Identification of a linear epitope in sortilin that partakes in pro-neurotrophin binding

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Sortilin acts as cell surface receptor for proneurotrophins (proNT) that upon complex formation with the p75 neurotrophin receptor (p75NTR) is able to signal neuronal cell death. Here we screened a sortilin peptide library comprising 16-mer overlapping sequences for binding of the pro-domains of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). We find that a linear surface exposed sequence, R163IFRSSDFAKNF174, constitutes an important proNT binding epitope in sortilin. Systematic mutational analysis revealed residues R163, F165, R166 and F170 to be critical for the interaction. Expression of a sortilin mutant in which these four amino acids were substituted by alanines disrupted proNT binding without affecting receptor heterodimerization with p75NTR or binding of ligands that selectively engages the centrally located tunnel in the β-propeller of sortilin. We furthermore demonstrate that a peptide comprising the ligand-binding epitope can prevent proNT-induced apoptosis in RN22 schwannoma cells.

Introduction

Sortilin is one of five members of the mammalian VPS10p-domain receptor family of neuronal type-1 receptors (1). In addition to sortilin, the family comprises sorLA, the sorting receptor for the amyloid precursor protein and major risk factor for late-onset Alzheimer’s disease (2-4), and three homologous receptors denoted sorCS1, -2, and -3 that have been genetically linked to type-2 diabetes and bipolar disorder (5, 6). Neurotrophins such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are trophic factors essential for development and maintenance of the nervous systems (7-11). They are produced as precursors, denoted proneurotrophins (proNTs), that can be processed to their mature form in the secretory pathway as well as outside the cell (12-14). It is well appreciated that proNTs may also have functions in their own right as they elicit activities opposite to those of their mature counterparts (15-20). While mature NTs can stimulate neuronal survival and long-term potentiation and synaptic strengthening, proNTs may induce apoptosis and long-term depression (21-23). Sortilin is required for the death signalling pathway as it forms a high affinity binding site together with the p75NTR neurotrophin receptor (24). Inhibiting binding of the growth factor pro-domains to sortilin prevents apoptosis by proNTs from occurring in cultured cells as well as in vivo, e.g. following spinal cord injury (25-32).

Recently, the structure of sortilin, the archetype receptor of the VPS10p-domain family, was solved at the atomic level in complex with the 13 amino acid long neuropeptide, neurotensin (33). The crystal structure revealed a unique 10-bladed β-propeller structure containing a centrally located tunnel with a diameter exceeding 25Å that harbours the binding site for neurotensin. Although proNT binding to sortilin is partly inhibited by neurotensin (25) the size of the proNT pro-domain (~100-110 amino acids) likely precludes entry of the entire domain into the tunnel. We therefore set out to study if a linear surface exposed amino acid sequence in sortilin might assist in proNT binding.

Experimental Procedures
**Materials and proteins** – To obtain tagged forms of the neurotrophin pro-domains readily for detection in SPOT analysis, we prepared constructs for each protein allowing for addition of N-terminal S-peptide and poly-histidine tags. Template cDNA for human NGF and BDNF was ATCC clones used for generation of fragments spanning residues E^1^R^102^ of NGF and A^1^R^110^ of BDNF using the primer pairs 5'-GGTATTGAAGGTCGCAACACCACACTCAGAGAGCAATGCCC-3', 3'-GGGGGAAGTTGTCCTGAGTGTCCTCGTTCGCACTCCGAGATTTGAGAGGAGA-5' and 5'-GGTTAGAAGGTCGCAACACCACACTCAGAGAGCAATGCCC-3', 3'-CACGTTTGTACAGGTACTCCCAGGCCGCGACTCCGAGATTTGAGAGGAGA-5' with compatible overhangs for ligation independent cloning into the pET-30 Xa/LIC vector from Novagen (cat.no. 70073-3) and amplification using Phusion DNA polymerase and following the protocol as provided by manufacturer. Proteins were expressed in the BL21/DE3 strain of *e.coli*, efficiently extracted from bacterial inclusion bodies using the *Bugbuster* reagent from Novagen (cat.no. 70921) with added benzonase (Novagen, cat.no. 70750), and purified by standard Ni^2+-NTA affinity chromatography in 500 mM NaCl, 5 mM Imidazole, and 20 mM Tris-HCl, pH 8.0. Protein eluation was done in buffer supplemented with 20 mM EDTA. Verification of the intact tagged versions of HisS-NGFpro and HisS-BDNFpro was carried out by SDS-PAGE analysis followed by coomassie staining or Western blotting using either antibody against the histidine tag from Sigma (H-1029) and secondary HRP-conjugated anti-mouse antibody from Calbiochem (cat.no. 401207), or alternatively by direct binding of HRP-conjugated S-protein from Novagen (cat.no. 69047-3).

For the production of the sortilin ectodomain, a construct encompassing the entire coding region of the N-terminal part of human sortilin including the endogenous signal peptide and followed by a C-terminal poly-histidine tag inserted in the pCEP-Pu vector was kindly provided by D. Militz, MDC-Berlin. The DNA was transferred into EBNA 293 cells that were selected by G418 (Gibco cat.no. 10131-027, 300 μg/mL) and Puromycin from Sigma (cat.no. P8833, 1 μg/mL) before proteins were collected from medium conditioned for 48 hr, and used for purification by applying to Ni^2+-NTA Sepharose. The secreted recombinant sortilin polypeptide chain spanning the intact extracellular domain of human sortilin is thus ending at S725 (+AMIEGRVGHHHHHH containing the FXa site and poly-histidine tag). The quality of the protein was tested by silver staining of SDS-PAGE analysis.

Peptides for binding and competition studies were synthesized in house at the Charite (Berlin), or purchased from Eurogentec. GST-NGFpro was prepared as previously described and unprocessed precursor proNGF was purchased from Millipore (cat.no. GF210).

**Sortilin mutants** - Single amino acid substitutions of ligand binding residues within the extracellular domain of sortilin were introduced using the Quickchange II XL site-directed mutagenesis kit from Stratagene (cat.no. 200521) and the wild-type sortilin pCEP-Pu expression construct as template. Mutagenesis primers were from BioTez (Berlin). Multiple rounds of mutagenesis allowed for generation of double, triple, quadruple mutant constructs knocking out several ligand binding residues in the sortilin domain.

To generate the quadruple mutations in a sortilin construct encoding the entire sortilin receptor including the transmembrane and cytoplasmatic domains, we took advantage of internal *Hpa*II and *Bsr*GI (NEB cat.no. R0105S and R0575S, respectively) restriction recognition sites in the sortilin sequence. Using both enzymes we cut out the sortilin fragment containing the 4A mutations from the pCEP-Pu expression vector for the soluble sortilin, and exchanged that for the corresponding fragment from a full-length sortilin expression vector in pcDNA3.1/zeo(+) thereby generating a mutant full-length (fl)-sortilin-4A construct. In parallel, the same fragment was also introduced into an endocytosis-deficient mutant of sortilin (34) to generate a version of full-length sortilin-4A that resides at the cell surface (fl-sortilin-4A<sub>Δendo</sub>).

**Circular Dichroism (CD) spectroscopy** – The purity of sortilin proteins were assessed by SDS-PAGE, and the purified proteins were dialyzed against 20 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4. Protein concentrations were determined using the absorbance at 280 nm and an estimated extinction coefficient of 133240 M<sup>-1</sup>cm<sup>-1</sup> (from the ExPaSy proteomics server tools). Ten CD spectra were recorded at 25 °C for each protein on a Jasco J-810 spectropolarimeter (Jasco Spectroscopic, Japan) using a polypeptide concentration of ~0.1 mg/mL and a cuvette of 2-mm path length. CD data were obtained in the
range from 260 to 200 nm at a resolution of 1 nm using a band width of 2.0 nm. The scan speed was 100 nm/min, and the response time was 1 sec.

**Surface plasmon resonance analysis** – Determination of direct binding of ligand to immobilized protein was performed on a biacore2000 instrument (Biacore, Sweden) using CaHBS as standard running buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2 mM CaCl$_2$, 1 mM EGTA, and 0.005% Tween-20). A biosensor chip from Biacore (CM5, cat.no. BR-1000-14) was activated using the NHS/EDC method as described by supplier followed by coating with sortilin to a protein density of 79 fmol/mm$^2$, and used for affinity measurements of the recombinant pro-domains of NGF and BDNF. Preparation of a biosensor surface with pro-sortilin followed an equal procedure. Regeneration of the flow cell after each cycle of ligand binding experiment was done by two 10 μL pulses of regeneration buffer (10 mM glycine-HCl, pH 4.0, 500 mM NaCl, 20 mM EDTA, and 0.005% Tween-20) and a single injection of 0.001% SDS. Fitting of sensorgrams for affinity estimations was done using the Biaevaluation version 3.1.

Following similar protocols, immobilization of HisS-NGFpro or HisS-BDNFpro was also done on a CM5 biosensor chip using the NHS/EDC coupling kit according to manufactures instructions (Biacore, Sweden), giving similar surface densities of immobilized protein (~300 fmol/mm$^2$). Purified peptides were applied to the chip at increasing concentrations to verify the direct binding of pro-neurotrophic domains to linear sortilin peptides. This chip was subsequent used to examine the binding of 390 nM wild-type sortilin domain in CaHBS buffer at a flow of 5 μL/min, in the absence or presence of competing sortilin peptide. In another competition assay, we applied the unprocessed versions of proNGF (50 nM) and proBDNF (50 nM) as well as RAP (90 nM) to immobilized sortilin and measured the binding in absence or presence of 200 μM competing peptide.

**Cellulose membrane preparation** – We generated peptide libraries for all members of the VPS10p-domain receptor gene family or specific peptide variations in terms of substitution or length analyses of identified ligand binding peptides. A total of 2181 peptides was used for representation of the sortilin gene family, corresponding to 273 peptides for sortilin (accession code: CAA66904), 734 peptides for sorLA (accession code: NP_003096), 389 peptides for sorCS1 (accession code: NP_001013049), 382 peptides for sorCS2 (accession code: Q96PQ0), and 403 peptides for sorCS3 (accession code: CAI64579), with a 13 amino acid overlap between 16-mers (35).

A cellulose support was prepared as a N-modified cellulose-amino-hydroxylpropyl ether membrane (N-CAPE), and all rounds of synthesis started with spot definition by 9-fluorenyl-methoxycarbonyl-β-alanine-pentafluorophenyl ester that created an alanine linker between peptide and membrane. Then followed an automated linear synthesis of stepwise addition of the different amino acids protected at their amino terminal by 9-fluorenyl-methoxycarbonyl and appropriate side chain protection for the growing peptide chain. The pattern of de-protection, activation, and coupling continued until 16-mer peptides were produced, resulting in an equally distributed array of covalently anchored peptides to the cellulose support at their C-terminal and an N-terminal free end (Details are given in ref. (36)). Removal of the side protection groups was done in two steps: First the membrane is treated with 90% TFA (in DCM, containing 3% TIBS and 2% H$_2$O) and secondly with 60% TFA (in DCM, containing 3% TIBS and 2% H$_2$O). To remove TFA salts the membrane was washed several times with H$_2$O, ethanol, TBS, ethanol and dried again. Finally, the membrane was blocked in blocking buffer from Sigma (cat.no. B6429) diluted in TBS (pH 8.0) and supplemented with 5% saccharose (Merck, cat.no. K32055087 422) for 2 hr before the predefined peptide library is ready for ligand binding analysis.

**Binding studies of cellulose-bound peptides** – The membrane-bound libraries were incubated with the combined S-peptide and poly-histidine-tagged pro-domains (10 μg/mL) in blocking buffer over night at 4°C, followed by a second incubation with 1 μg/mL of HRP-conjugated S-protein from Novagen (cat.no. 69047-3) also in blocking buffer but for 3 hr at room temperature. Subsequently, the membrane was washed three times for 10 min with TBS before quantitative characterization of bound ligand was carried out using the UptiLight chemoluminescence substrate from Uptima (cat.no. UP99619A) and the Lumilimeter instrument from Roche Diagnostics, providing the spot signal intensities in Boehringer Light Units (BLUs). Alternatively, detection of bound ligand was performed by an immunochemical assay with an antibody against the histidine tag from Sigma (cat.no. H-1029) and a secondary HRP-conjugated anti-mouse antibody from Calbiochem (cat.no. 401207). Incubations followed standard
Western blotting procedures and spot detection as above.

The method of substitution analysis and length analysis to identify unique single amino acid residues and to determine the minimal peptide sequence, respectively, for binding HisS pro-domains to the sortilin peptide, followed similar protocols as for the initial testing of ligand binding to the spotted membrane.

**Pull-down assay** – The expressed extracellular domains of sortilin-WT or sortilin-4A was incubated with either GST-tagged NGFpro or the propeptide of sortilin, and precipitated using 100 μL glutathione (GSH)-Sepharose beads (GE Healthcare, cat.no. 17-0756-01). The amount of applied receptor domains was determined by precipitation using Talon beads as control. Bound protein was separated by SDS-PAGE analysis and visualized using anti-histidine antibody by standard Western blotting.

**Ligand internalization by HEK 293 cells** – To study the effect of disrupting the identified binding site in full-length sortilin, HEK 293 cells were stably transfected with plasmids encoding either fl-sortilin-wt or fl-sortilin-4A. Receptor expression was confirmed by Western blotting using a sortilin-specific monoclonal antibody and by immunofluorescence. For immunofluorescence, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X100, and subsequently incubated with rabbit anti-sortilin polyclonal antibodies followed by donkey anti-rabbit Alexa-488 conjugated secondary antibodies (1:300). Internalization of bound ligands was studied by the incubation of cells for 30 min at 37 °C with anti-sortilin polyclonal antibodies (10 μg/ml), GST-NGFpro (1 μg/ml), or 100 nM BDNF pro-domain (Chemicon). Dead and live cells were subsequently quantified using the MultiTox-Fluor Multiplex cytotoxicity assay (Promega).

**p75<sup>NTR</sup> :sortilin co-immunoprecipitation** – HEK 293 cells stably transfected with constructs encoding fl-sortilin-WT<sup>endo</sup> or fl-sortilin-4A<sup>endo</sup> were incubated in PBS (with 1 mM CaCl<sub>2</sub> and MgCl<sub>2</sub>) for 90 min at RT and then treated with 5 nM reducible protein cross-linker dithiobis(succinimidylpropionate) (DSP) (Pierce) according to manufacturer’s instructions. After wash, cells were lysed on ice for 10 min in TNE buffer (20 mM TrisHCl, pH 8.0, 1% NP40, 10 mM EDTA) supplemented with complete protease inhibitor cocktail. Samples were immunoprecipitated ON at 4°C by use of Gammabind G-Sepharose beads (Amersham) coupled with anti-p75<sup>NTR</sup> (10494) from Abcam. Unspecific binding was removed by washing 5 times in TBS containing 0.05% Tween-20, and proteins eluted by boiling samples in reducing sample buffer (20 mM DTE, 2.5% SDS). Protein samples were subjected to SDS-PAGE and Western blotted using anti-sortilin from BD Transduction Laboratories (cat.no. 612100) and anti-p75<sup>NTR</sup>.

**Apoptosis assay** – The effect of sortilin-derived peptides on proNGF-induced cell death was studied in RN22 schwannoma cells. Cells were cultured in DMEM added 10% fetal bovine serum, and seeded in 96 well plates at a density of 10.000 cells/well. After 24 hr, the cells were washed twice in serum free medium and incubated for 72 hr in the presence of increasing concentrations of the sortilin-derived sort166-181 peptide diluted in DMEM without serum and phenol red, containing 30 nM selenium, 5 μg/ml insulin, 5 μg/ml transferine, 30 nM triiodothyronine, and 10 nM proNGF (Chemicon). Dead and live cells were subsequently quantified using the MultiTox-Fluor Multiplex cytotoxicity assay (Promega).

**RESULTS**

**Identification of a linear proNT binding sequence in sortilin**

We first produced the pro-domains of NGF and BDNF as recombinant fusion proteins containing two N-terminal tags, a polyhistidine and an S-peptide tag, HisS-NGFpro and HisS-BDNFpro respectively, in order to facilitate protein purification and detection of ligand binding to immobilized sortilin peptides (fig 1SA). The S-tag system is based on the interaction of the 15 amino acid S-tag peptide with a 104 amino acid long S-protein derived from pancreatic ribonuclease A (37). To confirm the receptor binding properties of the recombinant pro-domains we tested their binding to immobilized sortilin peptides using surface plasmon resonance (SPR) analysis. The calculated affinities of HisS-NGFpro (K<sub>D</sub> ~9 nM) and HisS-BDNFpro (K<sub>D</sub> ~1.6 nM) were in accordance with past findings (fig S1B) (25, 27).

To screen for potential linear ligand binding sites in the VPS10p-domain receptors, we synthesized...
a peptide library in which each of the receptors were dissected into consecutive 16-mer peptides overlapping by three amino acids and subsequently spotted onto filters. Binding of HisS-NGFpro and HisS-BDNFpro was then tested for their abilities to interact with the receptor peptide libraries by a so-called SPOT binding analysis (38-40). A specific sequential triplet of signals was observed for peptides derived from sortilin, suggesting that this particular VPS10p-domain contains a linear binding epitope for the neurotrophin pro-domains. Interestingly, the HisS-NGFpro and HisS-BDNFpro gave virtually identical results, suggesting that they may share a common (linear) binding site (fig 1).

We focused the subsequent studies on sortilin, and prepared a second peptide array comprising 273 immobilized sortilin-derived peptides. In this set of experiments we took advantage of both tags present in the HisS-BDNFpro ligand as we used either a peroxidase-conjugate of the S-protein or an immunoassay with an antibody against the polyhistidine tag for detection. The analysis of the two assays gave slightly different, but overlapping, results. Whereas detection with S-protein resulted in strong positive signals for peptides 67-69, spanning sortilin residues 166-187 (sort166-187) (fig 2A,C), the immunoassay identified peptides 64-66, comprising residues 157-178, as the ligand binding site (fig 2B,C). The shift in signal between the two detection methods likely represents different sterical hindrance from the S-protein and anti-polyhistidine antibody. We considered the signal corresponding to peptides 22-26 in the anti-histidine blot unspecific since a similar spot pattern was observed on membranes incubated with the secondary antibody alone (data not shown). Collectively, these experiments suggest that amino acids 163-174 in sortilin, corresponding to the overlapping residues in the peptide sequences, constitute a binding epitope for proNT (fig 2C).

To confirm binding of HisS-NGFpro and HisS-BDNFpro to sort163-174, we synthesized a peptide comprising these 12 amino acid residues. The peptide was subsequently subjected to SPR analysis (BIAcore) using a sensorchip coupled with HisS-NGFpro and HisS-BDNFpro, thereby reversing the system from immobilized peptides to peptides in solution. We found that the higher affinity of the BDNF pro-domain as compared to NGF pro-domain for sortilin (fig S1B and refs. (25, 27)) could be replicated when using the sortilin peptide. This suggests that the linear ligand recognition sequence sort163-174 may account for the difference in binding properties between proBDNF and proNGF (fig 2D).

Identification of key residues in sortilin for pro-domain binding
Using substitutional analysis we next set out to map key residues in the linear sortilin sequence that contribute to pro-neurotrophin binding. A variant of the SPOT synthesis method was applied to generate a new library in which every residue in the sequence R\(^{160}\)IFRSSDFAKNFVQTD\(^{178}\) had been replaced by all 20 naturally occurring L-amino acids residues. The SPOT filters were then incubated with ligand, as exemplified by HisS-BDNFpro (fig 3A), and the intensity of the binding signals were quantified and used to calculate the replacement variability for each amino acid.

To determine the minimal linear sortilin sequence capable of binding the proNT pro-domains we carried out a so-called length analysis. In practice, we synthesized the peptide sort163-178 and removed consecutive single residues from the N- and/or C-terminal ends of the peptide (table 1). Using this assay we narrowed down the length of the binding epitope to a central region of the sequence corresponding to the 12-mer fragment R\(^{160}\)IFRSSDFAKNF. This result is in accordance with the substitution analysis which also showed that residues V\(^{175}\)QTD\(^{178}\) do not significantly influence ligand binding (fig 3B).

Interestingly, by further trimming of the peptide we found that both halves of this fragment corresponding to the tetramer R\(^{160}\)IFR\(^{166}\) and the pentamer F\(^{170}\)AKNF\(^{174}\), respectively, were able to associate independently with HisS-BDNFpro (table 1) and HisS-NGFpro (data not shown).

This observation prompted us to repeat the substitution analysis, but this time with peptide libraries of R\(^{160}\)IFRSSDF\(^{170}\) and F\(^{170}\)AKNFVQTD\(^{178}\), because each of these fragments contains one of the two putative binding epitopes. Using this approach we confirmed that
an exchange of R163, F165, or R166 within the N-terminal half and F170, K172, or F174 within the C-terminal half significantly decreased binding of the HisS-BDNFpro ligand (fig 4A). Similar results were obtained for HisS-NGFpro (data not shown), further corroborating the presence of a shared binding site in sortilin for both proNTs. In further support of this notion, binding of HisS-BDNFpro to immobilized sortilin, as determined by SPR analysis, was completely prevented by pre-incubation with saturating concentrations of HisS-NGFpro (fig S2). Collectively, the above data suggest the existence of a common binding epitope comprising two interaction sites in which three residues in the second epitope may account for the stronger binding of HisS-BDNFpro as compared to HisS-NGFpro (fig 4B).

**Ligand binding to sortilin-4A**

Sortilin is produced with a propeptide that needs to be cleaved off to allow entrance of ligands into the tunnel (41). We therefore expressed a non-cleavable soluble sortilin variant as described (41), immobilized it on a biosensor chip and compared binding of unprocessed proBDNF and neurotensin to the mutant with that of the fully processed wild-type receptor. We found that both receptors bound proBDNF (fig 5A), in agreement with the existence of a binding site located away from the blocked central cavity. For comparison, binding of neurotensin was completely abolished in the non-processed sortilin variant (fig 5B), demonstrating that binding of this ligand entirely depends on access to the tunnel epitope.

To determine the impact of the linear proNT binding epitope in the full-length VPS10p-domain, we expressed in EBNA 293 cells a set of single residue alanine substitution mutants (R163A, F165A, R166A, F170A, K172A, and F174A) of the sortilin ectodomain. As a control, we included the mutation R160A, a residue outside the ligand binding peptide sequence. All mutants were effectively secreted from the cells in similar quantities and showed a migration pattern in non-reducing gel analysis identical to that of the non-mutated sortilin domain, suggesting correct disulfide bridging of the mutants (fig S3). To further demonstrate the intact structure of the VPS10p β-propeller, we determined the circular dichroism (CD)-spectra for each variant and found that none of the mutations resulted in aberrant folding of sortilin (fig S3). Binding GST-tagged NGFpro was subsequently tested using SPR analysis with each receptor mutant immobilized on individual flow cells at similar protein densities, thus allowing for direct comparison of bound ligand. However, none of the single residue mutants displayed any substantial reduction in ligand affinity (maximally 2-3-fold reduced binding) as deduced by fitting of series of ligand concentration sensorgrams (fig S3B, table 2).

Based on the existence of two independent motives within the linear binding site (cf. table 1) we speculated that failure of the single residue mutants to show reduced GST-NGFpro affinity was accounted for by binding to the non-mutated residues in the second site. We therefore hypothesized that disrupting an entire motif in combination with substitution of a single critical residue from the adjacent motif might be required to efficiently abolish ligand binding. To address this premise, we mutated the four most important residues within the R<sup>163</sup>FRSSDFAKNF<sup>174</sup>-motif (cf. fig 4) thereby generating a construct with a R163A, F165A, R166A, F170A quadruple mutation, denoted sortilin-4A. This receptor mutant was also efficiently expressed by EBNA 293 cells, able to be purified using Ni<sup>2+</sup>-affinity chromatography, and folded properly as demonstrated by CD analysis (fig S3C).

The interaction of sortilin-4A with NT pro-domains was studied by SPR analysis and using a ligand precipitation assay. HisS-BDNFpro and GST-NGFpro were immobilized on the sensor chip and equal amounts (100 nM) of wild-type sortilin (sortilin-WT) and sortilin-4A were applied. We found that HisS-BDNFpro and GST-NGFpro exhibited considerable stronger binding to sortilin-WT as compared to sortilin-4A (fig 6A, B). In another set of SPR experiments, where sortilin-WT and sortilin-4A were the immobilized parts, we found that GST-NGFpro bound to the mutant receptor with a >10-fold lower affinity, i.e. K<sub>d</sub> ~26 nM versus ~2 nM, corroborating an important role of the linear binding site also in the VPS10p holodomain (fig 7A, B). A similar decrease in GST-NGFpro binding to sortilin-4A was revealed when comparing the ability of GST-NGFpro to precipitate wild-type and mutant sortilin in solution. In practice, the two VPS10p-domains were incubated with GST-NGFpro, allowed to form complexes, and then precipitated by glutathione-beads. The precipitates were resolved by SDS-PAGE analysis and bound sortilin was subsequently detected by Western blotting (fig 7C). In line with the SPR analysis the mutated sortilin domain exhibited considerably less interaction with GST-NGFpro. To assure that binding of ligands that engage the tunnel of the β-
propeller far away from the site of the quadruple mutation was unperturbed, we repeated the experiment using the GST-tagged sortilin propeptide as a ligand (33). Importantly, this ligand efficiently precipitated sortilin-4A, confirming that the mutated domain is capable of interacting with other ligands independent of the linear binding site.

To provide evidence for a functional role for the linear proNT binding site in the full-length and membrane-associated receptor, we expressed the entire wild-type and mutated sortilin receptors, designated fl-sortilin-WT and fl-sortilin-4A, respectively, in HEK 293 cells. The mutated receptor was mainly localized in perinuclear vesicles in a pattern indistinguishable from that of wild-type sortilin (fig 8A). Identical expression levels of fl-sortilin-WT and -4A were documented by Western blot analysis of cell extracts (fig 8A). Normal internalization of an antibody against the sortilin extracellular domain confirmed that the mutant was also capable of endocytic uptake. Notably, the GST-propeptide of sortilin was equally well taken up by cells expressing wild-type and mutant receptor (fig 8B). However, whereas fl-sortilin-WT efficiently internalized the pro-domains and mutant receptor (fig 8A). This demonstrates that the linear proNT binding epitope that selective engages proNTs. In conclusion, we find that sortilin harbors a linear binding epitope that selective engages proNTs.

The linear sortilin epitope prevents proNT binding and death induction
The importance of the linear epitope for proNT binding was further substantiated in a competition assay where the pro-domains of BDNF and NGF were immobilized on a biosensor chip and the interaction with saturating concentrations of 200 nM soluble sortilin-WT was measured in the absence or presence of a competing peptide spanning the ligand-binding sequence. A clear decrease in receptor-ligand complex formation was observed in the presence of peptide. Thus, the response for sortilin-WT binding to HisS-BDNFpro was lowered from 300 RU in the absence of competitor to 100 RU in the presence of 200 μM sort166-181 (fig 9A). Quantification of the equilibrium response at increasing amounts of peptide showed a concentration dependent competition profile with the inhibition being stronger for the interaction of sortilin with HisS-BDNFpro than for HisS-NGFpro, in line with a higher affinity of the former pro-domain (fig 9B). Moreover, the peptide also blocked the interaction of proBDNF and proNGF with sortilin immobilized on the chip surface (fig 10A, B). As a control for the specificity of the inhibitor, binding of sortilin to an unrelated ligand, the receptor-associated protein (RAP) (42, 43), was not affected by the sortilin peptide (fig 10C).

We finally asked if the sortilin-derived peptide was capable of blocking proNGF induced apoptosis by the sortilin:p75NTR complex. To this end we incubated the schwannoma cell line RN22 with 10 nM proNGF in the absence or presence of the peptide sort166-181 and scored apoptosis after 72 hr. Indeed, the peptide inhibited proNGF-induced cell death in a concentration dependent manner with more than 60% reduction in the dead/live cell ratio at the highest peptide concentration used (fig 11A). As reduced killing could also arise from a deficient physical interaction between sortilin and p75NTR, we also examined if peptide sort166-181 would interfere with receptor hetero-oligomerization. We observed no effect on the ability of p75NTR to precipitate sortilin in the presence of 100 μM of the peptide inhibitor (fig 11B). These date show that blocking the interaction with the linear epitope in sortilin is sufficient to specifically prevent binding of proNTs to sortilin, leaving ligand binding inside the tunnel as well as receptor hetero-oligomerization intact.

In conclusion, we find that sortilin harbors a linear binding epitope that selective engages proNTs. We propose that this sequence may be used to derive a peptide antagonist or small organic molecule that can prevent apoptosis.

DISCUSSION
Pro-neurotrophins were initially considered inert precursors that merely serve as a reservoir for their mature counterparts, but it is now well
established that they also exhibit activities on their own (16, 23). While mature NTs stimulate neuronal survival and differentiation by engaging their specific tyrosine-receptor kinases (Trks) in conjunction with p75NTR, proNTs can elicit apoptosis in cells expressing sortilin and p75NTR (1, 24, 44, 45). Although the signaling pathway has not been fully elucidated, it has been shown that the simultaneous binding of the proNT pro-domain to sortilin and the mature part to p75NTR induces formation of a tripartite death-inducing complex capable of activating the Jun-kinase signaling pathway (46-49). We here identified a linear and surface exposed amino acid sequence in sortilin that partakes in proNT binding and plays a pivotal role for death-induction by pro-neurotrophins.

The molecular structure of the sortilin ectodomain in complex with the neuropeptide neurotensin was recently solved at atomic resolution. The structure revealed an unprecedented 10-bladed β-propeller encircling a large tunnel that encompasses the binding site for neurotensin, the sortilin propeptide and RAP (33, 41, 50). Intriguingly, we found that the surface of the receptor may engage in binding of proNTs. First, the soluble sortilin peptide sort163-174 bound to immobilized NT pro-domains and vice versa. Second, this interaction critically depended on the surface exposed amino acids R163, F165 and R166 for both NGFpro and BDNFpro whereas F170 and F174 also contributed to the binding of the BDNF pro-domain, an observation that may explain the higher affinity of sortilin for proBDNF than proNGF (fig 2d and S1; refs (25, 27)). Third, mutated sortilin harboring alanine substitutions in the surface exposed residues R163, F165, R166 and F170 showed only little interaction with proNTs as determined by SPR analysis, co-immunoprecipitation, and cellular uptake studies, while binding of the sortilin propeptide was unperturbed. Fourth, the peptide sort166-181 competitively inhibited sortilin binding of proNTs, but not of p75NTR, which likely engages an alternative surface epitope on the β-propeller, nor RAP or the sortilin propeptide that selectively target the tunnel. In agreement, the sortilin peptide was a potent inhibitor of proNT induced apoptosis in RN22 cells.

In the SPOT analysis, application of the two detection methods gave similar, though not identical, results confirming the specificity of the binding between the receptor peptides and the NT pro-domains. While HisS-NGFpro and HisS-BDNFpro binding to the sortilin peptides gave rise to the same spots when both were assayed with the S-protein (peptides 67-69) (fig 1), we saw a consistent shift towards earlier sequences (peptides 64-66) when developed with the anti-histidine antibody (fig 2). We speculate this difference could be accounted for by sterical hindrance of the detection system from the solid support. Thus, the S-protein is only 104 residues while the anti-histidine immunoassay requires binding of two large antibodies, each roughly of 1.500 amino acids.

Interestingly, the identification of two positive (R163 and R166) but no negative charged residues in the sortilin epitope, suggests that electrostatic interactions may contribute to the binding between the receptor and proNTs. In agreement with this model, the BDNF pro-domain contains 17 acidic residues many of which are located in a region suggested to be vital for the interaction with sortilin (51).

Sequence analysis of sortilin predicted the presence of several Asp-box motives throughout the VPS10p-domain (52). Indeed, X-ray analysis of sortilin revealed that Asp-boxes constitute a well-defined structure, that folds into a surface exposed hairpin-loop bridging strand 3 and 4 in each of the ten β-blades of the propeller (33). The Asp-box consensus sequence can be expressed as X-X-Ser-X-Asp-X-Gly-X-Thr-Trp/Phe-X where X represents any amino acid (53). Interestingly, this motif, R163FRRSSDFAKNF174, is part of the identified proNT binding in blade two of the receptor. Asp-boxes normally serve a structural function in β-propellers, where the conserved residues participate in folding and ensure the stability of the domain (54). The variable residues are free to carry out other functions, e.g. ligand binding as observed for nucleotide binding to microbial ribonucleases (55), in line with our finding that these amino acids are involved in binding of the NT pro-domain. It is noteworthy that although most β-propellers bind their ligands at the top face, as is also the case for neurotensin and the sortilin propeptide (33), binding to the bottom face or side domain has also been reported (55-57).

Our data do not exclude that NGFpro and BDNFpro may also target the epitope in the tunnel of the β-propeller. While the residues identified in the current study are exposed on the rim and outer surface of the barrel, it is possible that the >100 amino acids long NT pro-domains simultaneously
can associate with additional binding epitopes including the tunnel. This would be in accordance with the suggested highly flexible and extended conformation of the proNGF pro-domain (58, 59), and in line with the recently reported crystal structure of the proNGF:p75NTR complex that shows the pro-domain to accommodate an exposed and disordered conformation free to interact with sortilin (49).

By SPOT peptide mapping we only identified one linear binding site in sortilin comprising amino acids 163-174. We believe this fact is due to the tunnel surface binding site for neurotensin being comprised of residues located far away from each other in the primary sequence, e.g. K227, S283, R292, and Y318 (33). These residues are separated by up to 90 amino acids within the primary structure thereby representing a discontinuous binding epitope not suitable for detection by a peptide mapping approach.

There are experimental data to support as well to refute an additional proNT binding epitope in the tunnel. We found that following disruption of the four most critical amino acids (R163, F165, R166 and F170) for the interaction with proNT there was an ~25% residual binding still present, arguing for one or more additional interaction sites (fig 6). On the contrary, mutations in residue S283, that is indispensable for binding of the sortilin propeptide inside the tunnel, completely abrogated binding of this peptide while proNGF binding was unperturbed (33). Thus the experimental evidence in support of a composite proNT binding site that overlaps with that of the sortilin propeptide and neurotensin in the tunnel is contradictory. However, it is possible that proNT might engage the tunnel via an epitope in the vicinity of but independent of amino acid S283. This is supported by the observation that binding of the proNGF pro-domain is significantly better inhibited by the 13 amino acids long full-length neurotensin than by residues 11-13 that constitute the sortilin binding site in the neuropeptide (33). This data may suggest that neurotensin competes with proNGF for binding due to steric hindrance in the tunnel. Collectively, the above data underscore the requirement of elucidating the atomic structure of the sortilin:proNT complex in order to precisely and unanimously identify the number and location of all binding epitopes.

Recent evidence has suggested that proNT-induced cell deaths play an important role in conditions characterized by apoptosis such as aging, and in pathological conditions including seizures, spinal cord injury, retinal dystrophy, and Alzheimer’s disease (AD) (15, 18, 24, 28, 29, 60-65). In accordance, recent reports have described changes in the levels of proNTs in many of these conditions including early stages of AD (60, 66-68). Of particular interest, increased glycation and lipoxidation of proNGF in the hippocampus and entorhinal cortex of AD patients makes it less susceptible to processing into mature NGF. Furthermore, in these patients TrkA expression is down whereas the expression of p75NTR and sortilin are unchanged (refs (69, 70); Mufson et al., unpublished). Possibly, an imbalance between the relationship of receptor expression and pro versus mature NGF changes the equilibrium between survival and apoptosis signaling in favor of the latter (71, 72). Prompted by the biological significance of proNTs in cell death, we tested if the ligand binding peptide of sortilin might be capable of preventing apoptosis. Indeed, we found that the peptide offered efficient protection against proNGF-induced apoptosis in cultured RN22 schwannoma cells, a finding that holds promise to the idea that an antagonist that can prevent proNGF binding to sortilin might be an efficient target to prevent pro-apoptotic conditions.

The peptide used in the present work, is unlikely to be suitable for therapeutic use due to its modest affinity and accordingly high concentrations required to inhibit proNT binding. Yet, the peptide may serve as a lead for rational drug design aimed at increasing the binding affinity for NT pro-domains as well as to enhance the pharmacological properties in terms of solubility, stability, and efficacy. Ideally, such a compound should specifically prevent proNT-induced apoptosis, leaving the trophic actions of mature neurotrophins unchanged.
REFERENCES


FOOTNOTES

We thank Peder Madsen for the neurotrophin cDNAs, Lars Sottrup-Jensen for help doing the CD-analysis, and Claus M. Petersen for valuable discussions throughout the preparation of the manuscript. Søren Thirup is greatly acknowledged for preparing the picture in figure 4B. Anja Aagaard, Anne Marie Bundsgaard, and Marit Nyholm Nielsen are thanked for their excellent technical support. This work was supported by grants from the Danish Medical Research Council and the Lundbeck Foundation. P.B. is supported in part by the Deutsche Forschungsgemeinschaft (DFG. VO 885/3-1).

Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT, neurotrophin; proNT, proneurotrophin; RAP, receptor-associated protein; SPR, surface plasmon resonance; VPS10p, vacuolar protein sorting 10-protein; AD, Alzheimer’s disease;
FIGURE LEGENDS

Figure 1. Analysis of the NGF and the BDNF pro-domain binding to the mammalian VPS10p receptors by SPOT analysis.
A peptide library containing a total of 2181 peptides represented by 734 from sorLA, 403 from sorCS3, 389 from sorCS1, 382 from sorCS2, and 273 from sortilin as overlapping 16-mer peptides of the five human VPS10p-domain containing receptors sortilin (CAA66904; 831 aa), sorLA (AAC50891; 2214 aa), sorCS1(AAM43811; 1179 aa), sorCS2 (Q96PQ0; 1159 aa), and sorCS3(Q9U3P3; 1222 aa). The membrane was incubated either in the presence of 10 μg/mL HisS-NGFpro (A), 10 μg/mL HisS-BDNFpro (B) or in the absence of ligand (C). Detection was carried out by incubation of the membrane with a HRP-conjugate of S-protein that recognizes the S-peptide tag of bound NT pro-domains. Each receptor library is cornered at the upper left and lower right corner by three control peptides equivalent to the S-peptide tag (amino acid sequence KETAAAKFERQHMS). The specific binding site for both HisS-NGFpro and HisS-BDNFpro (A, B) which is not seen for the control experiment in absence of ligand (C) is indicated for the three sortilin peptides (SPOT numbers 67-69; red box).

Figure 2. Identification of a linear proNT binding site within sortilin.
(A, B) Sortilin represented as 273 overlapping peptides generated by SPOT synthesis on a cellulose membrane with peptides 64-69 binding to HisS-BDNFpro, as detected by either HRP-S-protein (A; SPOT numbers 67-69) or antibody immunoassay against the polyhistidine tag (B; SPOT numbers 64-66). (C) Peptide sequences of the six peptides 64-69 found to interact with the pro-domains of BDNF and NGF. Residues corresponding to the sortilin fragment 163-174 are boxed. (D) Surface plasmon resonance analysis of the 12-mer sortilin derived peptide (residues R163IFRSSDFAKNF174) binding to flow cells with immobilized HisS-BDNFpro and HisS-NGFpro.

Figure 3. Substitution analysis of the linear sortilin sequence.
(A) A representative substitutional binding analysis of HisS-BDNFpro to peptides with the wild-type sortilin sequence R163IFRSSDFAKNFVQTD178 listed to the left on the membrane and detection using the anti-histidine immunoassay. (B) Binding assays as demonstrated in panel A was carried out for both HisS-BDNFpro and HisS-NGFpro, and signal intensities, measured in Boehringer Light Units (BLU), were quantified for each peptide. The percentage of replacement variability (V) of each sequence position have been calculated (V=BLU/20 x 100%) and plotted against each amino acid within the peptide (residues 163-178).

Figure 4. Mutational binding analysis of HisS-proBDNF to short sortilin fragments.
(A) HisS-BDNFpro binding analysis to peptides with the wild-type sortilin sequences R163IFRSSDF170 and F170AKNFVQTD178 is listed to the left on each membrane. Binding to mutant peptides where each amino acid has been substituted with the 20 naturally occurring amino acids is used for identification of specific residues important for interaction with the immature part of proBDNF. The substitution analysis clearly identifies the two sequences R163IFR166 (upper part) and F170AKNF174 (lower part) as specific binding sites for HisS-BDNFpro. (B) Crystal structure presentation of sortilin showing the surface exposure of side chains from several identified key residues in purple (e.g. R163, R166, F170 and K172). Structure was drawn from coordinates present in the Protein Data Bank with accession code 3F6K.

Figure 5. Differential binding of proBDNF and neurotension to sortilin and pro-sortilin.
(A) Surface plasmon resonance analysis showing that unprocessed proBDNF (50 nM) binds nearly as efficiently to the receptor in the presence (pro-sortilin) as in the absence (sortilin) of the receptor propeptide. Pro-sortilin is a receptor variant unable to cleave off the propeptide due to mutation of the furin recognition site. (B) Binding of neurotensin (20 μM) is strongly ablated for pro-sortilin as compared with binding to sortilin without the presence of its propeptide.

Figure 6. Mutation of the linear binding site specifically impairs binding of both the NGF and the BDNF pro-domains.
SPR analysis showing reduced binding of equal amounts (analyte concentration: 200 nM) of the soluble extracellular domains of sortilin-4A compared to sortilin-WT when binding is tested to both the immobilized BDNF pro-domain (HisS-BDNFpro; A) and the immobilized NGF pro-domain (GST-NGFpro; B).

Figure 7. GST-NGFpro binding to sortilin-WT and sortilin-4A.
SPR analysis showing concentration series of GST-NGFpro (10, 20, 30, 40, and 50 nM) tested for binding to immobilized extracellular domains of sortilin-4A (A) and sortilin-WT (B), demonstrating a strong decrease in the binding capacity for the NGF pro-domain upon the quadruple mutation in sortilin-4A. A higher than 10-fold decrease in affinity (WT: $K_D \sim 2$ nM versus 4A: $K_D \sim 26$ nM) was estimated using the Biaevaluation software. (C) Medium from EBNA 293 cells producing either the soluble ectodomain of sortilin-WT or sortilin-4A were incubated with a GST-tagged variant of the receptor propeptide (GST-propept) or GST-NGFpro and precipitated by glutathione (GSH)-beads. The amount of total secreted sortilin-WT and sortilin-4A (i.e. input) was determined by precipitation using Talon-beads binding to the histidine-tag within the sortilin domains. The precipitated proteins were subjected to SDS-PAGE analysis and visualized by Western blot analysis for sortilin.

Figure 8. Decreased binding of proNT to sortilin-4A within cells.
(A) Immunostaining of HEK 293 cells transfected with constructs for full-length (fl)-sortilin-WT or sortilin-4A with an antibody against sortilin. Protein expression levels tested by Western blot analysis of cell lysates. (B, C) Cells were labeled with ligands at 4°C followed by endocytosis for 30 min at 37°C before fixation and internalized ligand visualization by staining. Ligands applied was either a sortilin antibody (against the receptor extracellular domain; α-sortilin), the receptor propeptide (GST-propept) (B), GST-NGFpro, or BDNFpro (C). (D) Cells co-transfected with constructs for sortilin variants devoid of endocytosis (fl-sortilin-WTΔendo or fl-sortilin-4AΔendo) together with p75NTR were immunoprecipitation using an antibody against p75NTR. Lysates and precipitated proteins were subjected to SDS-PAGE and analyzed by Western blotting for either p75NTR or sortilin as indicated.

Figure 9. Sortilin-derived peptide specifically competes binding of proNT to sortilin.
(A) Recombinant sortilin was purified from 293 cells (indicated to the right by silver stained SDS-PAGE analysis), and used for surface plasmon resonance studies to immobilized HisS-BDNFpro. The signal of 300 RU observed for binding in the absence of the peptide sort166-181 (− peptide) was significantly lowered to 100 RU in the presence of 200 μM linear sortilin antagonist (+ peptide). (B) Sortilin binding to either HisS-NGFpro or HisS-BDNFpro was determined by SRP analysis (as exemplified in A) and the inhibition by increasing amounts of sort166-181 (at 2, 20, and 200 μM) was plotted relative to the observed interaction in the absence of competitor (in percentage). Values represent the mean ± standard errors of the mean from three experiments. *P=0.0007; two-tailed Student’s t-test.

Figure 10. Selective competition of ligands by sortilin-derived peptide antagonist.
SPR binding analysis of 50 nM unprocessed proBDNF (A), 50 nM unprocessed proNGF (B), and 90 nM RAP (C) to immobilized sortilin in the absence and presence of the sort166-181 peptide (100 μM). Specific inhibition of proNGF and proBDNF is observed whereas the interaction between sortilin and RAP is largely intact.

Figure 11. A sortilin-derived peptide blocks proNGF-induced cell death.
(A) RN22 schwannoma cells were incubated in the presence of 10 nM proNGF and increasing concentrations (0.2, 2.0, and 200 μM) of the sort166-181 peptide. The amount of proNGF-induced cell death after 72 hr were quantified using a fluorescence-based assay, and plotted against competitor concentrations as the ratio between dead and live cells in percentage. (B) Cells co-transfected with constructs for a sortilin variant devoid of endocytosis (fl-sortilin-WTΔendo) together with p75NTR were incubated in the absence or presence of 100 μM of the sort166-181 peptide, and immunoprecipitated using an antibody against p75NTR. Lysates and precipitated
proteins were subjected to SDS-PAGE and analyzed by Western blotting for either p75\textsuperscript{NTR} or sortilin as indicated.
Table 1. Length analysis for trimming the R\textsuperscript{163}FRRSSDFAKNFVQTD\textsuperscript{178} peptide.
The quantification of a representative experiment testing the influence of the length of the sort163-178 peptide binding to HisS-BDNFpro in a modified SPOT analysis. Peptides with a high ligand affinity are shown on a grey background. For this presentation, an intensity threshold for the signal intensity of 30,000 Boehringer Light Units (BLU) have been chosen as indication for high affinity. The presence of the two identified minor binding sites R\textsuperscript{163}IFR\textsuperscript{166} and F\textsuperscript{170}AKN\textsuperscript{174} is presented on a black background.

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Table 2. Binding kinetics of GST-NGFpro binding to sortilin mutants. Concentration series of 10, 20, 30, 40, 50 nM of GST-NGFpro was applied to biosensor chips with immobilized sortilin single residue mutants. Affinities and rate constants were estimated using the biaevaluation software, using the 1:1 Langmuir binding isotherm.

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<td>$1.47 \times 10^5$</td>
</tr>
<tr>
<td>R163A</td>
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<td>$1.85 \times 10^5$</td>
</tr>
<tr>
<td>F165A</td>
<td>RGGRIARSSDFAKNF</td>
<td>$1.10 \times 10^5$</td>
</tr>
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<td>$6.48 \times 10^4$</td>
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<td>F174A</td>
<td>RGGRIFRSSDFAKNF</td>
<td>$2.35 \times 10^5$</td>
</tr>
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</table>
Figure 1
Andersen et al.
Figure 2
Andersen et al.
Figure 3
Andersen et al.

A

Variable amino acids

B

HisS-BDNFpro

Variance [%]

amino acids

HisS-NGFpro

Variance [%]

amino acids
Figure 4
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Figure 5
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Figure 6
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Figure 7
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Figure 8
Andersen et al.
Figure 9
Andersen et al.
Figure 10
Andersen et al.
Figure 11
Andersen et al.