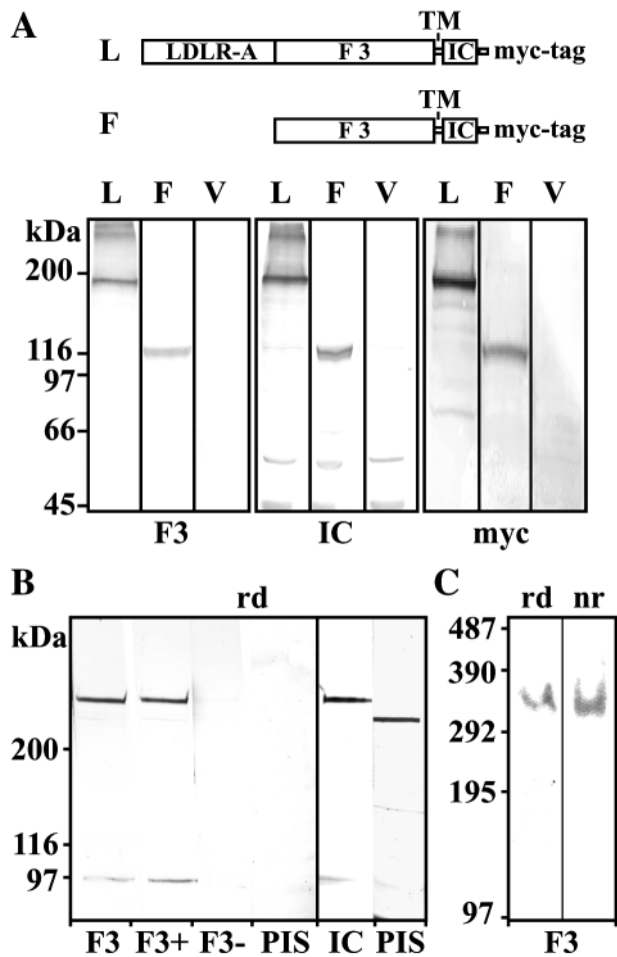


Fig. 3. Characterization of antisera against SorLA and recognition of endogenous SorLA. (A) Detection of heterologously expressed SorLA. Cos7 cells were transfected with the indicated SorLA constructs (L,F) or with the vector alone (V). After 3 days cell membranes were subjected to western blotting and probed with antisera against the extracellular (F3) or the intracellular (IC) domains of SorLA or against the myc-tag. (B) SorLA in mouse brain membranes. Both antisera detected a band at >250 kDa in western blots corresponding to full-length SorLA. Specificity was shown by using the antiserum against the extracellular domain preabsorbed with the bacterially expressed SorLA-antigen (F3-) or, as control, with bovine serum albumin (F3+). The respective preimmune sera (PIS) did not detect the >250 kDa band. (C) Apparent molecular mass of SorLA. Mouse brain membranes were subjected to 3.5% polyacrylamide/urea gels under reducing (rd) and non-reducing (nr) conditions. After western blotting the apparent molecular mass of SorLA was calculated to be 330 kDa using crosslinked phosphorylase B as marker.

Ectodomain shedding of SorLA is regulated by a metalloprotease and HA

Soluble SorLA could be generated by alternative splicing or by protein processing. If alternative splicing would take place, a SorLA-mRNA variant should be found which differs from the known mRNA in the part coding for the transmembrane domain or the preceding fibronectin type III repeats. Of the

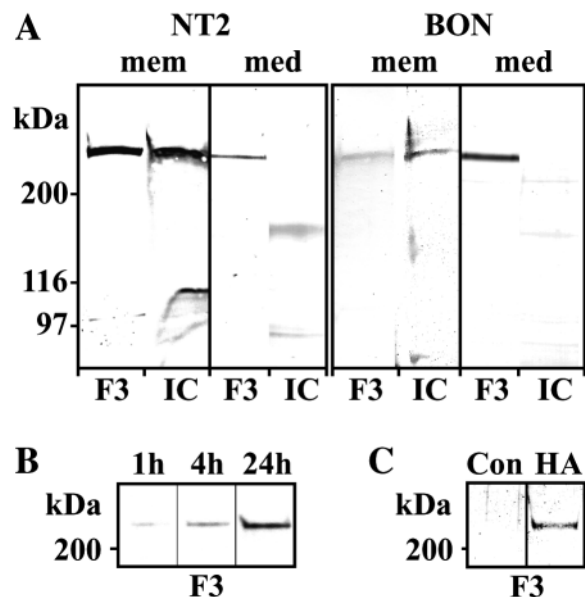


Fig. 4. SorLA exists as a transmembrane and as a soluble protein which binds HA. The amount of SorLA was analyzed by western blotting using antisera directed against the extra- (F3) or intracellular (IC) domains. (A) Soluble SorLA lacks the intracellular domain. In NT2 cells SorLA is found in the membrane fraction (mem), whereas it is primarily released into the medium (med) by BON cells. (B) Soluble SorLA accumulates in conditioned medium from BON cells with time showing high levels after 24 hours of cultivation. (C) Soluble SorLA binds to HA Sepharose. Conditioned medium from the cell line BON was incubated with Sepharose coupled to HA or a control (Con) without peptide. Bound proteins were subjected to western blotting after extensive washing of the Sepharoses.

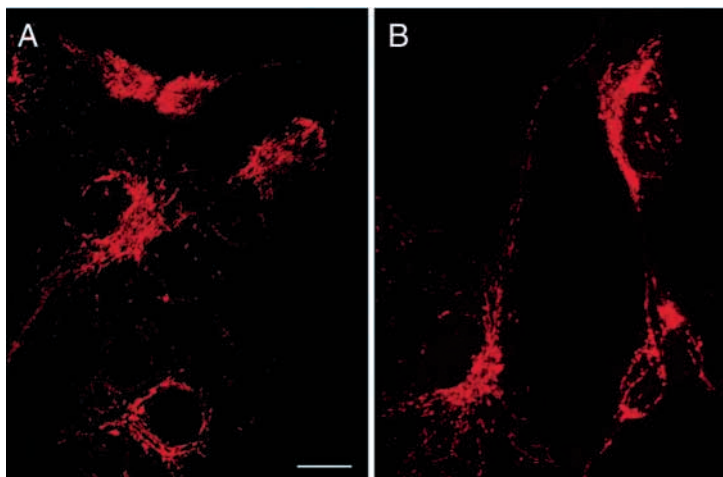


Fig. 5. Intracellular localization of SorLA in NT2 cells. After fixation NT2 cells were incubated (A) with the antiserum against the extracellular domain (F3, diluted 1:1,000) and (B) with the antiserum against the intracellular domain (IC, diluted 1:3,000). For visualization Cy3-labeled secondary antisera and confocal microscopy were used. Bar, 5 μ m.

more than 80 EST SorLA sequences in the database, 35 encode this region. Only two (AA053631 and AA465100) differ from the reported mRNA. They lack nucleotides at or near a splice site just in front of the coding region for the transmembrane domain leading to a shift in the reading frame and a premature stop. To analyze whether this change is responsible for the production of soluble SorLA, we amplified this part of the SorLA mRNA by PCR from BON cells which primarily release SorLA. Since we could only find the original in-frame SorLA mRNA (data not shown), we assume that the differences in the EST database entries are due to errors in sequencing or cDNA generation.

Alternatively, the soluble form of SorLA might be generated by proteolytic removal of the ectodomain from a transmembrane precursor. To test this hypothesis we expressed a SorLA-cDNA construct encoding the extracellular fibronectin type III domains, the transmembrane, and the intracellular domain in BON cells using a recombinant adenovirus. After two days of infection the SorLA fragment was found both in BON membranes and in the conditioned medium with a size difference of about 10 kDa (Fig. 7A). Since the viral SorLA gene contained no introns, proteolysis must be responsible for shedding of the ectodomain. Probably due to improper folding of the overexpressed SorLA fragment, only a minor fraction was cleaved from its transmembrane anchor. Endogenous 330 kDa SorLA was shed to a larger extent (Fig. 7A).

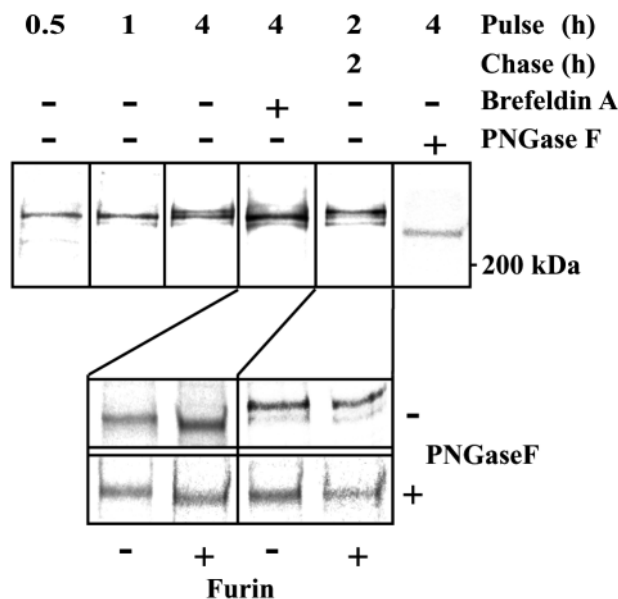
For shedding of transmembrane receptors predominantly proteases are responsible which act extracellularly. Especially transmembrane metalloproteases of the ADAM family fulfil such functions (Primakoff and Myles, 2000). To test their possible role in SorLA shedding, we treated BON cells with

the broad spectrum metalloprotease inhibitor BB-3103 and found that production of soluble SorLA was blocked (Fig. 7B). Lower concentrations of the inhibitor were less effective. SorLA release into the medium was reduced at 20°C as compared to 37°C (Fig. 7C). This may indicate that processing was prevented, because translocation of the metalloprotease or of SorLA to the cell surface did not occur. Involvement of a metalloprotease is supported by the finding that SorLA release was stimulated by PMA (Fig. 7D). This phorbol ester activates protein kinase C, which is known to upregulate ectodomain shedding by metalloproteases (Schlöndorff and Blobel, 1999).

In the notch signaling cascade ligand binding induces the shedding of its extracellular receptor domain by a metalloprotease (Brou et al., 2000; Mumm et al., 2000). Similarly, a 1 hour treatment of BON cells with HA increased the amount of soluble SorLA in the conditioned medium (Fig. 7E). The stimulated release of SorLA by PMA and HA was dependent on metalloproteases, since it was blocked by BB-3103 (Fig. 7D,E).

HA enhances proliferation of BON (Kayser et al., 1998) and NT2 cells (Fig. 1), and SorLA binds HA (Fig. 4C). If SorLA

Fig. 6. SorLA is posttranslationally processed. NT2 cells were incubated for various periods of time with 35 S-labeled amino acids (pulse), followed as chase in unlabeled medium. The apparent molecular mass of SorLA was determined by immunoprecipitation with the antiserum against the extracellular domain, SDS-page, and autoradiography. Brefeldin A (0.1 μ g/ml) was added during the pulse to prevent protein transport from the ER to the Golgi. Treatment of immunoprecipitated SorLA with PNGaseF resulted in a reduced molecular mass of SorLA. In the lower panel the indicated precipitates were treated with and without furin and PNGaseF prior to analysis.



mediated the HA-induced stimulation of cell proliferation, release of its ectodomain from the cell membrane might

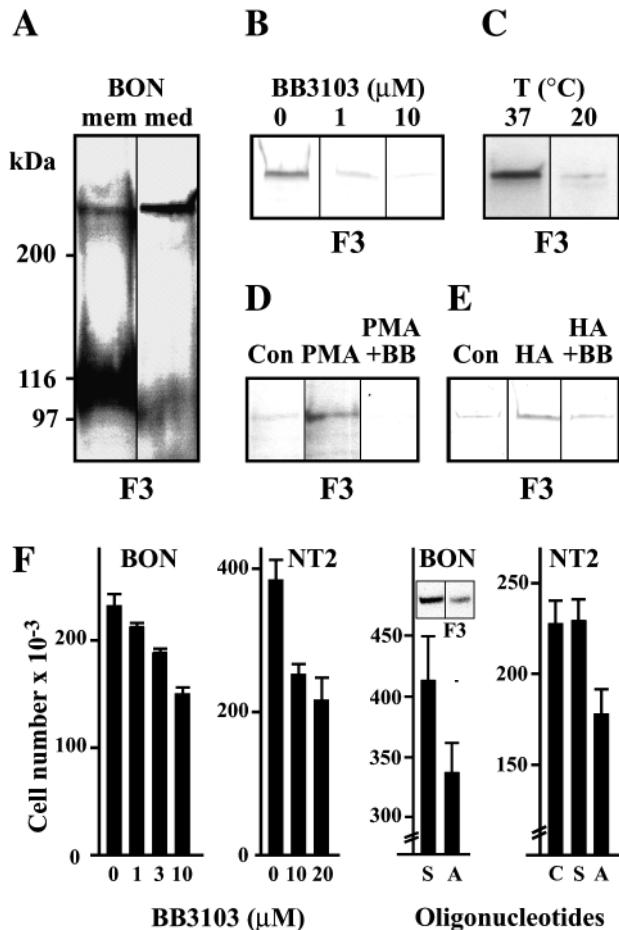


Fig. 7. SorLA shedding from BON cells involves a metalloprotease and is stimulated by HA. (A) Release of the ectodomain of a partial transmembrane SorLA construct. BON cells infected with a recombinant adenovirus encoding SorLA construct F as described in Fig. 3A produced a 110 kDa membrane-bound (mem) and a 100 kDa soluble released form (med) of partial SorLA. (B) SorLA release is dependent on metalloprotease action. Addition of the metalloprotease inhibitor BB-3103 to the growth medium of BON cells strongly reduced the amount of full-length SorLA shed overnight. (C) Inhibition of shedding at low temperature. Release of soluble SorLA from BON cells was strongly decreased at 20°C compared to 37°C. (D) PMA stimulates SorLA shedding. The protein kinase C activator PMA (0.1 μg/ml) led to enhanced SorLA release as measured after 1 hour. Addition of the metalloprotease inhibitor BB-3103 (BB) reduced the PMA-induced SorLA release. (E) SorLA release is stimulated by HA. Addition of 2 nM monomerized HA for 1 hour stimulated SorLA release in a metalloprotease-dependent fashion. (F) The metalloprotease inhibitor BB-3103 and antisense oligonucleotides against the SorLA start site have a negative effect on cell proliferation. BON and NT2 cells were grown in medium without (0, C) or with increasing concentrations of the metalloprotease inhibitor BB-3103 or with 1 μM antisense (A) or complementary sense (S) oligonucleotides. The total number of BON cells was determined after 4 days, that of NT2 cells after 2 days. For each treatment 3-6 samples were counted, and the results presented as means ± s.e.m. To control the reduction in SorLA synthesis, we analyzed the amount of SorLA protein shed from BON cells after treatment with the oligonucleotides by western blotting (inset).

influence cell growth. Indeed we found an inhibiting effect of the metalloprotease inhibitor BB-3103 on the proliferation of BON and NT2 cells (Fig. 7F). To assay an involvement of SorLA in cell proliferation directly, we used antisense oligonucleotides directed against its translational start site. We found that incubation of BON cells with micromolar antisense oligonucleotides not only led to a reduction of shed SorLA (Fig. 7F, inset), but also to an inhibition of cell proliferation

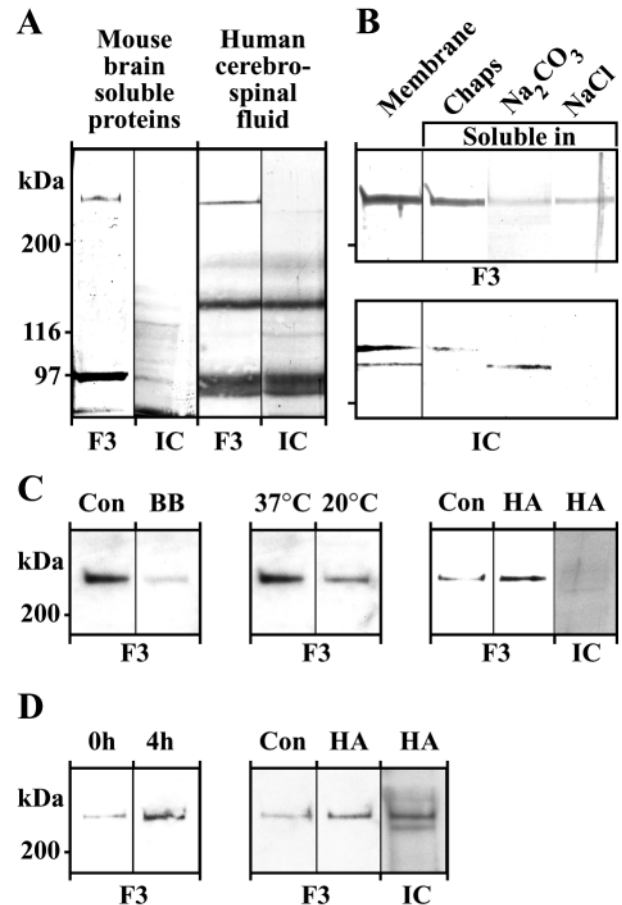


Fig. 8. Release of soluble SorLA from brain slices and SorLA synthesis are stimulated by HA. The amount of SorLA was analyzed by western blotting using antisera directed against its extra- (F3) or intracellular (IC) domain. (A) Soluble SorLA in brain. SorLA devoid of the intracellular domain was detected in low amounts as soluble protein in a mouse brain homogenate and in human cerebrospinal fluid. (B) Release of SorLA from mouse brain membranes. Proteins were solubilized using 1% CHAPS, 0.1 M Na₂CO₃ pH 11.5, or 1 M NaCl. After a high-speed centrifugation soluble SorLA devoid of the cytoplasmic tail was detected in the Na₂CO₃ and in the NaCl extract. (C) Soluble SorLA is released from brain slices. A freshly prepared murine brain was cut into 200 μm slices. SorLA release into the medium (Con) during a 2 hour period was inhibited by 10 μM BB-3103 (BB), or by incubation at 20°C instead of 37°C, and it was stimulated by 3 nM monomerized HA. Released SorLA was devoid of the intracellular domain. (D) HA induces SorLA production. The amount of SorLA in membranes from murine brain slices was higher after a 4 hour incubation in medium (4h) than directly after cutting (0h). Addition of 3 nM monomerized HA to the medium during the 4 hour incubation period further increased SorLA production (HA). SorLA in the membrane fraction reacted with the antiserum directed against the intracellular domain.

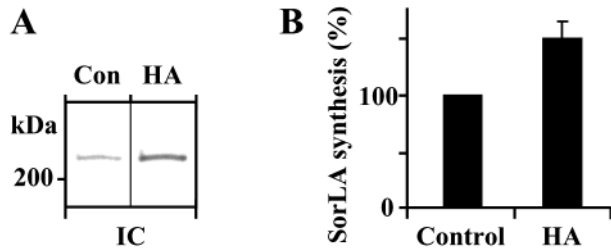


Fig. 9. HA stimulates SorLA production in NT2 cells. NT2 cells were metabolically labeled for 1 hour in the absence or presence of 2 nM monomerized HA and solubilized. (A) SorLA was immunoprecipitated using the antibody directed against the intracellular domain and visualized by autoradiography. (B) Quantification of SorLA production with a phosphoimager. SorLA was immunoprecipitated as shown in (A) using both SorLA antisera. SorLA production in the absence of HA was taken as 100%, and the increase caused by HA is presented as means \pm s.e.m. of 4 experiments.

resulting in an 18% reduction in total cell number. The number of NT2 cells treated with antisense oligonucleotides was reduced by 22% (Fig. 7F).

Soluble SorLA is present in brain and released from mouse brain slices

To corroborate the results from the cell culture experiments, we looked for soluble SorLA in the mammalian brain. The antiserum against the extracellular domain detected low levels of SorLA in a fraction of soluble proteins from murine brain homogenate and in human cerebrospinal fluid (Fig. 8A). As in the cultured cells, the apparent molecular mass of soluble SorLA appeared to be slightly smaller than that of the membrane-bound form. In contrast to membrane-bound SorLA, the soluble form was not recognized by the antiserum

against the intracellular domain (Fig. 8A). The amount of soluble SorLA was estimated to be about 1% of total SorLA in the brain. Treatment of mouse brain membranes with 1 M NaCl or 0.1 M Na₂CO₃, pH 11.5, which are used to remove peripherally attached membrane proteins, resulted in release of 5–10% of the membrane-bound SorLA into the solute (Fig. 8B). As expected, the soluble form of SorLA lacked the carboxy tail and did not react with the antiserum against the intracellular domain (lower panel in Fig. 8B). The detergent CHAPS was much more potent in the solubilization of SorLA indicating that more integral than peripherally attached SorLA is present in mouse brain membranes.

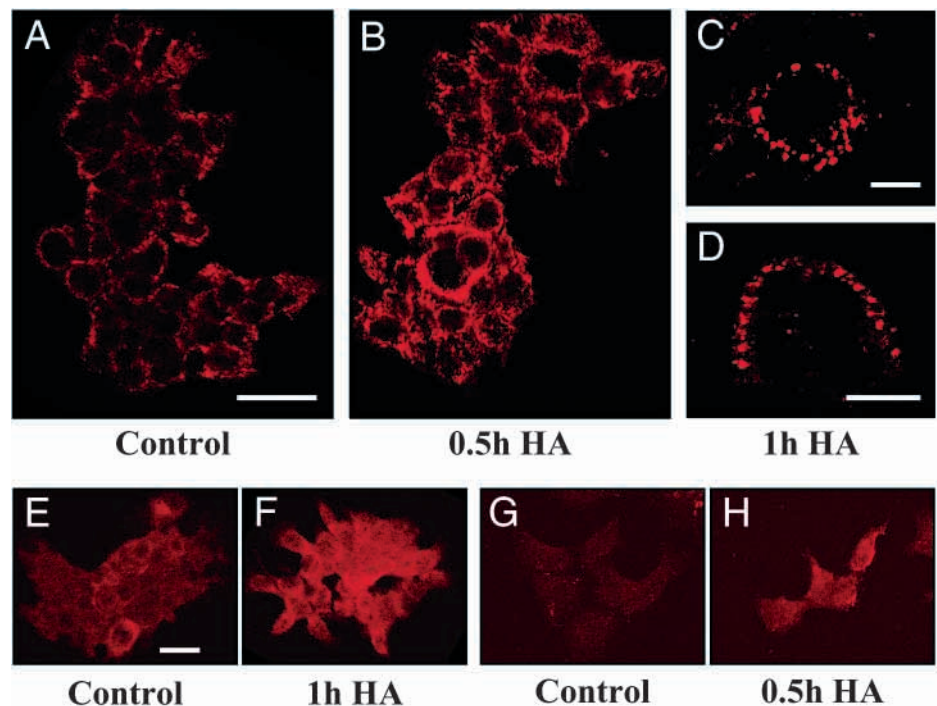
For hydra we had found that HAB was released very rapidly into the medium after wounding to induce regeneration (Hampe et al., 1999b). Similarly, from mouse brain slices about 3% of the total SorLA was shed during the first 4 hours of incubation (Fig. 8C). Incubation of the slices with HA upregulated the shedding, whereas the metalloprotease inhibitor and reduction of the incubation temperature from 37°C to 20°C decreased it.

The amount of SorLA was also determined in the membrane fraction prepared from the slices. It showed an increase from 0–4 hours of incubation indicating that SorLA synthesis was stimulated in the slices (Fig. 8D). Residual SorLA in the brain slices was further elevated after incubation with HA, suggesting that not only the release, but also the production of SorLA was enhanced by HA (Fig. 8D). As expected, released SorLA lacked the intracellular tail, whereas SorLA in the membrane fraction of the slices still contained it, as shown by reaction with the antiserum directed against the cytoplasmic tail.

Synthesis and compartmental shuttling of SorLA are influenced by HA

To verify the effect of HA on SorLA production, we quantified

Fig. 10. HA induces SorLA translocation, internalization, and production. Cells were cultured for 0.5 or 1 hour in the absence or presence of 2 nM monomerized HA. SorLA was detected in (A–F) by the antiserum directed against the extracellular domain (diluted 1:1,000) and in (G–H) by that against the intracellular domain (diluted 1:3,000) of SorLA and visualized using Cy3-labeled secondary antibodies and confocal microscopy. (A–D) HA leads to increased SorLA presence on the outer cell membrane and to SorLA internalization. After incubation with or without HA, living BON cells were incubated with the antiserum against the extracellular domain for 20 minutes. At a higher magnification vesicular structures close to the cell surface were visible (C,D). (E–H) SorLA immunoreactivity is enhanced by HA. BON (E,F) and NT2 cells (G,H) were fixed and permeabilized with icecold 1% acetic acid in ethanol prior to incubation with the SorLA antisera. Bars: 10 μ m (A,B, E–H); 2.5 μ m (C,D).



newly synthesized SorLA in NT2 cells after metabolic labeling. An increase in SorLA concentration was observed already 1 hour after addition of HA to NT2 cells (Fig. 9A). The amount of immunoprecipitated labeled SorLA was quantified by phosphoimaging and showed a 50% increase in SorLA production caused by the presence of HA (Fig. 9B).

The effects of HA on SorLA in cultured cells were also analyzed by immunocytochemistry. A 30-60 minutes treatment with HA increased the SorLA immunoreactivity at the surface of living BON cells showing a translocation of SorLA from internal to outer membranes (Fig. 10A,B). Presence of SorLA in vesicular structures close to the cell surface after a 1 hour HA treatment as observed after incubation of living cells with the primary antiserum pointed at an increased reuptake and internalization of SorLA (Fig. 10C,D). Immunocytochemistry of permeabilized BON and NT2 cells showed that HA treatment increased the SorLA immunoreactivity within cells (Fig. 10E-H), supporting the metabolic labeling experiments presented in Fig. 9.

DISCUSSION

SorLA is the only mammalian homologue to hydra HAB. Like HAB, SorLA is precipitable by HA Sepharose showing that it binds HA. In this paper we chose to work with two human cell lines, NT2 cells, which are neurogenic embryonal carcinoma cells, and BON cells, a neuroendocrine cell line derived from a pancreas carcinoid. Both cell lines respond to HA by entry into mitosis and increased cell proliferation, and produce HA as autocrine growth factor (Kayser et al., 1998; Niemann and Schaller, 1996). Binding of HA occurs with nanomolar affinity, as we had found for hydra HAB.

We present evidence that processing of SorLA is highly regulated. As a first step, probably cotranslationally, the signal peptide is cleaved off, and the protein is N-glycosylated in the ER. After transport to the Golgi, the glycosylation is modified, and the propeptide is removed. The propeptide consists of 53 amino acids and ends with RRKR in human and RRRR in murine SorLA, both perfect cleavage sites for furin (Molloy et al., 1999). We could show that furin *in vitro* is able to process SorLA to its mature form indicating that furin or a similar protease process SorLA *in vivo*. Propeptides play a role in activation of enzymes, ensuring e.g. that proteases only become active in the right compartment and at the right time. All VPS10 domain-containing receptors have one or two furin motifs for propeptide cleavage. This includes SorCS (Hervey et al., 1999) and the neurotensin receptor sortilin (Petersen et al., 1997). For the latter it was shown that the propeptide has to be cleaved off, before the ligand neurotensin can bind to the receptor (Petersen et al., 1999). Interference of SorLA's propeptide with HA-stimulated mitosis and proliferation (data not shown) hints at a similar function.

SorLA and HAB are so far the only VPS10-domain containing receptors which occur both as transmembrane and soluble proteins. Soluble SorLA is created by proteolysis. We could show that specific inhibitors of metalloproteases blocked SorLA ectodomain shedding. SorLA release was stimulated by HA and by phorbol esters, the latter indicating involvement of protein kinase C, which is known to activate shedding of receptor ectodomains by metalloproteases (Schlöndorff and

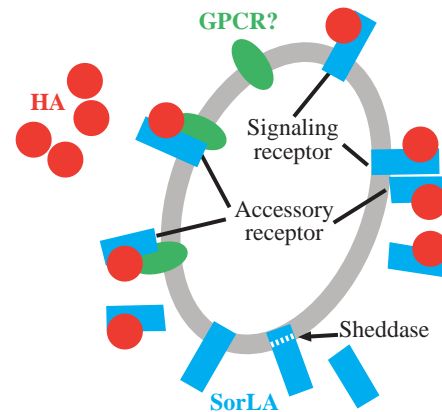


Fig. 11. Possible functions of SorLA in HA signal transduction. SorLA as transmembrane receptor is itself a signaling receptor and as such able to transduce the HA signal into the interior of the cell. Alternatively, transmembrane or shed SorLA are accessory receptors which present HA to a signaling receptor, possibly belonging to the family of G-protein coupled receptors (GPCR). As third variant shed and transmembrane SorLA dimerize after HA binding to form a complex active in signal transduction.

Blobel, 1999). Presence of the motif Ala-Val 18 amino acids apart from the transmembrane domain suggests TACE (ADAM 17) as possible candidate for this function (Brou et al., 2000). Shedding of SorLA from cells and brain slices did not occur at 20°C, indicating that translocation of either the protease or of SorLA to the plasma membrane is prevented. Similarly, at this temperature the amyloid precursor protein APP was not shed (Parvathy et al., 1999). APP like many other receptors is processed by metalloproteases on the plasma membrane indicating that soluble SorLA might also be released from the external face of the cell surface.

SorLA synthesis, its translocation from intracellular membranes to the plasma membrane, its shedding, and its subsequent internalization were stimulated by nanomolar HA concentration. The sortilin/neurotensin receptor 3, one of the few other VPS10 domain-containing receptors, also translocated to the cell surface after application of its neuropeptide ligand neurotensin (Chabry et al., 1993; Mazella et al., 1998). The fact that the ectodomain of notch is shed by a metalloprotease after binding to its ligand may hint at a similar mechanism (Brou et al., 2000; Chan and Jan, 1998; Mumm et al., 2000). In case of notch, a peptide is released intracellularly from the remaining membrane-bound notch fragment, enters the nucleus, and acts as a transcriptional regulator. Future experiments have to elucidate the function of the intracellular domain of SorLA.

SorLA contains in its 55 amino acid long carboxy tail several motifs which in other proteins are involved in protein sorting, internalization, and in the activation of second messenger pathways (Hampe et al., 1999b; Jacobsen et al., 1996). Such motifs are also present in the intracellular domains of other members of the LDL-R family, some of which activate heterotrimeric G proteins or bind adaptor proteins capable of initiating kinase cascades (Goretzki and Mueller, 1998; Gotthardt et al., 2000; Stockinger et al., 2000; Trommsdorff et al., 1998). Prominent examples are the very low density lipoprotein receptor and the apolipoprotein E receptor 2, which

transmit the reelin signal in the developing brain to ensure proper neuronal migration (Rice and Curran, 1999). A signaling cascade is initiated by binding of LDL to the LDL-R, leads to increased intracellular $[Ca^{2+}]$, and is inhibitable by pertussis toxin (Allen et al., 1998). This pathway is very reminiscent to that observed after application of HA to stimulate mitosis in HA-responsive cells (Kayser et al., 1998; Ulrich et al., 1996). SorLA contains in its carboxy-terminal tail a motif of basic amino acids which in other single transmembrane-spanning receptors mediates coupling to heterotrimeric inhibitory G proteins (Anand-Srivastava et al., 1996; Okamoto et al., 1990). This indicates that SorLA itself might function as a signaling receptor (Fig. 11). In this scenario soluble SorLA could compete with transmembrane for LA for HA binding. Stimulation of SorLA shedding by HA would then induce a negative feedback loop leading to downregulation of HA signaling.

BON and NT2 cells differ in their capacity to shed SorLA. While NT2 cells contain high amounts of transmembrane SorLA, BON cells shed SorLA predominantly into the medium. Nevertheless, cell proliferation in both cell lines is stimulated by HA and reduced by metalloprotease inhibitors or SorLA antisense oligonucleotides. We interpret this to mean that soluble and membrane-bound SorLA do not differ in function. This would imply that both act as accessory receptors which in their HA-bound state are able to activate a signaling receptor. Since HA-stimulated entry into mitosis is inhibited by pertussis toxin, a blocker of the inhibitory G protein, the signaling protein could be a classical heptahelical G protein-coupled receptor (GPCR, Fig. 11). Heterodimerization of GPCRs with other receptors has been demonstrated (Rocheville et al., 2000). If a complex of soluble SorLA and HA would bind to transmembrane SorLA, SorLA would be both, signaling and accessory receptor, depending on the presence of the intracellular tail. The presence of fibronectin type III modules, which in other proteins function as protein binding or dimerization domains (Bork et al., 1996), may confirm this notion.

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