Mapping of the Interaction Site between Sortilin and the p75 Neurotrophin Receptor Reveals a Regulatory Role for the Sortilin Intracellular Domain in p75 Neurotrophin Receptor Shedding and Apoptosis*

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Background: Sortilin and p75NTR induce neuronal apoptosis by binding pro-neurotrophins during development and following neuronal injury. Sortilin interacts with an extracellular juxtamembrane 23-amino acid sequence of p75NTR. Despite binding being mediated through extracellular interactions, the intracellular domain of sortilin regulates p75NTR shedding and apoptosis. Mapping may allow design of compounds inhibiting neuronal cell death by blocking the interaction between sortilin and p75NTR.

Neurotrophins comprise a group of neuronal growth factors that are essential for the development and maintenance of the nervous system. However, the immature pro-neurotrophins promote apoptosis by engaging in a complex with sortilin and the p75 neurotrophin receptor (p75NTR). To identify the interaction site between sortilin and p75NTR, we analyzed binding between chimeric receptor constructs and truncated p75NTR variants by co-immunoprecipitation experiments, surface plasmon resonance analysis, and FRET. We found that complex formation between sortilin and p75NTR relies on contact points in the extracellular domains of the receptors. We also determined that the interaction critically depends on an extracellular juxtamembrane 23-amino acid sequence of p75NTR. Functional studies further revealed an important regulatory function of the sortilin intracellular domain in p75NTR-regulated intramembrane proteolysis and apoptosis. Thus, although the intracellular domain of sortilin does not contribute to p75NTR binding, it does regulate the rates of p75NTR cleavage, which is required to mediate pro-neurotrophin-stimulated cell death.

Neurotrophins, including NGF, BDNF, and neurotrophin-3 (NT3)3 contribute a group of neuronal growth factors that are important for the development, maintenance, and differentiation of the nervous system (1). However, neurotrophins can also be released in an immature form known as pro-neurotrophins (proNTs), which display distinct and often opposing biological activities to those of their mature counterparts (2–4). Hence, by forming a heterotrimeric complex with sortilin and the p75 neurotrophin receptor (p75NTR), proNGF and proBDNF induce apoptosis during development and cell senescence, as well as following neuronal injury (5–7). In addition, proNT3 has recently been found to promote apoptosis in superior and spiral ganglion neurons by binding sortilin and p75NTR (8, 9). Binding of proNTs is achieved by binding of the pro-domain by sortilin, whereas p75NTR interacts with the mature part of the neurotrophin (5). However, sortilin and p75NTR also exist as a preformed receptor complex, interacting in the absence of ligand (5).

Sortilin is a neuronal type 1 receptor belonging to the Vps10p family (10, 11). Extracellularly, sortilin contains a ligand-binding 10-bladed β-propeller followed by a 10CC module (12). A short cytoplasmic tail contains several internalization and sorting motifs, which facilitate the trafficking and sorting functions of sortilin. p75NTR is a member of the TNF receptor superfamily and is best known for its role in neuronal apoptosis and neurodegeneration (14). Its extracellular domain consists of four tandemly arranged ligand-binding domains followed by a ~60-amino acid juxtamembrane stalk region. As for other members of the TNF receptor superfamily, a death domain is found in the cytoplasmic domain linked to the transmembrane sequence via a 60-amino acid p75NTR-spe-
cific “chopper” module (15). The p75NTR intracellular domain (ICD) has been reported to promote apoptosis, as well as cell survival in a cell type-specific manner if released into the cytosol (16–19). This is achieved by regulated intramembrane proteolysis (RIP) whereby the stalk region of p75NTR is first cleaved by the α-secretase TACE/ADAM17, generating the C-terminal fragment. The remaining membrane-tethered stub is subsequently cleaved by preselinin-dependent γ-secretase, releasing the p75NTR ICD to the cytosol for signaling (20).

We have previously demonstrated that sortilin increases p75NTR affinity for proNTs. For example, cells expressing p75NTR alone bind proNGF with an approximate $K_d$ value of $\sim 15$ nm, whereas co-expression of sortilin increases affinity for proNGF $\sim 100$-fold to $K_d = \sim 160$ pm (5). This dramatically decreases the effective concentration of proNTs required for apoptotic signaling by p75NTR. Here we identify the extracellular domains in sortilin and p75NTR that are responsible for receptor heterodimerization. Although the intracellular domain of sortilin is not involved in receptor interactions, we found that it regulates RIP of p75NTR and proNT-stimulated apoptotic signaling, suggesting additional roles for sortilin in p75NTR-mediated apoptosis.

**EXPERIMENTAL PROCEDURES**

**DNA Work**—The pcDNA3.1/Zeo(−) containing cDNA encoding human wild type sortilin, sortilinmut (Y792A, L795A, and Leu829 to Leu830 deleted), IL2Rtm/sortilinmut,icd0 and sortilintailless (truncated at position Cys783) have previously been described (13, 21, 22). For construction of sortilin-ecd-IL2Rmin,icd, a fragment was amplified by standard PCR techniques using the $\alpha$-subunit of the human interleukin-2 receptor (IL2R)/pcDNA3.1/Zeo(−) as the template and an upstream primer encoding part of the transmembrane domain of IL2R and part of the luminal domain of sortilin, and a downstream primer containing a cytosolic sequence of IL2R. Using the native luminal BspE1 site of sortilin and a 3′ primer-generated AfII site, the fragment was ligated into predigest sortilinmut/pcDNA3.1/Zeo(−). PCR-mediated overlap extension was used to fuse the extracellular and transmembrane domains of sortilin and HA-tagged p75NTR with the β-galactosidase $\Delta$α and $\Delta$ω, respectively, generating sortilin−β-galα and HA−p75NTR−β-galω. An upstream fragment encoding part of the extracellular and transmembrane domains of sortilin in combination with part of β-galactosidase $\Delta$α was amplified using sortilinmut/pcDNA3.1/Zeo(−) as the template. A downstream fragment encoding β-galactosidase$\Delta$α was amplified using the template Δω/pwzl/Neo (23). The upstream fragment containing HA−p75NTR extracellular and transmembrane domains and part of β-galactosidase $\Delta$ω was generated using HA−p75NTR/pcDNA3.1/G418 as the template. A downstream fragment encoding β-galactosidase $\Delta$ω was amplified using Δω/pwzl/Hygro (23) as the template. Following amplification of overlapping PCR products, sortilin fusion protein was inserted into sortilinmut/pcDNA3.1/Zeo(−) using the native luminal BspE1 site and the 3′ primer-generated AfII site, whereas HA−p75NTR fusion protein was ligated into predigested pcDNA3.1/G418(−) using a primer-generated 5′-NotI and the 3′ AfII sites.

To make deletion and truncation expression constructs of p75NTR (p75NTR $\Delta$C1 (1–29, 66–425), p75NTR $\Delta$C1,2 (1–29, 109–425), p75NTR $\Delta$c1,2,3,4 (1–29, 190–425), p75NTR $\Delta$stalk (1–227, 251–425), p75NTR $\Delta$stalk,tm/icd (228–425), and p75NTRICD (274–425)) and YFP- or CFP-tagged versions of these (34), a modified pCDNA3 (Invitrogen) backbone was used. The rat p75NTR signal peptide including a Kozak sequence (nucleotides −29 to +87) was inserted between the KpnI and EcoRV restriction sites, generating the vector pCDNA3-SP. p75NTR coding sequences were amplified under standard PCR conditions and cloned into pCDNA3-SP using the primer-generated EcoRV and Nhel sites. In cases where p75NTR variants were fused to a fluorophore, YFP and CFP were amplified by PCR from peYFP-N1 and peCFP-N1 (Clontech), using primers incorporating 5′ EcoRV and Nhel restriction sites and a 3′ stop codon and a HindIII site. Enhanced YFP and CFP were cloned in frame between the EcoRV and HindIII restriction sites of pCDNA3-SP, generating the vectors pCDNA3-YFP/CFP.

p75NTR coding sequences were amplified by PCR with a 5′ EcoRV and a 3′ Nhel restriction site. p75NTR coding sequences were then cloned between the EcoRV and Nhel restriction sites of pCDNA3-YFP/CFP to generate in-frame fusion proteins. $p_{75NTR}$tm/icd (251–425) was constructed as previously described (24) and fused to YFP as described above. To generate sortilin-YFP, full-length human sortilin, including the sequences encoding the signal and pro-peptides, and the Kozak sequence were amplified by PCR, thereby generating a 3′-Kpn site and a 5′-Nhel site. The fragment was inserted into pCDNA3-YFP. The p75NTR-Gal4 reporter construct for the p75NTR cleavage reporter assay was cloned by inserting rat p75NTR cDNA into the pcDNA3-GVP vector containing Gal4 DNA binding and VP16 transcription activating domains and a neomycin selection cassette. The plasmid was digested with Mulu, and a Gal4-responsive 9×UAS Luc2P cassette from the pGL4.35 reporter vector (Promega) was inserted by blunt end cloning after filling in the recessed 3′ ends with Klenow fragment.

**Cell Lines and Culturing**—HEK293 cells and rat Schwannoma cells (RN22 cells) were cultured in DMEM (Lonza) supplemented with 10% FCS, 100 units of penicillin, and 100 μg/ml streptomycin (Invitrogen) in 5% CO2 at 37 °C. Transfections were carried out with FuGENE 6 transfection reagent (Roche Applied Science), and stably transfected clones were selected in medium containing 150 (HEK293 cells) or 300 (RN22 cells) μg/ml Zeocin (Invitrogen) and/or 400 μg/ml Geneticin (Invitrogen).

**Antibodies and Proteins**—Anti-neurotensin receptor-3, recognizing the extracellular domain of sortilin, was purchased from BD Transduction Laboratories (612100) and anti-p75NTR antibody (ab10494) from Abcam. Anti-p75NTR ICD 9992, for detecting p75NTR ICD, was a generous gift from Professor Moses V. Chao (Skirball Institute of Biomolecular Medicine, New York University School of Medicine). Anti-IL2Ra (I6152) and anti-HA tag (H6908) antibodies were from Sigma. Anti-GFP (11814460001) was purchased from Roche Applied Science. Recombinant His$\alpha$-tagged soluble human sortilin (sol-sortilin) was produced and purified as previously described (22). The luminal domain of human p75NTR (Met1–Asn290) and...
human RET receptor tyrosine kinase (Met1–Arg635), all fused to the Fc region of IgG1, were purchased from R & D Systems. Recombinant human furin cleavage-resistant proNGF, proNT3, and wild type proBDNF were a gift from Professor Elisabeth Schwarz (Institute for Biotechnology, Martin-Luther-Universität, Halle-Wittenberg) (25). Recombinant mouse proNGF used for RN22 cell death assays was purchased from Chemicon. Mouse NGF was from Austral Biologicals (GF-022) and neurotensin from Sigma (N6383).

Co-immunoprecipitation—Stably or transiently transfected HEK293 cells were incubated with or without ligand in PBS (with 1 mM CaCl2 and MgCl2) for 90 min at room temperature and then treated with the reducible protein cross-linker dithio-bis succinimidylpropionate (Pierce) according to the manufacturer’s instructions. After washing, the cells were lysed on ice for 10 min in TNE buffer (20 mM Tris, pH 8, 10 mM EDTA) supplemented with 1% Nonidet P-40 and complete protease inhibitor mixture (Roche Applied Science). Samples were immunoprecipitated overnight at 4 °C by use of Gammabind inhibitor mixture (Roche Applied Science). Samples were then treated with the reducible protein cross-linker dithiol, 2.5% SDS). Protein samples were subjected to reducing SDS-PAGE, Western blotting, and visualization using the Fuji infrared imaging system. The blots were stripped in 62.5 mM Tris-HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS. Protein samples were subjected to reducing SDS-PAGE, Western blotting, and visualization using the Fuji film LAS1000 imaging system. The blots were stripped in 62.5 mM Tris–HCl, pH 6.7, 100 mM 2-mercaptoethanol, and 2% SDS for 30 min at 50 °C under rotation. Co-immunoprecipitation performed with p75NTR variants was visualized with the Odyssey infrared imaging system.

β-Galactosidase Complementation Assay—HEK293 cells were seeded into white 96-well tissue culture plates with clear bottoms (Sigma) at a density of 30,000 cells/well. The cells were then transfected with sortilin-β-galΔα and/or HA-p75NTR-β-galΔα and after 48 h incubated with or without proNTs for 30 min at 37 °C. β-galactosidase activity was assessed by adding 100 μl of Gal-Screen substrate (50 mM from Pierce) solution A and solution B) (Applied Biosystems) to each well. After 30 min at 50 °C, the relative optical density was measured with a Victor3 1420 multilabel counter. Surface Plasmon Resonance Analysis—Analyses were performed on a Biacore 3000 instrument equipped with CM5 sensor chips and activated as previously described (22). sortilin was immobilized at a density of ~72 fmol/ml/mm2 in 10 mM sodium acetate, pH 4.0, and the remaining sites were blocked with 1 mM ethanolamine, pH 8.5. Injection of proteins was done in running buffer at 25 °C (10 mM HEPES, pH 7.4, containing 150 mM NaCl, 1.5 mM CaCl2, 1 mM EGTA, and 0.005% P20). Binding was expressed as the fractional increase in YFP emission caused by FRET (26) according to the formula described in Ref. 34.

Receptor Processing Experiments—HEK293 cells or stably transfected polyclonal RN22 cells were seeded into poly-l-lysine-coated 24-well culture tissue plates at a density of 40,000 cells/well. HEK293 cells were transfected after 24 h and used for shedding experiments after an additional 48 h. Prior to treatment with 200 nM phorbol 12-myristate 13-acetate (PMA) (Calbiochem) cells were incubated for 1 h with 200 mM compound E (Alexis Biochemicals) or 1 μM epoxomicin (Sigma) in DMEM supplemented with 10% FCS. The cells were then incubated for 3 h (HEK293 cells) or 6 h (RN22 cells), after which they were lysed in TNE buffer supplemented with 1% Nonidet P-40 and complete protease inhibitor mixture and subjected to Western blotting.

Primary cultures of superior cervical ganglion neurons were established from postnatal day 3–5 sortilin knock-out mice (7) as described in Ref. 27. Dissociated neurons were seeded into poly-l-ornithine (Sigma) and 3 μg/ml laminin (Invitrogen) coated wells in neurobasal A medium supplemented with 1% nonessential amino acids, 1% Glutamax (all from Gibco), 0.2% B27 (Invitrogen), and 10 ng/ml NGF. 24 h post-seeding, the cells were starved for 4 h and subsequently added 1 μM epoxomicin, 10 ng/ml NGF and 10% FCS for 1 h followed by the addition of 200 nM PMA for 3 h. The cells were harvested in TNE buffer supplemented with 1% Nonidet P-40 and complete protease inhibitor mixture and subjected to Western blotting using HRP-conjugated secondary antibodies and Supersignal West Femto Sensitivity Substrate from Pierce.

RN22 Cell Death Assay—Polyclonal stable RN22 cell lines were seeded into poly-l-lysine-coated black 96-well tissue culture plates (PerkinElmer) at a density of 7500 cells/well. After 24 h, the cells were washed three times in DMEM without phenol red, supplemented with 100 units of penicillin and 100 μg/ml streptomycin, 1% Glutamax (Invitrogen), 1.5 μg/ml insulin, 50 μg/ml transferrin, 30 nM selenium, and 30 nM triiodothyronine (all from Sigma) and incubated with or without proNGF in a final volume of 100 μl of the same medium. Following 72 h of incubation, the number of dead and live cells was scored with the MultiTox-Fluor multiplex cytotoxicity assay (Promega) measuring fluorescence with a Victor3 1420 multilabel counter. To correct for differences in cell numbers among wells, the death signal was related to the life signal from the same well.

Luciferase Assay—HEK293 cells stably co-expressing p75NTR-Gal4 and luciferase under the UAS promoter were seeded in 24-well tissue culture plates at a density of 30,000 cells/well. After 24 h, some cells were transiently transfected
with variants of sortilin. 72 h post-seeding, the cells were analyzed for luciferase activity using the luciferase assay from Promega. Prior to analysis, the cultures were treated 1 h with epoxomicin (1 μM) and in some cases with the α-secretase inhibitor TAPI-2 (20 μM) followed by the addition of PMA (200 nm) for 3 h. Luminescence was measured with a POLARstar OPTIMA plate reader.

**Statistics**—The results were analyzed by Student’s t test, with p < 0.05 being considered a significant level of difference. In Figs. 5 and 6, the effect of wild type sortilin was compared with control and with the effect of sortilin mutants.

**RESULTS**

**ProNGF Increases Affinity between Sortilin and p75NTR**—To map binding between sortilin and p75NTR, we established a co-immunoprecipitation protocol using stably or transiently transfected HEK293 cells. These cells were then treated with the cell-permeable, reducible protein cross-linker dithiobis succinimidylpropionate and lysates subjected to co-immunoprecipitation, reducing SDS-PAGE, and Western blotting. For our studies, we used the well characterized sortilin variant sortilinmut, which contains two disrupted internalization motifs, one being the tyrosine-based YXXØ sequence (792YSVL) and the other being a deletion of the C-terminal dileucine motif (829LL) (13). Whereas wild type sortilin is mainly localized in perinuclear compartments (10, 28), sortilinmut is predominantly expressed on the cell surface (13). Results obtained using this construct are therefore directly comparable with those obtained with chimeric receptor constructs lacking intracellular domain sequences, which are also mainly expressed on the cell surface. First, we established that sortilinmut and p75NTR bind and evaluated the effect of both proNGF and NGF on this interaction. Following cross-linking and immunoprecipitation of p75NTR, sortilinmut was clearly co-immunoprecipitated, showing that sortilinmut and p75NTR interact in the absence of ligand (Fig. 1) consistent with previous reports (5, 7). Treatment with 25 nM NGF, prior to cross-linking, had no effect on sortilinmut-p75NTR complex formation. In contrast, 25 nM proNGF increased the interaction 2.3-fold ± 0.4 (n = 4, p < 0.04), consistent with the notion that proNGF is a shared ligand between sortilin and p75NTR, and the interaction is stabilized by NGF. The constructs were then transiently transfected into HEK293 cells and β-galactosidase activity measured using a chemiluminescent substrate. Whereas expression of each construct alone only resulted in a weak signal, co-expression increased enzyme activity ~13-fold, suggesting that the extracellular and/or the transmembrane domain of sortilin and p75NTR also interact in this paradigm (Fig. 2, C and D). The addition of increasing amounts of proNGF, proBDNF, or proNT3 from as low as 40 pm proNT increased the interaction of sortilin with p75NTR (Fig. 2E). These results further show that proBDNF and proNT3 bind with very high affinity to sortilin and p75NTR, similar to what we previously have reported for proNGF (5).

**Sortilin and p75NTR Interact through Their Extracellular Domains**—To investigate whether p75NTR interacts with the extracellular domain of sortilin, we constructed a sortilin-interleukin-2 receptor (IL2R) chimeric receptor designated sortilinecd-IL2Rtm,icd that combined the extracellular domain of sortilin and the transmembrane region and intracellular domain of IL2R (Fig. 3A). The binding properties of sortilinecd-IL2Rtm,icd with p75NTR were then tested in transfected HEK293 cells by co-immunoprecipitation experiments. We found that sortilinecd-IL2Rtm,icd readily interacted with p75NTR and that proNGF increased this interaction 1.9-fold ± 0.1, n = 5, p < 0.002 (Fig. 3B). These findings were further substantiated by surface plasmon resonance analysis using the commercially available fusion protein of the extracellular domain of p75NTR linked to...
the constant region of human IgG (p75<sup>NTR</sup> <sub>ecd</sub>-Fc). When p75<sup>NTR</sup> <sub>ecd</sub>-Fc was injected onto a Biacore sensor chip containing immobilized soluble sortilin (sol-sortilin), binding was clearly evident (Fig. 3C). To ensure that binding did not stem from the Fc region of the fusion protein, binding to a RET-Fc tyrosine-kinase fusion protein was probed under similar conditions. RET-Fc did not bind sol-sortilin (Fig. 3C), confirming that the binding between sortilin and p75<sup>NTR</sup> relies on residues within these extracellular domains.

We next addressed whether p75<sup>NTR</sup> engages the ligand-binding site in the 10-bladed β/β9252-propeller domain of sortilin. To do this, we carried out surface plasmon resonance competition studies with excess amounts of the tridecapeptide neurotensin. Neurotensin binds the tunnel of the β-propeller of sortilin and thereby sterically blocks access of all known ligands to sortilin (11, 12). Because neurotensin has a low molecular weight, binding to immobilized sortilin elicits only a minor signal, in terms of response units. However, compared with the signal obtained from binding of higher molecular weight proteins, competitive binding of neurotensin results in a greatly reduced signal overall. However, we were only able to block binding between sortilin and p75<sup>NTR</sup> <sub>ecd</sub>-Fc to a minor extent, suggesting that p75<sup>NTR</sup> predominantly binds sortilin outside the neurotensin binding area (Fig. 3D).

We next determined whether p75<sup>NTR</sup> also interacts with the sortilin intracellular domain. To address this, we made an addi-
Mapping of the Sortilin-p75NTR-binding Site

Defining a Sortilin-binding Region of the p75NTR Extracellular Domain—To map the interaction site(s) of the extracellular domain of IL2R with that from sortilin, we used to co-immunoprecipitate using sortilin ecd-IL2Rtm,icd (Fig. 3A) except for p75NTRΔstalk, which had greatly reduced binding to sortilin ecd-IL2Rtm,icd. However, in the presence of 25 nM proNGF, p75NTRΔstalk was co-immunoprecipitated with sortilin ecd-IL2Rtm,icd (Fig. 3C). Because p75NTRΔstalk retained the ability to associate with sortilin via the shared ligand proNGF, reduced binding in the absence of proNGF cannot be ascribed to incorrect folding or improper cell surface expression of p75NTRΔstalk. Rather, these data suggest that the stalk region of p75NTR is responsible for its constitutive interaction with sortilin.

To substantiate these results, we subjected p75NTRΔstalk and sortilin to FRET analysis. To do this, full-length p75NTR and p75NTRΔstalk were C-terminally fused with CFP to generate p75NTR-CFP and p75NTRΔstalk-CFP, respectively. Constructs were transiently transfected into HEK293 cells together with sortilin fused to YFP (i.e., sortilin-YFP). Because constitutive ligand-independent homodimerization of p75NTR is not mediated through the intracellular domain (29), FRET originating from cells co-transfected with the ICD of p75NTR fused to either CFP or YFP (i.e., p75NTRICD-CFP and p75NTRICD-YFP) served as a negative control. The extent of FRET between p75NTR-CFP and sortilin-YFP was increased 3-fold relative to background; however, the extent of FRET between p75NTRΔstalk-CFP and sortilin-YFP was significantly lower, being equivalent to that of p75NTRICD-CFP and p75NTRICD-YFP (Fig. 4D). We also assessed the binding properties of YFP-tagged p75NTR receptor constructs by co-immunoprecipitation using sortilin ecd-IL2Rtm,icd as a binding partner. Similar to FRET analysis, p75NTRΔstalk-YFP had a reduced binding to sortilin ecd-IL2Rtm,icd compared with p75NTR-YFP (Fig. 4E). However, it is noteworthy that a deletion of the stalk region of p75NTR abrogates binding to sortilin as measured by FRET, indicating that the extracellular domains alone mediate the binding between sortilin and p75NTR.

Next, we sought to determine whether the 23 amino acids in the stalk region of p75NTR alone are sufficient for binding to the sortilin extracellular domain. Co-immunoprecipitation experiments were therefore performed using a truncated variant of p75NTR-YFP designated p75NTRstalktm,icd-YFP containing only the stalk, transmembrane, and intracellular domains of p75NTR. Because sortilin ecd-IL2Rtm,icd was used as a binding partner, only the 23 amino acids of the p75NTR stalk region overlap with sortilin (Fig. 4A). We found that the presence of the stalk region fully rescued binding of the p75NTR extracellular domain with sortilin ecd-IL2Rtm,icd (Fig. 4F). This strongly suggests that the residues Thr228–Asp250 within the C-terminal stalk region of p75NTR constitute a core-binding surface of the p75NTR extracellular domain for sortilin.

Sortilin Intracellular Domain Regulates RIP of p75NTR and proNGF-dependent Apoptosis—Given that the interaction between sortilin and p75NTR maps to the extracellular domain, we sought to determine whether this domain is sufficient to affect p75NTR function. Depending on the molecular context,
Mapping of the Sortilin-p75NTR-binding Site

A

B

C

D

E

F
Mapping of the Sortilin-p75\textsuperscript{NTR}-binding Site

one function of p75\textsuperscript{NTR} relates to its ability to induce apoptosis, which requires RIP of p75\textsuperscript{NTR} (16, 17). Because sortilin is essential for proNT-dependent apoptosis through p75\textsuperscript{NTR} (5–9), we measured the accumulation of p75\textsuperscript{NTR} ICD by Western blotting within HEK293 cells transfected with wild type sortilin, sortilin\textsubscript{mut}, and sortilin\textsubscript{tailless} in the presence of proteasome inhibitors. To induce p75\textsuperscript{NTR} RIP, experiments were carried out in the presence of phorbol ester (PMA), which increases \(\alpha\)-secretase activity via the protein kinase C pathway (30). Expression of any sortilin construct caused a nonsignificant increase in the amount of p75\textsuperscript{NTR} C-terminal fragment. In contrast, the sortilin constructs had significant but differential effects on p75\textsuperscript{NTR} ICD production. p75\textsuperscript{NTR} control-transfected cells produced a low but obvious amount of p75\textsuperscript{NTR} ICD. Co-transfection with wild type sortilin significantly increased the amount of p75\textsuperscript{NTR} ICD after 3 h (Fig. 5, A and B). Sortilin\textsubscript{mut} increased formation of p75\textsuperscript{NTR} ICD to an intermediate nonsignificant level, and formation of p75\textsuperscript{NTR} ICD in the presence of sortilin\textsubscript{tailless} was even more reduced.

To confirm these findings in another cellular context, we assessed the effect of sortilin on p75\textsuperscript{NTR} ICD release using the Ga4/UAS system (31). This assay is based on binding of the nuclear protein Ga4 to the UAS element, which controls the nuclear transcription of luciferase. Because Ga4 is fused to the C-terminal end of p75\textsuperscript{NTR}, only shedding and release of p75\textsuperscript{NTR} ICD-Ga4 and subsequent translocation to the nucleus induces transcription of luciferase, which can be measured by a chemiluminescent substrate (Fig. 5C). To validate our assay, HEK293 cells were stably co-transfected with p75\textsuperscript{NTR}--Gal4 and UAS-luciferase cDNA and treated with PMA. PMA significantly increased luciferase activity compared with that observed in untreated cells, consistent with the notion that PMA increases RIP of p75\textsuperscript{NTR} (Fig. 5D). Furthermore, the effect of PMA was fully inhibited in the presence of an \(\alpha\)-secretase inhibitor (TAPI-2), showing that RIP of p75\textsuperscript{NTR} is required for up-regulation of luciferase transcription and activity (Fig. 5D).

To test the effect of sortilin variants, p75\textsuperscript{NTR}--Gal4 and UAS-luciferase stably transfected HEK293 cells were transiently transfected with wild type sortilin, sortilin\textsubscript{mut} or sortilin\textsubscript{tailless} and treated with PMA. Wild type sortilin significantly increased luciferase activity compared with that in control transfected cells, an effect observable even without PMA stimulation (Fig. 5E and F). Again, sortilin\textsubscript{mut} and sortilin\textsubscript{tailless} did not significantly induce p75\textsuperscript{NTR} ICD-Ga4-mediated transcription above that of the control plasmid.

We next assessed the accumulation of endogenous p75\textsuperscript{NTR} ICD in rat RN22 Schwannoma cells, which naturally express high levels of p75\textsuperscript{NTR} and have previously been demonstrated to undergo RIP (16). Because RN22 cells only express very low amounts of endogenous sortilin, we generated polyclonal stable RN22 cell lines for wild type sortilin, sortilin\textsubscript{mut} and sortilin\textsubscript{tailless} and measured the accumulation of p75\textsuperscript{NTR} ICD by Western blotting following addition of PMA and proteasome inhibitors. As for HEK293 cells, we found that wild type sortilin significantly increased p75\textsuperscript{NTR} ICD formation, whereas sortilin\textsubscript{mut} induced a less pronounced nonsignificant accumulation of p75\textsuperscript{NTR} ICD (Fig. 6, A and B). Sortilin\textsubscript{tailless} did not affect p75\textsuperscript{NTR} ICD accumulation, which was again comparable with that observed in untransfected RN22 cells.

Because sortilin apparently regulates RIP of p75\textsuperscript{NTR} and given that RIP has been associated with neuronal apoptosis (16), we next determined the ability of wild type sortilin, sortilin\textsubscript{mut} and sortilin\textsubscript{tailless} to regulate proNT-dependent apoptosis. We established a proNGF-induced cell death assay using polyclonal stable RN22 sortilin-expressing cell lines. Cells were then incubated with increasing amounts of proNGF, and death was assessed after 72 h using the MultiTox-Fluor multiplex cytotoxicity assay, which measures activity of proteases released from necrotic cells. Untransfected RN22 cells did not die upon proNGF stimulation. However, proNGF significantly stimulated cell death in cells transfected with wild type sortilin in a dose-dependent manner (Fig. 6C). Cells expressing sortilin\textsubscript{mut} exhibited an intermediate rate of death, whereas cells transfected with sortilin\textsubscript{tailless} were largely unaffected. This strongly suggests that the intracellular domain of sortilin regulates proNGF-dependent cell death through p75\textsuperscript{NTR} in RN22 cells.

To test the effect of sortilin on p75\textsuperscript{NTR} RIP under physiological conditions, we measured accumulation of p75\textsuperscript{NTR} ICD within primary cultures of SCG neurons isolated from sortilin knock-out mice. SCG neurons endogenously express high amounts of sortilin and p75\textsuperscript{NTR}, which has previously been shown to undergo RIP upon exposure to PMA (16). In the presence of PMA, neurons devoid of sortilin showed reduced rates of p75\textsuperscript{NTR} RIP compared with SCG neurons isolated from wild type littermates (Fig. 6D). These findings, together with the results obtained from HEK293 and RN22 cells, collectively suggest that sortilin promotes RIP of p75\textsuperscript{NTR}, resulting in increased release of p75\textsuperscript{NTR} ICD and ultimately in apoptosis.

DISCUSSION

Here we have mapped the binding site between sortilin and p75\textsuperscript{NTR} and found that the interaction relies on residues within their extracellular domains. Although we found no evidence
FIGURE 5. The intracellular domain of sortilin regulates RIP of p75<sub>NTR</sub>. A, representative (n = 6) Western blot (WB) of HEK293 cell lysates, which were transiently co-transfected with p75<sub>NTR</sub> and sortilin constructs as indicated and treated for 3 h with 200 nM PMA, 200 nM γ-secretase inhibitor compound E, or 1 μM proteasome inhibitor epoxomicin as indicated. B, densitometric quantification of the amount of p75<sub>NTR</sub>-ICD in each condition in A relative to the amount of full-length p75<sub>NTR</sub>. The data represent the means ± S.E. of six experiments. C, schematic representation of the Gal4/UAS assay. D, luminescence resulting from luciferase activity present in lysates of HEK293 cells stably co-expressing cDNAs encoding p75<sub>NTR</sub>-Gal4 and luciferase under the UAS promoter. The results were obtained following 3 h of treatment with 200 nM PMA and/or 20 μM α-secretase inhibitor TAPI-2 as indicated. PMA induced a ~60% increase in the amount of p75<sub>NTR</sub>-ICD-Gal4-mediated luciferase activity (the data represent the means ± S.E. of three experiments). E, luciferase luminescence mediated by p75<sub>NTR</sub>-ICD-Gal4 generated in HEK293 cells stably expressing p75<sub>NTR</sub>-Gal4 and luciferase under the UAS promotor and transiently transfected with sortilin variants as indicated. 48 h post-transfection, the cells were treated with 200 nM PMA for 3 h and analyzed for luciferase activity (the data represent the means ± S.E. of three experiments). F, luciferase activity in untreated HEK293 cells stably expressing p75<sub>NTR</sub>-Gal4 and luciferase and transiently transfected with wild type sortilin or control DNA. Cont. transf., control transfected; CTF, C-terminal fragment.
that the sortilin intracellular domain interacts with p75NTR, we found that it does regulate RIP of p75NTR and p75NTR-dependent cell death signaling.

The present results confirm previous findings that co-expression of sortilin and p75NTR generates a high affinity receptor complex for proNTs, which increases p75NTR affinity for proNTs by more than 2 orders of magnitude (5). Furthermore, the current study is the first demonstration that picomolar concentrations of proBDNF and proNT3 (40 pM) increase complex formation between sortilin and p75NTR, similar to what has been reported for proNGF (5). Mapping of the interaction site between sortilin and p75NTR revealed that the extracellular domain of sortilin alone is sufficient to mediate an interaction with full-length p75NTR. Unfortunately, because of the structure of the sortilin extracellular domain (12), it is not possible to truncate the receptor further and still maintain the structural integrity of either the 10-bladed β-propeller or the 10CC domain. Thus, we were unable to further map the region of the extracellular domain of sortilin required for its constitutive interaction with p75NTR. However, based on the finding that addition of a non-neurotrophin sortilin ligand, neurotensin, had little effect on the direct interaction between the extracellular domain of sortilin and p75NTR, we conclude that the ligand-binding tunnel of the 10-bladed β-propeller domain of sortilin is unlikely to contain the p75NTR interaction site and that the 10CC domain is more likely to mediate this interaction.

Using a variety of truncated p75NTR receptor constructs, we determined that the extracellular domain of p75NTR is alone sufficient to mediate the co-association with sortilin in the absence of ligand. Deletion of the cysteine-rich ligand-binding domains of p75NTR had no effect on sortilin binding; however, deletion of 23 amino acids within the membrane-proximal region of the p75NTR stalk domain significantly reduced the ability of p75NTR to interact with sortilin. Remarkably, a construct containing only the most membrane-proximal 23 amino acids of the extracellular domain was sufficient to restore binding between p75NTR and sortilin, indicating that this is the major sortilin-binding site of p75NTR. These findings further support our notion that the 10CC domain of sortilin is likely to contain the p75NTR-binding site because the 10CC domain is

FIGURE 6. Sortilin intracellular domain promotes RIP of p75NTR and proNGF-dependent cell death in RN22 cells. A, representative (n = 6) Western blots (WB) of lysates from RN22 cells that were stably transfected with sortilin constructs as shown and treated for 6 h with 200 nM PMA, 200 nM compound E, or 1 μM epoxomicin as indicated. B, densitometric quantification of the amount of p75NTR ICD in each condition in A relative to the amount of full-length p75NTR. The data represent the means ± S.E. of six experiments. C, quantification of proNGF-induced cell death relative to no proNT addition in polyclonal RN22 cell lines stably expressing various sortilin constructs. The cells were incubated with proNGF as indicated for 72 h, whereupon cell death was quantified using the MultiTox-Fluor multiplex cytotoxicity assay. *, p < 0.05; **, p < 0.01. D, Western blot (n = 3) of lysates from SCG neurons isolated from wild type and sortilin knock-out mice cultured in the presence of 1 μM epoxomicin for 1 h followed by the addition of 200 nM PMA for 3 h. CTF, C-terminal fragment; Untransf., untransfected.
expected to be located relatively close to the plasma membrane surface (12).

By use of Western blotting and an assay that measures p75NTR ICD-dependent transcription of luciferase, we analyzed the effect of sortilin on p75NTR RIP. We found that sortilin increases RIP of p75NTR, leading to significantly increased levels of p75NTR ICD. Moreover, the increased RIP of p75NTR was accompanied by an increase in cell death signaling mediated by p75NTR. Our finding that sortilin expression facilitates the generation of the p75NTR ICD is consistent with our data and published reports that proNTs facilitate both an interaction between p75NTR and sortilin and the generation of a p75NTR ICD-dependent cell death signal (5, 7, 8, 16). Importantly, the YXXO and dileucine trafficking motifs of sortilin were required for sortilin to increase the rate of p75NTR RIP and to promote cell death in the presence of proNGF in RN22 cells. Interestingly, the dileucine motif together with the YXXO motif are both required for sortilin endocytosis (13), and the sortilin mutant used herein has both of these motifs specifically mutated. The tailless sortilin mutant also lacks these sequences.

In summary, we have mapped the binding site between sortilin and p75NTR to the extracellular juxtamembrane stalk region of p75NTR. The interaction between sortilin and p75NTR is further strengthened by the pro-apoptotic proNT ligands, which promote generation of the p75NTR ICD (16). Furthermore, sortilin-dependent release of the p75NTR ICD correlates with proNT-dependent apoptosis facilitated by sortilin. We therefore conclude that sortilin mediates cell death not only by interacting with p75NTR as a co-receptor for proNTs but also through generation of p75NTR cleavage fragments and release of p75NTR ICD.

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