Functional Organization of the Sortilin Vps10p Domain*

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A Vps10p domain makes up the entire luminal part of Sortilin, and this type of domain is the hallmark of a new family of neuronal receptors that target a variety of ligands, including neurotrophins and neuropeptides. We have shown that two structural features of the Vps10p domain, the N-terminal propeptide and the C-terminal segment of ten conserved cysteines (10CC), are key elements in the function of Sortilin. The propeptide has two functions. (i) It binds the mature part of Sortilin and prevents ligands in the biosynthetic pathway from binding to the uncleaved proreceptor, and (ii) it facilitates receptor transport in early Golgi compartments by a mechanism that does not depend on its ability to prevent ligand binding. In contrast, other Vps10p domain receptors, such as SorLA and SorCS3, do not need their propeptide for normal and swift processing. The 10CC segment constitutes an exchangeable module containing five conserved disulfide bridges, and using module-shuffling and truncations, we have shown that the 10CC segment is a major ligand-binding region in Sortilin.

The mammalian Vps10p domain (Vps10p-D) receptors constitute a newly defined family of heterogeneous type-1 receptors that are expressed in a number of organs but particularly in developing and adult neuronal tissue. The five currently known family members are Sortilin (~100 kDa) (1), SorLA (~250 kDa) (2, 3), and the three more recently identified receptors SorCS1–3 (~130 kDa) (4–6). Their common characteristic and the hallmark of the family is the so-called Vps10p-D, named after the yeast sorting protein Vps10p, which contains two copies of a similar domain in its luminal part. Each family member contains a single Vps10p-D situated at the receptor N terminus. In Sortilin, which can be considered the archetypal member, the Vps10p-D makes up the entire luminal receptor, whereas additional unrelated ectodomains are found in the other four. Thus, the mutually very homologous SorCS1–3 all comprise a leucine-rich segment between the Vps10p-D and the plasma membrane (4), whereas SorLA contains a cluster of the ligand-binding low density lipoprotein receptor class A repeats, additional elements typical of the low density lipoprotein receptor family, and adjacent to the membrane, a domain of fibronectin type-3 repeats also found in neuronal adhesion molecules (2, 3, 7). Following the ectodomains and the transmembrane segment, each receptor carries a short (50–80 amino acids) cytoplasmic domain comprising typical motifs for interaction with cytosolic adaptor molecules. Functional sorting sites, e.g. dileucines, acidic clusters, and tyrosine-based motifs, involved in endocytosis and intracellular transport have so far been established in Sortilin (8) and SorLA (9), and at least one of the receptors, SorCS1, is transcribed with alternative cytoplasmic tails, giving rise to differentially expressed and sorted receptor isoforms (10).

The physiological role(s) of the Vps10p-D receptors is far from clarified, but recent findings indicate that the receptors serve important and diverse functions inside as well as outside of the nervous system. Thus, Sortilin may be part of the machinery that governs cell survival in developing neuronal tissue and a key determinant in the induction of post-traumatic neuronal apoptosis (11), whereas SorLA seems implicated in the generation of Alzheimer’s disease (12) as well as in atherosclerotic plaque formation (13, 14). Information at the molecular and cellular level confirms that the receptors are multifunctional, bind several different ligands, and they engage in intracellular sorting as well as in endocytosis and signal transduction. Sortilin, for instance, mediates rapid endocytosis of lipoprotein lipase (15), neurotensin (16), and the proform of nerve growth factor (proNGF) (11) but may also target proteins in Golgi for transport to late endosomes (8, 17) and is furthermore essential to proNGF induction of neuronal death via complex formation with p75NTR on the cell membrane (11). The existence of differentially expressed SorCS1 isoforms and the fact that both Sortilin and SorLA bind, and to some extent share, a variety of ligands, including receptor-associated protein (RAP), neurotensin, lipoprotein lipase, apolipoproteins, and elements of the plasminogen activator system, adds to the picture of considerable functional diversity (1, 7, 15, 18, 19).

Although the composite family receptors may have more than one ligand-binding domain, SorLA, for instance, has at least two (7). The Vps10p-D is the only luminal domain in Sortilin and is a key element in the function and processing of the Vps10p-D receptors (20). Overall, there is only a modest sequence similarity between the Vps10p-Ds from Sortilin, SorLA, and the three SorCS molecules, but they all share two distinct structural features: (i) an N-terminal propeptide outlined by a consensus sequence for cleavage by proteases of the subtilisin family of protein convertases and (ii) a ~120

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† The abbreviations used are: Vps10p-D, Vps10p domain; Vps10p-SorLA, the Vps10p-D of SorLA; 10CC, ten conserved cysteines; BFA, brefeldin A; BSS, balanced salt solution; CHO, Chinese hamster ovary; Endo-H, endoglycosidase H; GDNF, glia-derived neurotrophic factor; GST, glutathione S-transferase; NGF, nerve growth factor; NGFpro, prodomain of NGF; RAP, receptor-associated protein; s-, indicates soluble minireceptors consisting of the luminal part of given receptors (e.g. s-Sortilin); wp, without propeptide; wt, wild-type; HPLC, high pressure liquid chromatography; ER, endoplasmic reticulum; SPR, surface plasmon resonance.
amino acid, C-terminal segment (designated 10CC) containing ten conserved cysteines. Previous studies have established that Sortilin is synthesized as a precursor (pro)protein. The inert precursor is converted in the trans-Golgi to the mature ligand-binding receptor by furin-mediated cleavage and subsequent dissociation of a 44-residue propeptide (20). The Vps10p-Ds of SorLA, SorCS1, and SorCS3 are similarly processed, and similar to Sortilin, the Vps10p-D of SorLA needs propeptide cleavage to expose its ligand-binding region, which is inaccessible in the unprocessed precursor (7, 10, 20). Thus, propeptide cleavage conditions both Sortilin and SorLA for ligand binding, and their propeptides bind the mature receptors with high affinity and inhibit binding of all currently known Vps10p-D ligands. These findings imply that the propeptides might be types of “intrinsic” chaperones that assist in protein folding or serve to prevent the newly synthesized Vps10p-D receptors from premature interaction with ligands in the biosynthetic pathway.

The 10CC segment is situated at the C terminus of the Vps10 domain. Compared with the rest of Vps10p-D, 10CC is very similar in all the receptors, both in terms of primary structure and with respect to the number (ten) and spacing of the contained cysteines. In a functional context, it is remarkable that many of the ligands that target Vps10p-Ds interact with more than one receptor, e.g. neurotensin, RAP, and apolipoprotein. Moreover, all ligands compete for binding, and the propeptides of Sortilin and SorLA both inhibit binding of any known ligand to either of the two receptors (7, 20). This implies that the binding sites within the Vps10p-Ds are closely situated and located in a region with a relatively high degree of structural conservation. The 10CC segment complies with these requirements better than any other part of the Vps10p-D and is therefore a candidate structure for interaction with ligands.

The present study was undertaken to map key elements in the functional organization of Sortilin (in particular) and of Vps10p-Ds (in general). We have determined the disulfide bridge pattern of Sortilin and of the SorLA Vps10p-D and have shown that the conserved cysteines form five disulfide bonds within the 10CC module. We present strong evidence that the 10CC module constitutes the major binding region of the Vps10p-D, and using mutational analysis of recombinant peptides, we have mapped the receptor-binding segment in the Sortilin propeptide. Finally, by analysis of soluble minireceptors in transfected cells, we demonstrated that Sortilin, in contrast to SorLA and SorCS3, depends on its propeptide to expedite transport in the biosynthetic pathway.

**EXPERIMENTAL PROCEDURES**

**Receptor cDNA Constructs, Protein Expression, and Purification—** The human cDNA constructs encoding full-length Sortilin and the soluble luminal domain of Sortilin (s-Sortilin, residues Met1–33-Ser723) (20), and the construct encoding the Vps10p-D of SorLA (Vps10p-SorLA, Met26–Glu721) (7) have been described elsewhere. Similar constructs for the expression of the luminal part of SorCS3 (s-SorCS3, Met41–Ser1084) (5) and of the following receptor proteins were generated (residue numbers refer to the primary structure of corresponding wild-type receptors): (i) minireceptors without propeptide, i.e. s-Sortilin (Ser45–Ser723), Vps10p-SorLAwp (Ser54–Glu731), s-SorCS1wp (Ser78–Glu731), and s-SorCS3wp (Ser1084–Glu731); (ii) propeptide fragments Gly22–Arg28 was produced as a synthetic GST fusion part was achieved by thrombin-mediated cleavage. The GST-propeptide fragment Gly22–Arg28 was produced as a synthetic peptide.

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**Propeptides and Ligands—** The wt propeptides of Sortilin (20), SorLA (7), and SorCS1 (10) were constructed as GST fusion proteins. The Sortilin propeptide (Gly1–Arg28) as well as the SorLA mutant GST-propeptides (covering Glu1–Leu21, Gly19–Ala38, and Gly36–Arg53) were generated by a similar approach. Truncated Sortilin-propeptides (Gly1–Arg41, Gly1–Pro74, Gly1–Ala26, Gly1–Arg26, Gly1–Gly22, and Gly1–Arg37) were generated from the pGEX-T-1 Sortilin wild-type construct (Gly1–Arg28) using a GEX forward primer and appropriate C-terminal deletion primers, and the resulting PCR fragments were ligated back into the pGEX-4T-1 vector. The construct harboring residues Trp37–Pro57 was generated from the Gln1–Pro57 construct using a primer deleting the 16 N-terminal residues. All constructs were expressed in the Escherichia coli BL21 strain and affinity-purified on glutathione-agarose beads (Amersham Biosciences). Separation of generated peptides from their GST fusion part was achieved by thrombin-mediated cleavage. The Sortilin propeptide fragment Gly28–Arg53 was produced as a synthetic peptide.

**Disulfide Bridge Mapping—** Cysteine-labeled s-Sortilin (107 counts/min) was purified from the medium of biolabeled CHO transfectants, mixed with 250 μg of unlabeled s-Sortilin in 100 ml NH4HCO3, and digested with trypsin (6 h) at an enzyme:substrate ratio of 1:50 (w/w). The digest was separated by reverse-phase HPLC on a Vydac C18 column using an Amersham Biosciences system comprising of a 2249-gradient pump connected to a 2510 UVicord S.D. detector (22). The peptides were separated in 0.1% trifluoroacetic acid and eluted with a stepwise linear gradient of acetonitrile developed over 50 min (0–5 min, 0%; 5–40 min, 0–50%; 40–50 min, 50–95%) at a flow rate of 0.85 ml/min. The column was operated at 40°C, and the peptides were monitored in the effluent by measuring the absorbance at 226 nm.

**Metabolic Labeling, Affinity Precipitation, and Deglycosylation—** CHO transfectants were biolabeled with [35S]cysteine-containing dipeptides and subsequently characterized by Edman sequence analysis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. A major fraction eluting at the end of the gradient contained a high proportion of the radioactivity. This fraction was subjected to digestion (6 h, 55°C) with trypsin, digested with trypsin (6 h) at an enzyme:substrate ratio of 1:50 (w/w) enzyme:substrate ratio and separated as described above.

**Automated Edman sequence analyses were performed on an Applied Biosystems 477A sequencer equipped with an on-line high pressure liquid chromatograph. Part of the phenylthiohydantoin-derivative sample, left over after on-line injection, was used for identification of the dipeptide-phenylthiohydantoin-cysteine by liquid scintillation. Mass spectra were acquired with Bruker BIFLEX matrix-assisted laser desorption ionization time-of-flight mass spectrometer. A major fraction eluting at the end of the gradient contained a high proportion of the radioactivity. This fraction was subjected to digestion (6 h, 55°C) with trypsin, digested with trypsin (6 h) at an enzyme:substrate ratio of 1:50 (w/w) enzyme:substrate ratio and separated as described above.
RESULTS

Mapping of Binding Segments in the Sortilin Propeptide—
The propeptide of Sortilin (Gln1–Arg44) binds to the mature receptor with high affinity (K_D ≈ 5 nM) and inhibits binding of all known ligands, and early in the synthetic pathway, the propeptide moiety is thought to interact with segments of the Vps10p-D to prevent premature ligand binding (20). To identify binding segments, we expressed a series of GST-propeptide fusion proteins and analyzed their binding to immobilized luminal Sortilin (s-Sortilin) by surface plasmon resonance (SPR).

Initial results demonstrated that deletion of the C-terminal third of the propeptide (Ala28–Arg44) had little or no effect on the binding affinity. In contrast, more extensive truncation, including Glu25–Arg28, caused a substantial decrease in affinity, and a peptide comprising most of the remaining N terminus (Gln1–Arg16) exhibited almost no binding (Fig. 1A). Accordingly, the segment Trp17–Pro37 showed a high affinity for mature Sortilin, and the heptapeptide Gly22–Arg28 was further more found to inhibit binding of the wt propeptide by about 70% (Fig. 1B). The heptapeptide was equally effective in inhibiting the previously reported interaction between the Sortilin propeptide and the Vps10p-D of SorLA (7) (not shown), confirming that it constitutes an important part of the binding segment (likely to include Trp17–Arg28) in the propeptide and suggesting the presence of similar target sites in the two Vps10p-Ds.

The propeptide of SorLA (Glu1–Arg53) was analyzed because it binds to the Vps10p-Ds of SorLA and Sortilin with similar affinities (7). Both the N-terminal (Glu1–Leu21) and the central part (Gly19–Ala38) of this propeptide bound, but the latter appeared most predominant as it retained most of the affinity for SorLA (Fig. 1C). Interestingly, no common motif is apparent in the two propeptides, and because they compete for binding to the Vps10p-Ds of both Sortilin and SorLA (7), it is likely that they target separate but closely situated sites in the two receptors. In contrast to Sortilin and SorLA, neither SorCS1, as previously described (10), nor SorCS3 bind their own propeptides (not shown).

Lack of Propeptide Inhibits the Secretion of s-Sortilin—To determine whether the propeptide is necessary for normal processing of Sortilin and of Vps10p-D receptors as such, we generated a construct of s-Sortilin without propeptide (s-Sortilin_{np}) and analogous constructs of the Vps10p-D of SorLA (Vps10p-SorLA) and of the luminal part of SorCS3 (s-SorCS3). The wt and the truncated constructs were then expressed and chased in culture medium and cell lysates of biolabeled CHO transfectants. The results depicted in Fig. 2A show that, although newly synthesized s-Sortilin is rapidly secreted, s-Sortilin_{np} is subject to a considerable delay. By 4 h, >91.1 ± 1.1% (mean ± S.D., n = 3) of the wt minireceptor was found in the medium as opposed to only 24.9 ± 3.4% (mean ± S.D., n = 3) of the truncated species, demonstrating that the Sortilin propeptide facilitates secretion. On the other hand, neither Vps10p-SorLA nor s-SorCS3 depends on their respective propeptides for rapid secretion (Fig. 2B). It follows that a propeptide, whether or not it binds to its mature receptor, is not a general requirement for the processing of Vps10p-D receptors.

The Sortilin Propeptide Facilitates Transport without Influencing Receptor Folding—To clarify the reason for its delay in secretion, we first examined whether newly synthesized s-Sortilin is subject to degradation, which is a likely result of major misfolding. Prolonged (>24 h) chase in biolabeled CHO transfectants showed that, given enough time, all newly synthesized s-Sortilin_{np} was released into the medium (not shown). Moreover, treatment with BFA for 4 h prior to chase, to provide additional time for folding in the ER, did not enhance

proteinase inhibitor mixture (CompleteMini; Roche Applied Science). Alternatively, for chase experiments, the labeled cells were washed and reincubated in unsupplemented full medium for given periods of time prior to recovery of cell lysates and medium (Roche Applied Science) was performed as described previously (20). Deglycosylated and untreated precipitates were prepared for SDS-PAGE by boiling the cells for 3 min in 100 μl of 10 mM dithiothreitol, 2.5% SDS sample buffer.

Binding of ligands to soluble receptors of biolabeled CHO cultures was assessed by pull-down experiments. Medium and cell lysates were diluted as described above and precipitated with uncoupled CNBr-activated Sepharose 4B beads (Amersham Biosciences) or glutathione-Sepharose (Amersham Biosciences) prior to incubation with RAP-Sepharose or GST fusion peptide (prodomain of NFG) bound to glutathione-Sepharose, respectively. After 4 h at 4 °C, the beads were pelleted, and unbound receptors were recovered from the supernatants by immunoprecipitation (described above). The beads were washed in 0.05% Tween 20 BSS and resuspended in reducing SDS sample buffer, and the precipitated proteins were analyzed by SDS-PAGE using di-phenylalloxazole-fluorographed gels exposed at −70 °C. Quantitation of 35S-labeled protein bands was performed by phosphorimaging (FLA-3000/LAS 1000, Fuji imager system).

Biochip Measurements—All measurements were performed on a Biacore 2000 instrument using CM5 sensor chips maintained at 20 °C. The sensor surface was under a continuous flow (5 μl/min) of 10 mM HEPES, pH 7.4, 3.0 mM EDTA, 150 mM NaCl, 0.05% surfactant P20. The carboxylated dextran matrix of flow cells 1–4 was activated by injection of 0.2 M N-ethyl-N-(3-dimethylaminopropyl)carbodiimide and 0.05 M N-hydroxy-succinimide in water (240 μl). A 10 mM sodium acetate solution (pH 4.0) of 10–30 μg/ml purified receptor (s-Sortilin, Vps10p-SorLA, s-SorCS3, or similar constructs) was then injected over flow cells 1–4 (10 μl/min) of the remaining binding sites in all four flow cells, which were subsequently blocked by injection of 1 mM ethanolamine, pH 8.5. The amount of immobilized protein, as determined by the relative response, varied between 60 and 80 fmol/mm2. Binding of ligands was determined by injecting 40–150 μl aliquots (0.01–5 μl ligand, 5 μl/min) through all flow cells. Unless otherwise stated, the samples were dissolved in 10 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM CaCl2, 1 mM EDTA, and 0.05% surfactant P20, which was also used as running buffer. The surface plasmon resonance signal is expressed in relative response units, i.e. the difference in response between the immobilized protein flow cell and the corresponding control flow cell (i.e. flow cell 1, activated and blocked but without protein). Regeneration of the sensor chip after each cycle of analysis was performed with 10 mM glycine/HCl, pH 4.0, 1.0 mM NaCl, 0.005% EDTA, and 0.005% surfactant P20. The BIAevaluation 3.1 software was used to determine kinetic parameters.

Immunocytochemistry and Cross-linking—CHO transfectants expressing s-Sortilin minireceptors were incubated in medium supplemented with 10 μg/ml BFA. After 1 h, the cells were washed and then either fixed immediately in 4% formaldehyde buffer (pH 7.0) or reincubated for an additional h in medium containing 10 μg/ml cycloheximide, but not BFA, prior to fixation. The fixed cells were permeabilized in 0.5% Triton X-100 buffer, washed in BSS, and subsequently incubated with primary and secondary antibodies diluted in BSS. Sortilin was labeled using protein A-purified rabbit anti-sortilin Ig (20) as primary and Alexa 488-conjugated goat anti-rabbit Ig as secondary antibodies. Mouse anti-GLB28 (Stressgen Biotechnologies) was used as a cis-Golgi marker and was stained by Cy3-conjugated goat anti-mouse Ig. Fluorescence microscopy was performed using a laser scanning confocal unit (LSM510; Carl Zeiss) attached to an Axiovert microscope (Carl Zeiss). Immunostaining was visualized with a 10×/1.4 NA objective to delineate the nuclear and Golgi compartments, and the cells were resuspended in fresh medium (without BFA) for 1.5 h at 37 °C prior to wash and incubation with the cross-linker at room temperature.
the efficiency of secretion. The functional state of the mutant minireceptor was subsequently assessed by SPR analysis, showing that purified samples of s-Sortilin and s-Sortilinwp had indistinguishable affinities of RAP, neurotensin, and the Sortilin propeptide (not shown). Finally, RAP affinity precipitation of s-Sortilinwp from medium and cell lysates was found to be equally efficient (Fig. 3A), signifying that both secreted and retained receptors were fully functional. When taken together, the findings establish that the propeptide is not essential for correct folding of Sortilin or of Vps10p-Ds in general.

We next analyzed where in the biosynthetic pathway s-Sortilinwp was delayed. Following culture in the presence of BFA, transfectants expressing s-Sortilin and s-Sortilinwp were fixed and stained either immediately (time zero) or after reincubation for 1 h in BFA-free medium that was supplemented with cycloheximide to minimize continuous receptor synthesis. It appeared (Fig. 3B) that by 1 h both receptors, which were broadly distributed in the endoplasmic reticulum at zero time, had accumulated in paranuclear compartments and colocalized with the cis-Golgi marker GS28. After an additional 1–2 h of incubation, s-Sortilinwp was still mainly paranuclear, but colocalization with GS28 was far less pronounced (not shown). The results demonstrated that in the absence of its propeptide, Sortilin is subject to normal export from the ER and is not, to any major extent, trapped in cis-Golgi. Additional studies showed that, in contrast to secreted s-Sortilinwp, the intracellular pool of s-Sortilinwp is entirely Endo-H-sensitive (Fig. 3C), signifying that the receptors are rapidly secreted upon the addition of terminal sugars in the distal part of the biosynthetic pathway. We concluded that the transport of Sortilin without propeptide is delayed mainly between the cis- and trans-Golgi compartments.

To assess whether the facilitating effect of the Sortilin propeptide relates to its ability to block ligand-binding sites in the Vps10p-D, we produced a hybrid receptor, combining the mature part of s-Sortilin with the propeptide of SorCS3 (s-Sort/SorCS3-pro). The hybrid was expressed and secreted both on a
cleavable wt form and on a noncleavable proform containing a disrupted propeptide cleavage site. Initial SPR analysis (not shown) showed that binding of RAP, neurotensin, and the Sortilin propeptide to the purified cleavage-resistant proform of s-Sort/SorCS3-pro was no different from binding to mature s-Sortilin, i.e. the SorCS3 propeptide did not deny access to the ligand-binding region of the unprocessed hybrid receptor. The secretion of cleavable s-Sort/SorCS3-pro was then compared with that of s-Sortilin and s-Sortilinwp, and as shown in Fig. 4, the presence of the SorCS3 propeptide (or SorCS1 propeptide, not shown) partially restored secretion. Sortilin transport can therefore be facilitated by a propeptide for reasons other than providing protection against premature ligand binding. This notion was supported by other experiments (not shown) demonstrating that secretion of s-Sortilinwp was unaffected in double transfectants overexpressing RAP or the NGF precursor proNGF. Also, attempts to identify endogenous interactors by chemical cross-linking were unproductive (not shown). We suggest that in Sortilin, but not in other Vps10p-D family members, the propeptide covers a site, which when exposed, leads to a delayed passage through the biosynthetic pathway.

Identification of the Ligand-binding Region in Mature Sortilin—To generate relevant receptor constructs for mapping of the ligand-binding region, we first determined the disulfide bridge pattern of the Vps10p-D. Soluble Sortilin was purified by RAP affinity chromatography from the culture medium of [35S]cysteine-labeled and unlabeled CHO transfectants and digested by trypsin and thermolysin. Labeled (d)peptides separated by reverse HPLC were then identified by microsequencing and mass spectrometry. As outlined in Fig. 5A, all sixteen cysteines in s-Sortilin (the Vps10p-D) were found to engage in intramolecular disulfide bonding. The ten consecutive cysteines in the C-terminal part of the protein (cysteines 7–16) contribute to the formation of five intertwined bridges within the 10CC segment, whereas the remaining six cysteines form...
two “small loops” (Cys\textsuperscript{224}-Cys\textsuperscript{244} and Cys\textsuperscript{415}-Cys\textsuperscript{425}) within a “large loop” (Cys\textsuperscript{53}-Cys\textsuperscript{523}) (Fig. 5B). Similar results were obtained from an incomplete analysis of the fourteen cysteines in Vps10p-SorLA, and as can be seen (Fig. 5B), the bridge patterns identified within the 10CC segments of Sortilin and SorLA match. The finding predicts a similar arrangement in SorCS1–3 and strongly indicates that the 10CC segment constitutes a conserved and separate module within the Vps10p-D.

Sortilin and the corresponding Vps10p-D of SorLA both bind several ligands. All of the ligands of a given domain compete for binding, and several are shared by the two receptors; e.g., RAP, neurotensin, the Sortilin and SorLA propeptides, and apo-lipoprotein E3. Because this suggests that the binding region constitutes a compact and conserved structure, we decided to focus on the 10CC module as the prime candidate. To separate 10CC from the large N-terminal part of s-Sortilin, we first tried to introduce enzymatic cleavage sites in the short stretch (Trp\textsuperscript{524}-Asn\textsuperscript{578}) between the sixth and seventh cysteine. This implied the exchange of only two or three residues, but none of the resulting constructs were secreted by transfected cells. As an alternative approach, we then generated a chimeric minireceptor composed of an s-Sortilin construct in which the C-terminal 10CC module of Sortilin (Cys\textsuperscript{579}-Ser\textsuperscript{723}) was exchanged for that of SorLA (Cys\textsuperscript{593}-Glu\textsuperscript{731}) (Fig. 6A). The construct (Sort/SorLA10CC) was expressed in CHO cells and was secreted into the medium in mature form; i.e., after cleavage of the propeptide. Affinity precipitation using RAP beads showed that the chimeric receptor was functional and capable of ligand binding. However, whereas s-Sortilin was efficiently precipitated by RAP beads, pull-down of Vps10p-SorLA and the Sort/SorLA chimera was less productive (Fig. 6B, right panel), displays that s-Sortilin, but neither the chimera nor the Vps10p-SorLA, bound NGF\textsuperscript{pro}, suggesting that the binding site resides in the Sortilin 10CC module. For further evidence, we also analyzed binding of the glia-derived neurotrophic factor (GDNF), which in the process of screening for binding of various growth factors, was identified as a ligand that specifically targets SorLA (Fig. 6C, panel 1). Initial tests strongly indicated binding of GDNF to immobilized Sort/SorLA10CC (Fig. 6C, panel 2). The fact that the binding of GDNF was modest can be ascribed to the instability of the chimera during the coupling procedure inasmuch as binding of RAP was equally reduced (not shown). Additional experiments using immobilized GDNF confirmed the interaction and showed that the chimera possessed a capacity and affinity for GDNF similar to that of Vps10p-SorLA (Fig. 6C, panel 3). Thus, exchange of the Sortilin 10CC module with that of SorLA results in loss, as well as gain, of function with a change to a “SorLA-like” ligand-binding profile.

To further dissect the function of the 10CC module, we finally expressed mutant constructs of s-Sortilin (Ser\textsuperscript{1}-Glu\textsuperscript{661}) and of Vps10p-SorLA (Ser\textsuperscript{1}-Pro\textsuperscript{675}) comprising C-terminal deletions encompassing the last three cysteines of the respective 10CC segments. Only the mutated Vps10p-SorLA construct was secreted by CHO transfectants, and it was subsequently purified and tested (in parallel with wt Vps10p-SorLA) for its ability to bind RAP and the SorLA-propeptide. The SPR anal-
ysis clearly demonstrated that, although the truncated and the wild-type minireceptor bound the SorLA-propeptide equally well ($K_d = 20 \text{ nM}$), only the wild-type construct was capable of RAP binding (Fig. 7). The results show that the 10CC module constitutes a major binding segment in the Vps10p-D and harbors separate sites for binding of individual ligands.

**DISCUSSION**

The Vps10p-D is named after the vacuolar protein-sorting 10 protein (Vps10p), which targets carboxypeptidase Y to lysosomes in yeast (24). In mammals, corresponding domains are only found in the five members of the Vps10p-D receptor family that are widely expressed in neuronal tissues (25–27). The domain constitutes one of the two known ligand-binding domains of Sortilin (7), it is the major constituent of SorCS1–3 (10), and makes up the entire luminal part of Sortilin (1). The receptors are found on the cell surface as well as in intracellular vesicles (7, 8, 10), and recent findings in Sortilin indicate that the Vps10p-D may bind both pro- and mature forms of target molecules and engage ligands at the surface membrane as well as in vesicles and compartments of the distal biosynthetic pathway (11, 17).

Here we have analyzed the functional organization of Sortilin with particular reference to the two characteristic features of the Vps10p-D, i.e., the N-terminal propeptide and the C-terminal segment of 10 conserved cysteines (10CC).

**The Propeptide of Sortilin Is an Endogenous Chaperone with More than One Function—** Sortilin is converted to its mature form by furin-like proprotein convertase-mediated cleavage in the trans-Golgi network (20). We have shown previously that mature Sortilin (and the Vps10p-D of SorLA) binds its own propeptide and that the unprocessed Sortilin precursor is vir-
turally incapable of ligand binding (20). On the other hand, the actual function of the propeptide and the implications of its interaction with the ligand-binding region has not been clarified. Our present findings demonstrated that Sortilin depends on its propeptide for normal passage of the biosynthetic pathway. The propeptide is not a prerequisite for normal folding of the receptor or its exit from ER, but promotes receptor transport through early and/or medial Golgi compartments before the addition of terminal sugars. This appears to be independent of the ability of the propeptide to block binding of ligands to uncleaved Sortilin, because the facilitating effect is at least partly preserved when its own propeptide (that of Sortilin) is exchanged for that of SorCS3, which does not interfere with binding. One function of the Sortilin propeptide is therefore most likely to conceal sites that hamper trafficking in the Golgi compartments and that are situated outside of the ligand-binding region. This scenario differs from that of SorLA and SorCS3 and from other chaperone-mediated mechanisms reported to facilitate transport. For example, the propeptide of furin functions as an intramolecular chaperone, which remains bound to the endoprotease after cleavage and permits its exit from ER (28), and the escort molecule RAP binds to low density lipoprotein receptor-related protein and prevents premature aggregation of the receptor resulting from premature ligand binding (29, 30).

Neither SorLA nor SorCS3 depend on their propeptides for transport, but unlike SorCS3, the Vps10p-D of SorLA binds its own propeptide, and similar to Sortilin, its ability to interact with ligands depends on cleavage and removal of the propeptide. These differences indicate that propeptides may cover more than one function and suggest that binding between a propeptide and its corresponding Vps10p-D may be a function in its own right. We have recently demonstrated that Sortilin can mediate neuronal cell death by forming a complex with the proform of NGF-Alpha (H9252) and the neurotrophin receptor p75 (11). The NGF precursor predominates in the biosynthetic pathway, and in this context, it could be speculated that the Sortilin propeptide functions as a safeguard that protects the cells against the formation of death-signaling intracellular complexes. Propeptide binding may therefore serve to prevent unwarranted premature binding of particular ligands and reflects that some Vps10p-Ds are targeted by “critical” ligands, whereas others are not. Thus, it is likely that the propeptide has at least two separate functions in Sortilin. On the other hand, it cannot be excluded that propeptides in some Vps10p-D family members may be redundant.

The 10CC Segment Constitutes a Separate Module and Is Imperative for Binding of Ligands—The primary structures of neurotensin and of the mapped binding segments in the propeptides of Sortilin and SorLA show no similarity or basis for a common motif. Yet, all three peptides compete with high affinity for binding to Sortilin and SorLA (7, 20). Other ligands, such as RAP and apoE, also bind to the Vps10p-D of both receptors, which altogether indicates that ligands target separate but closely situated sites in a conserved region of the Vps10p-D. The C-terminal 10CC segment is the best conserved region in the family receptors, and our present analysis of s-Sortilin and Vps10p-SorLA establishes that it represents a separate module within the Vps10p-D. Notably, mapping of the disulfide bridges also demonstrates that cysteines 1 and 6 in Sortilin connect and thereby seem to bring the propeptide within close proximity of the 10CC module. This position would form an ideal basis for interaction between the two and agrees with our subsequent observations of ligand binding to chimeric and truncated minireceptors. These findings provide strong evidence that ligand binding originates in the 10CC module. Thus, exchange of the Sortilin 10CC module for that of SorLA was found to be accompanied by a corresponding shift in affinity for receptor-specific ligands, demonstrating that binding of the NGF prodomain depends on the Sortilin 10CC, whereas determinants for GDNF binding resides in the SorLA 10CC. Moreover, additional analysis of Vps10p-SorLA showed that a truncation, deleting a C-terminal segment in 10CC, could completely eliminate binding of one ligand (RAP) without affecting the affinity of another (SortLA-propeptide). This supports the notion that different ligands bind separate sites and provides further evidence that the 10CC module constitutes the (major) binding region in the Vps10p-Ds. It does not exclude, however, that binding to some extent, or in some cases, may involve segments outside of the 10CC module. In fact, the prodomain of the conotoxin TxVI binds to a segment in the N-terminal part of Sortilin, and this interaction is not inhibited by the Sortilin propeptide (31).

In summary, we have shown that two common structural features, i.e. the N-terminal propeptide and the C-terminal 10CC module, are key elements in the functional organization of Sortilin and the related Vps10p-D receptors. The 10CC module appears as an independent and exchangeable region with a conserved arrangement of five disulfide bridges. The module is decisive to the binding of GDNF, RAP, and GDNF in Sortilin and Vps10p-SorLA and is likely to comprise most, if not all, ligand-binding structures in Vps10p-Ds in general. Finally, our results show that although a propeptide may be redundant (expendable) in some Vps10p-D receptors, it serves in others to prevent undesirable premature binding of ligands and, at least in Sortilin, to facilitate the transport of proreceptors in the biosynthetic pathway.

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