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SorCS3 does not require propeptide cleavage to bind nerve growth factor

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Abstract The functional properties of the Vps10p-domain receptor SorCS3 are undescribed. Here, we examine its processing and sorting in cellular transfectants, and analyze the binding of potential ligands to the purified receptor. We show that SorCS3 is synthesized as a proprotein and converted to its mature form by N-terminal propeptide cleavage in distal Golgi compartments. The propeptide is not a requirement for normal processing of the receptor and does not prevent ligands from binding to the SorCS3 precursor form. Expression of wt and chimeric receptors further suggests that SorCS3 predominates on the plasma membrane, exhibits slow internalization and does not engage in intracellular trafficking. SorCS3 emerges as a new neurotrophin binding Vps10p-domain receptor functionally distinct from its relatives Sortilin and SorLA.
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Keywords: Sortilin; Vps10p; Nerve growth factor; Propeptide

1. Introduction

The five transmembrane proteins Sortilin, SorLA and SorCS1-3 [1–5] constitute a family of heterogeneous type-1 receptors that are particularly expressed in embryonic and adult neuronal tissue [6–9]. Each receptor is characterized by having a single, N-terminal copy of the so-called Vps10p-domain, a domain-type not found in other mammalian proteins, and originally identified in the yeast vesicular sorting protein Vps10p [10]. The Vps10p-domain is the only ectodomain in Sortilin, whereas the other four family members have more composite luminal parts. Thus, SorLA comprises structural elements typical of the low density lipoprotein receptor as well as a domain of fibronectin type III repeats, and SorCS1-3 contains a leucine-rich segment bordering the transmembrane domain. Apart from the ectodomains, each receptor has a short cytoplasmic domain (cd; 50–80 amino acids) and, at least in Sortilin and SorLA, the cds contain functional sites (e.g., dileucines, acidic clusters and tyrosine-based signals) for endocytosis as well as intracellular trafficking [11–13]. The N-termini of mammalian Vps10p-domains comprise dibasic target-motifs for proprotein convertase activity and are functionally important propeptides in Sortilin and SorLA. The two receptors bind their respective propeptides, which in return prevents binding of other ligands, and they therefore depend on propeptide cleavage for full functional activity [12,14]. Following cleavage, the mature receptors convey binding and endocytosis of a variety of ligands, e.g., neurotrophins, lipoprotein lipase and neurotensin [12,15–17], but may also target proteins in the biosynthetic pathway for Golgi-endosome transport [11,18]. Moreover, Sortilin in complex with $p75^{NTR}$ interacts with the proform of nerve growth factor-β (proNGF) and is essential to proNGF-mediated induction of neuronal death [16]. This suggests that Vps10p-domain receptors may also partake in transmembrane signalling and adds to the picture of a multifunctional receptor family.

In contrast to Sortilin and SorLA little is known about the SorCS molecules that form a subgroup of particularly closely related Vps10p-domain receptors. SorCS1, which is expressed as different isoforms with alternative cds, appears to be involved in differential, isoform-dependent trafficking in cells [19]. Recent findings further demonstrate that although it is subject to propeptide cleavage it does not bind its own propeptide [19], a finding that might reflect a difference between the functional organization of the SorCS proteins and that of Sortilin and SorLA. However, SorCS-specific ligands have not been identified, the role of the propeptide is unclarified and functional expression of SorCS1 and -2 has not been described.

The present study was undertaken to provide new information on the molecular function of the SorCS-subgroup. We have examined the processing and trafficking of SorCS3 in transfected cells by expression of wt SorCS3 and of chimeric receptors carrying the SorCS3 cytosolic domain. In addition, we have tested potential ligands for binding to purified SorCS3 and analyzed the ligand binding activity of its uncleaved precursor.

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Abbreviations: s-SorCS3, luminal part of SorCS3 as secreted minireceptor; s-$SorCS3_{pro\text{-}mut}$, cleavage-resistant minireceptor; TGN, trans-Golgi network; NGF, nerve growth factor; proNGF, proform of NGF; NGFpro, propeptide of proNGF; IL2R, interleukin 2 receptor; cd, cytoplasmic domain

2. Materials and methods

2.1. DNA constructs

The coding sequence of human full-length SorCS3 (M^{-41}–V^{1181}) was excised from the original KIAA 1059 cDNA clone (Genbank Accession No. AB028982) [5] and ligated into the pcDNA3.1/zeo(−) expression vector (Invitrogen). SorCS3 without its transmembrane and cds

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(M^{-41}–S^{1084}; luminal part of SorCS3 as secreted minireceptor (s-SorCS3)) was constructed from the original cDNA and expressed using the pcDNA4/myc-his C expression vector (Invitrogen). Cleavage-resistant minireceptor (s-$SorCS3_{pro\text{-}mut}$) was generated by site-specific mutations in the propeptide cleavage motif ($R^{88}RSRR^{92}$/$R^{88}RSGG^{92}$) and expressed in pcDNA4/myc-his C. Chimeric receptors (IL2R/SorCS3) combining the luminal and transmembrane domains of interleukin 2 receptor (IL2R) (CD25/Tac) or cation-independent mannose 6-phosphate receptor (MPR300) with the cd (wt or mutant) of SorCS3 was constructed and inserted into the pcDNA3.1/zeo(−) vector as described [11]. Human nerve growth factor-β (NGF) was from Austral Biologicals, brain-derived neurotrophic factor (BDNF), neurotrophin3 (NT3), neurotrophin4/5 (NT4), neurturin and glial cell-line derived neurotrophic factor (GDNF) were from R&D Systems, and neurotensin was from Sigma. The prodomain-coding regions of human NGF (E^{1}–R^{102}), BDNF (S^{40}–R^{108}), NT3 (N^{1}–R^{79}), NT4 (S^{1}–R^{56}), neurturin (I^{1}–R^{76}) and GDNF (F^{1}–R^{58}) were amplified from the Genbank-clones AW770784, AI242172, AA448311, BC012421 and AW050617, or GDNF from cDNA generated from fetal brain by RT-PCR. The peptides were expressed in *Escherichia coli* BL21 as GST-fusion proteins and purified using glutathione–Sepharose beads (Amersham Biosciences). Receptor-associated protein (RAP) was generated as described [20] and the NGF precursor (proNGF) was a gift from Scil Proteins Gmbh.

2.2. Cells and transfection

CHO-K1 cells were cultured in serum-free HyQ-CCM5 medium (HyClone, Logan UT) and mouse embryonic fibroblasts deficient in both mannose 6-phosphate receptors [21] were grown in Dulbecco's modified eagles medium with 10% fetal calf serum. Cells were transfected using FuGENE 6 (Roche). Stable transfectants were selected at 500 μg/ml Zeocin and identified by Western blotting using a mouse anti-IL2R (anti-CD25/Tac; Roche) and a rabbit antibody raised against a peptide segment (P^{137}–T^{234}) in the luminal part of SorCS3. s-SorCS3 and s-$SorCS3_{pro\text{-}mut}$ were purified using TALON-matrix affinity chromatography. β-Hexosaminidase activity in medium and cell lysates was determined as described [22]. Mannose 6-phosphate was from Sigma.

2.3. Biolabelling and precipitation

Metabolic cell labelling was performed with L-[^{35}S]-methionine and L-[^{35}S]-cysteine (pro-mix™, Amersham Biosciences) in Met and Cys-free medium at 100 μCi/ml and 10 μg/ml brefeldin A (BFA, Sigma). After 2–5 h, the cells were washed and reincubated in unsupplemented HyQ-CCM5 medium. At given times, incubation was stopped and labelled SorCS3 constructs were precipitated from the medium and/or cell lysates using Gammabind G-Sepharose beads (Amersham Biosciences) coated with anti-SorCS3 antibodies [23]. Following wash, the beads were resuspended in reducing sample buffer (20 mM dithiothreitol, 2.5% SDS) either immediately or after treatment with 2.5 U N-glycosidase F (PNGase-F; Roche) or 20 mU Endoglycosidase H (Endo-H; Boehringer) as described. Boiled samples were analyzed by SDS–PAGE and autoradiography.

2.4. Immunocytochemistry and internalization

CHO cells expressing IL2R/SorCS3 chimeras were cultured on cover slides and incubated (2 h, 4 °C) with mouse anti-IL2R prior to wash and reincubation in prewarmed medium. At given time points the cells were fixed (4% formaldehyde, pH 7), permeabilized (0.5% Triton X-100) and stained with Alexa 488 conjugated goat anti-mouse Ig (Molecular Probes). Staining was analyzed by confocal microscopy (LSM510, Carl Zeiss, Germany). Quantization of IL2R/SorCS3-receptor mediated internalization of ^{125}I-labelled anti-IL2R was performed as previously described [16]. To determine the relative amount of SorCS3 that was expressed on the plasma membrane, transfected cells were biotinylated with the membrane-impermeable sulfo-N-hydroxy-succinimidobiotin. After cell lysis and precipitation with streptavidin–Sepharose, the relative amounts of surface-associated (biotinylated and precipitable) receptors and of intracellular (not precipitated) SorCS3 were estimated by Western blotting as described [15].

2.5. BIAcore

Surface plasmon resonance measurements were performed on a Biacore 2000 instrument (Biacore, Uppsala, Sweden) equipped with CM5 sensor chips activated as described [14]. s-SorCS3 and s-$SorCS3_{pro\text{-}mut}$ were immobilized to densities 62–82 fmol/mm^2 and samples for binding (40 μl) were injected at 5 μl/min at 25 °C in 10 mM HEPES, 150 mM NaCl, 1.5 mM $CaCl_2$, 1 mM EGTA, and 0.005% Tween 20, pH 7.4. Binding was expressed in relative response units as the response obtained from the flow cell with immobilized receptor minus the response obtained when using an activated but uncoupled chip. The overall K_d values were determined by BIAevaluation 3.0 software using a Langmuir 1:1 binding model and simultaneous fitting to all curves in the concentration range considered (global fitting).

3. Results

3.1. SorCS3 is synthesized as a proreceptor

The N-terminal part of SorCS3 contains a classical sequence-motif for furin-mediated cleavage, suggesting that SorCS3 similar to other examined Vps10p-domain receptors, is synthesized as a precursor molecule. To examine this, s-SorCS3 minireceptors secreted into the medium or contained in the cells, were precipitated from cultures of biolabelled CHO-transfectants. The SDS–PAGE analysis (Fig. 1A) shows that in deglycosylated samples, but not in untreated samples, s-SorCS3 from the medium was less retained in the gel than

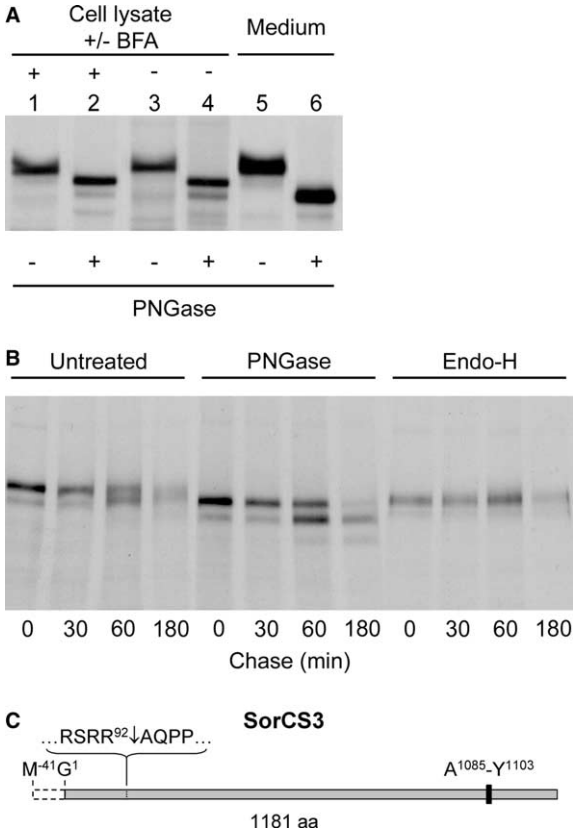

Fig. 1. Propeptide cleavage of the secretable minireceptor s-SorCS3 (A) and of full-length SorCS3 in CHO-transfectants (B). The cells were biolabelled in the presence of BFA prior to wash, reincubation in unsupplemented medium and chase of labelled receptors. At the times indicated, labelled receptors were immuno-precipitated from medium and cell lysates (A) or from cell lysates only (B). Untreated precipitates, and samples treated with PNGase-F or Endo-H were subsequently analyzed by SDS–PAGE. Autoradiographs of 8–16% gels are shown. Panel C shows a schematic presentation of full-length SorCS3. The positions of the propeptide cleavage site and the transmembrane segment are indicated.

minireceptors from cell lysates, signifying cleavage of the secreted species. Similar results were obtained by chase experiments in cells expressing full-length SorCS3 (Fig. 1B). It appears that nearly all newly synthesized receptors were converted from the pro- to the mature form within 3 h (PNGase-panel). The results further show that after 60 min, when all receptors are resistant to Endo-H, only ~50% have been converted, which strongly suggests that cleavage takes place in late Golgi-compartments.

N-terminal sequencing of s-SorCS3 purified from culture supernatants confirmed cleavage between R^{92} and A^{93} and thus establishes G^{1}–R^{92} as the SorCS3 propeptide.

3.2. SorCS3 binds NGF and its prodomain

Binding of potential ligands to immobilized purified s-SorCS3 was assessed by plasmon resonance analysis. A series of known Sortilin and SorLA Vps10p-domain ligands and related proteins/peptides were tested. Initial experiments demonstrated that unlike Sortilin and SorLA, but similar to its close relative SorCS1, SorCS3 bound neither RAP, neurotensin nor its own propeptide. Since the Vps10p-domain of Sortilin binds both the prodomain and the mature form of NGF, and as SorLA interacts with GDNF [23], we next tested a series of neurotrophic factors and their prodomains for binding. As depicted in Fig. 2, SorCS3 interacted with NGF as well as with its prodomain. Both ligands bind with an estimated K_d of about 35 nM, which means that mature NGF binds equally well to SorCS3 and Sortilin, whereas the prodomain has a 5–10-fold higher affinity for Sortilin as determined from the present and previous results [16]. Additional experiments (Fig. 2, lower panel) show that SorCS3 also interacts with the (uncleaved) NGF-precursor with an affinity which is 5–10-fold lower than that of Sortilin. In contrast, SorCS3 exhibits little or no binding of BDNF, NT3, NT4 and GDNF, or of their respective prodomains (K_d's > 1 μM).

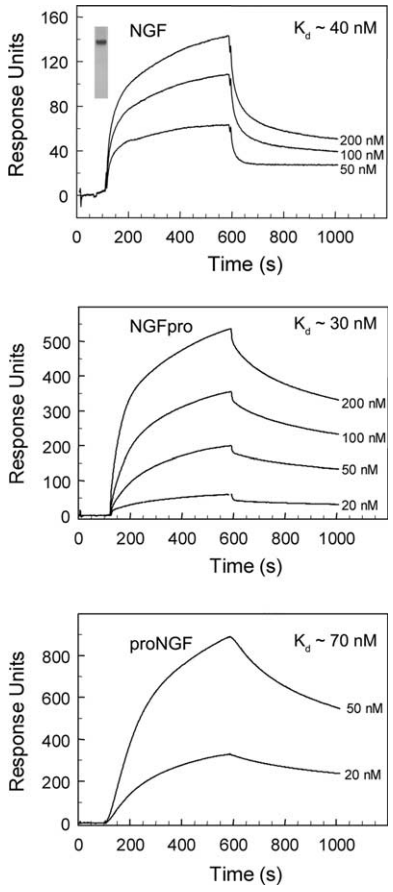

Fig. 2. Binding of mature NGF, the NGF prodomain (GST-fusion protein) and the uncleaved proform of NGF to the luminal part of SorCS3. Purified s-SorCS3 was immobilized on chips and binding at pH 7.4 of various concentrations of ligands in solution was determined by plasmon resonance analysis. Estimated K_d-values are given. The purity of the s-sorCS3 preparation was verified by reducing SDS–PAGE and silverstaining (inset, upper panel).

3.3. Propeptide cleavage is not a requirement for ligand binding to SorCS3

Sortilin and SorLA bind their respective propeptides and depend on propeptide cleavage for interaction with ligands. In contrast, SorCS3 does not bind its own propeptide and might therefore bind ligands irrespective of cleavage. To clarify this, we expressed a mutant s-SorCS3 carrying a disrupted motif for cleavage. The convertase-resistant precursor, s-$SorCS3_{pro\text{-}mut}$, was purified from culture medium and tested for ligand binding in parallel with wt s-SorCS3. The plasmon resonance analysis (Fig. 3) shows that the two s-SorCS3 species bind the NGF-propeptide with similar affinities. It can be concluded that cleavage and removal of the propeptide is not a requirement for SorCS3 mediated ligand binding.

In separate experiments the rates of secretion of wt s-SorCS3 and of a corresponding construct missing the propeptide (truncated at A^{93}) were compared by chase of minireceptors in cells and medium of biolabelled cultures (not shown). The cells were labelled in the presence of BFA, washed and reincubated in fresh unsupplemented medium (zero time), and at given times labelled minireceptors were precipitated from medium and lysed cells. As determined by this procedure the two constructs were secreted at similar rates. Thus, the propeptide serves neither to prevent ligand binding nor to facilitate processing of the newly synthesized SorCS3.

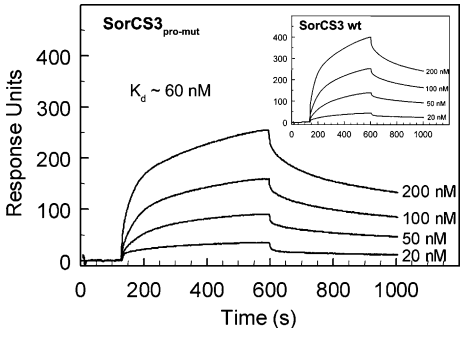

Fig. 3. SorCS3 propeptide cleavage is not required for ligand binding. Cleavage-resistant s-$SorCS3_{pro\text{-}mut}$ and wt s-SorCS3 (inset) were purified, immobilized and tested in parallel for binding of the NGF (GST-)prodomain. Sensorgrams obtained by plasmon resonance analysis at various concentrations of ligand are shown and estimated K_d-values are indicated.

3.4. SorCS3 predominates on the surface membrane

Full-length SorCS3 is mainly expressed on the cell membrane and after biotinylation of proteins exposed on transfected cells 64.3% ± 18 ($n = 3$) of the cellular SorCS3 was

precipitated by streptavidin beads. In accordance, immunocytochemical analysis of SorCS3 transfectants shows a pronounced receptor-specific staining on the plasma membrane (Fig. 4A, left panel). IL2R/SorCS3 chimeric receptors comprising the wt SorCS3 tail shows a similar distribution (Fig. 4A, right panel) and as CHO-cells neither express IL2R nor react with anti-IL2R (anti-CD25), this construct was used for analysis of receptor mediated endocytosis. It appears that anti-IL2R bound to the IL2/SorCS3 chimera at 4 °C is internalized by CHO-transfectants at 37 °C (Fig. 4B). As determined by ^{125}I-labelled anti-IL2R about 30% (29.4% ± 2.8, $n = 3$) of the antibody was internalized (i.e., not dissociable at pH 2.5) within 60 min (Fig. 4C). Surprisingly, disruption of the potential sorting sites $Y^{10}AQV^{13}$ (to $A^{10}AQV$ or $YAQA^{13}$) and $E^{45}PEELL^{50}$ (to $EPEEA^{49}A^{50}$) gave similar results, i.e., 27.3% ± 4.9, 36.1% ± 7.0 and 37.3% ± 5.8, respectively. Thus, it can be concluded that although the cd of SorCS3 may mediate endocytosis, it is much less efficient than the Sortilin and SorLA cds [11,24], and does not depend on classical motifs for internalization.

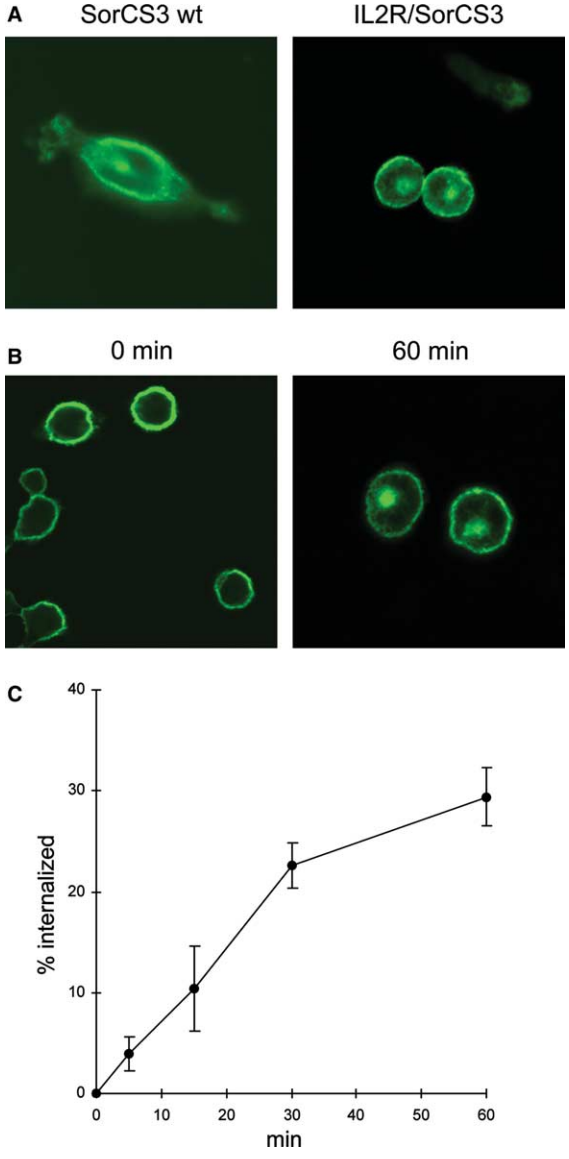

Fig. 4. Subcellular distribution of full-length SorCS3 and chimeric IL2R/SorCS3 (A) and internalization of IL2R/SorCS3 chimeras (B and C). (A) CHO-transfectants expressing wt SorCS3 or IL2R/SorCS3 (carrying the cd of SorCS3) were fixed and stained using rabbit anti-SorCS3 or mouse anti-IL2R as primary antibodies and Alexa 488 conjugated-goat anti-rabbit or anti-mouse, respectively, as secondary antibodies. After binding of anti-IL2R (B) or ^{125}I-labelled anti-IL2R (C) at 4 °C, CHO-cells transfected with IL2R/SorCS3 were washed and reincubated (zero time) in warm medium. (B) At the times indicated, the cells were fixed and receptor:antibody complexes were stained by secondary anti-mouse antibodies, and analyzed by confocal microscopy. (C) At the times indicated, internalization was determined as the amount of cell-associated radioactivity that was not released upon incubation at pH 2.5. Each point represents mean of triplicates and all values are shown relative to total cell-associated radioactivity at zero time.

Finally, a MPR300/SorCS3 chimera was expressed in mannose 6-phosphate receptor deficient cells that, in the absence of sorting receptors, release their newly synthesized lysosomal enzymes to the medium. The chimera binds enzymes via its MPR300 luminal domain, but in the presence of mannose 6-phosphate, which prevents (re)uptake from the medium, it failed to restore sorting and to prevent secretion of the MPR300 ligands β-glucoronidase and β-lactosidase. Thus, after 20 h of culture both transfected (MRP300/SORCS3) and untransfected mpr$^-$-cells had released most of their β-glucoronidase (72.1% and 81.6%) and, respectively, β-lactosidase (71.1% and 87.1%) to the medium, whereas less than 22% was released by mpr$^-$-cells transfected with wt-MRP300. It follows that SorCS3, unlike Sortilin, is not engaged in Golgi-endosome trafficking.

4. Discussion

The functions of the SorCS-subgroup of Vps10p-domain receptors are unclarified and current knowledge is based on a single report on SorCS1 expression [19]. Here, we report the first study of expressed SorCS3. We find that SorCS3 similar to previously examined Vps10p-domain receptors is synthesized as a precursor molecule that is converted to its mature form by N-terminal propeptide cleavage. In contrast to Sortilin and SorLA, however, SorCS3 does not bind its own propeptide and, notably, does not depend on propeptide cleavage for interaction with ligands. In addition, we demonstrate that SorCS3 like SorLA, but unlike Sortilin, is normally processed in the absence of a propeptide [23]. These findings suggest that the SorCS3 propeptide may be functionally redundant and establish that the function of the propeptide varies between the family receptors.

Another interesting difference between SorCS3, Sortilin and SorLA concerns receptor localization and sorting. Unlike its two relatives [11,12], SorCS3 predominates on the cell surface, its cd conveys only slow internalization and it does not partake in Golgi-endosome trafficking. These results evidence that SorCS3 is not involved in intracellular sorting or transport of proteins but, more likely, participates in functional events at the surface membrane. In that respect, its binding of NGF and proNGF is particularly interesting. Thus, Sortilin also binds the prodomain of the NGF precursor and this interaction was recently shown to facilitate proNGF induction of $p75^{NTR}$ mediated neuronal cell death [16]. Future studies will determine if SorCS3 similar to Sortilin can engage in a complex with $p75^{NTR}$, but in view of its high expression on the cell

membrane it is conceivable that SorCS3 is implicated in proNGF mediated signalling.

In summary, we find SorCS3 to be a neurotrophin binding receptor that differs distinctly from its relatives Sortilin and SorLA in terms of propeptide function and intracellular trafficking.

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