THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 52, pp. 43798–43809, December 21, 2012

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*

Received for publication, April 22, 2012, and in revised form, October 24, 2012 Published, JBC Papers in Press, October 26, 2012, DOI 10.1074/jbc.M112.374710

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Background: Sortilin and $p75^{NTR}$ induce neuronal apoptosis by binding pro-neurotrophins during development and following neuronal injury.
Results: Sortilin interacts with an extracellular juxtamembrane 23-amino acid sequence of $p75^{NTR}$.
Conclusion: Despite binding being mediated through extracellular interactions, the intracellular domain of sortilin regulates $p75^{NTR}$ shedding and apoptosis.
Significance: Mapping may allow design of compounds inhibiting neuronal cell death by blocking the interaction between sortilin and $p75^{NTR}$.

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 ($p75^{NTR}$). To identify the interaction site between sortilin and $p75^{NTR}$, we analyzed binding between chimeric receptor constructs and truncated $p75^{NTR}$ variants by co-immunoprecipitation experiments, surface plasmon resonance analysis, and FRET. We found that complex formation between sortilin and $p75^{NTR}$ 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 $p75^{NTR}$. Functional studies further revealed an important regulatory function of the sortilin intracellular domain in $p75^{NTR}$-regulated intramembrane proteolysis and apoptosis. Thus, although the intracellular domain of sortilin does not contribute to $p75^{NTR}$ binding, it does regulate the rates of $p75^{NTR}$ cleavage, which is required to mediate pro-neurotrophin-stimulated cell death.

Neurotrophins including NGF, BDNF, and neurotrophin-3 (NT3)[3] constitute 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 ($p75^{NTR}$), 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 $p75^{NTR}$ (8, 9). Binding of proNTs is achieved by binding of the pro-domain by sortilin, whereas $p75^{NTR}$ interacts with the mature part of the neurotrophin (5). However, sortilin and $p75^{NTR}$ also exist as a preformed receptor complex, interacting in the absence of ligand (5).

Sortilin is a neuronal type I receptor belonging to the Vps10p domain receptor 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 (13). $p75^{NTR}$ 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 $p75^{NTR}$-spe-

* This work was supported by the by the Lundbeck Foundation, the National Health and Medical Research Council of Australia, the Finnish Graduate School of Neuroscience, the Sigrid Juselius Foundation, and Academy of Finland Center of Excellence program.
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[3] The abbreviations used are: NT3, neurotrophin-3; ecd, extracellular domain; ICD, intracellular domain; IL_2R, interleukin-2 receptor; $p75^{NTR}$, p75 neurotrophin receptor; proNT, pro-neurotrophin; RIP, regulated intramembrane proteolysis; SCG, superior cervical ganglion; sol-sortilin, soluble sortilin; tm, transmembrane domain; UAS, upstream activating sequences; PMA, phorbol 12-myristate 13-acetate.

cific "chopper" module (15). The p75^{NTR} 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 p75^{NTR} is first cleaved by the α-secretase TACE/ADAM17, generating the C-terminal fragment. The remaining membrane-tethered stub is subsequently cleaved by presenilin-dependent γ-secretase, releasing the p75^{NTR} ICD to the cytosol for signaling (20).

We have previously demonstrated that sortilin increases p75^{NTR} affinity for proNTs. For example, cells expressing p75^{NTR} alone bind proNGF with an approximate K_d value of ~15 nM, whereas co-expression of sortilin increases affinity for proNGF ~100-fold to K_d = ~160 pM (5). This dramatically decreases the effective concentration of proNTs required for apoptotic signaling by p75^{NTR}. Here we identify the extracellular domains in sortilin and p75^{NTR} 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 p75^{NTR} and proNT-stimulated apoptotic signaling, suggesting additional roles for sortilin in p75^{NTR}-mediated apoptosis.

EXPERIMENTAL PROCEDURES

DNA Work—The pcDNA3.1/Zeo(−) containing cDNA encoding human wild type sortilin, sortilin$_{mut}$ (Y792A, L795A, and Leu^{829} to Leu^{830} deleted), IL$_2$R$_{ecd,tm}$-sortilin$_{mut,icd}$, and sortilin$_{tailless}$ (truncated at position Cys^{783}) have previously been described (13, 21, 22). For construction of sortilin$_{ecd}$-IL$_2$R$_{tm,icd}$, a fragment was amplified by standard PCR techniques using the α-subunit of the human interleukin-2 receptor (IL$_2$R)/pcDNA3.1/Zeo(−) as the template and an upstream primer encoding part of the transmembrane domain of IL$_2$R and part of the luminal domain of sortilin, and a downstream primer containing a cytosolic sequence of IL$_2$R. Using the native luminal BspEI site of sortilin and a 3′ primer-generated AflII site, the fragment was ligated into predigested sortilin$_{mut}$/pcDNA3.1/Zeo(−). PCR-mediated overlap extension was used to fuse the extracellular and transmembrane domains of sortilin and HA-tagged p75^{NTR} with the β-galactosidase Δα and Δω, respectively, generating sortilin-β-galΔα and HA-p75^{NTR}-β-galΔω. An upstream fragment encoding part of the extracellular and transmembrane domains of sortilin in combination with part of β-galactosidase Δα was amplified using sortilin$_{mut}$/pcDNA 3.1/Zeo(−) as the template. A downstream fragment encoding β-galactosidaseΔα was amplified using the template Δα/pwzl/Neo (23). The upstream fragment containing HA-p75^{NTR} extracellular and transmembrane domains and part of β-galactosidase Δω was generated using HA-p75^{NTR}/pcDNA3.1/G418 as the template. A downstream fragment encoding β-galactosidase Δω was amplified using Δω/pwzl/Hygro (23) as the template. Following amplification of overlapping PCR products, sortilin fusion protein was inserted into sortilin$_{mut}$/pcDNA3.1/Zeo(−) using the native luminal BspEI site and the 3′ primer-generated AflII site, whereas HA-p75^{NTR} fusion protein was ligated into predigested pcDNA 3.1/G418(−) using a primer-generated 5′-NotI and the 3′ AflII sites.

To make deletion and truncation expression constructs of p75^{NTR} (p75^{NTR}Δ$_{C1}$ (1–29, 66–425), p75^{NTR}Δ$_{C1,2}$ (1–29, 109–425), p75^{NTR}Δ$_{C1,2,3,4}$ (1–29, 190–425), p75^{NTR}Δ$_{stalk}$ (1–227, 251–425), p75^{NTR}$_{stalk,tm,icd,}$ (228–425), and p75^{NTR}ICD (274–425)) and YFP- or CFP-tagged versions of these (34), a modified pCDNA3 (Invitrogen) backbone was used. The rat p75^{NTR} signal peptide including a Kozak sequence (nucleotides −29 to +87) was inserted between the KpnI and EcoRV restriction sites, generating the vector pCDNA3-SP. p75^{NTR} coding sequences were amplified under standard PCR conditions and cloned into pCDNA3-SP using the primer-generated EcoRV and NheI sites. In cases where p75^{NTR} 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 NheI 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. p75^{NTR} coding sequences were amplified by PCR with a 5′ EcoRV and a 3′ NheI restriction site. p75^{NTR} coding sequences were then cloned between the EcoRV and NheI restriction sites of pCDNA3-YFP/CFP to generate in-frame fusion proteins. p75^{NTR}$_{tm,icd}$ (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′-Nhe site. The fragment was inserted into pCDNA3-YFP. The p75^{NTR}-Gal4 reporter construct for the p75^{NTR} cleavage reporter assay was cloned by inserting rat p75^{NTR} 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 MunI, 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% CO_2 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-p75^{NTR} antibody (ab10494) from Abcam. Anti-p75^{NTR} ICD 9992, for detecting p75^{NTR} ICD, was a generous gift from Professor Moses V. Chao (Skirball Institute of Biomolecular Medicine, New York University School of Medicine). Anti-IL$_2$Rα (I6152) and anti-HA tag (H6908) antibodies were from Sigma. Anti-GFP (11814460001) was purchased from Roche Applied Science. Recombinant His$_6$-tagged soluble human sortilin (sol-sortilin) was produced and purified as previously described (22). The luminal domain of human p75^{NTR} (Met^1–Asn^{250}) and

human RET receptor tyrosine kinase (Met^{1}–Arg^{635}), all fused to the Fc region of IgG_1, 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 $CaCl_2$ and $MgCl_2$) for 90 min at room temperature and then treated with the reducible protein cross-linker dithiobis 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 G-Sepharose beads (Amersham Biosciences) coupled with anti-p75^{NTR} (10494) or anti-GFP antibodies. Nonspecific binding was removed by washing beads five times in TBS supplemented with 0.05% Tween 20, and the proteins were eluted by boiling samples in reducing sample buffer (20 mM dithioerythritol, 2.5% 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 p75^{NTR} 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-p75^{NTR}-β-galΔω 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 μl of both solution A and solution B) (Applied Biosystems) to each well. The plates were incubated at room temperature for 1 h, and luminescence was measured with a Victor^3 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). sol-sortilin was immobilized at a density of ~72 fmol/mm^2 in 10 mM sodium acetate, pH 4.0, and the remaining sites were blocked with 1 M 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 $CaCl_2$, 1 mM EGTA, and 0.005% P20). Binding was expressed in relative response units as the response obtained from the flow cell containing immobilized receptor minus the response obtained when using an activated and blocked but uncoupled flow cell.

Cell Population FRET Assay—The FRET of p75^{NTR} molecules within a population of HEK293 cells was determined 48 h post-transfection of triplicate wells of transiently transfected cells and untransfected cells (to determine background fluorescence). The cells were washed once in PBS, pH 7.4, harvested in PBS, and centrifuged for 30 s at 10,000 × *g*. Cell pellets were resuspended in 200 μl of PBS and loaded into 96-well black microtiter plates (Greiner) for immediate analysis. Fluorescence was recorded simultaneously for the donor fluorophore at 430 ± 10 nm/480 ± 10 nm and the acceptor fluorophore at 485 nm/530 ± 10 nm, and FRET was measured at 430 ± 10 nm/530 ± 10 nm on a POLARstar OPTIMA multidetection microplate reader (BMG Labtech). FRET was calculated on cells as a FRET ratio, which is 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 nM 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 Victor^3 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 p75^{NTR}-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 p75^{NTR}—To map binding between sortilin and p75^{NTR}, 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 sortilin$_{mut}$, which contains two disrupted internalization motifs, one being the tyrosine-based Y*XXØ* sequence (^{792}YSVL) and the other being a deletion of the C-terminal dileucine motif (^{829}LL) (13). Whereas wild type sortilin is mainly localized in perinuclear compartments (10, 28), sortilin$_{mut}$ 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 sortilin$_{mut}$ and p75^{NTR} bind and evaluated the effect of both proNGF and NGF on this interaction. Following cross-linking and immunoprecipitation of p75^{NTR}, sortilin$_{mut}$ was clearly co-immunoprecipitated, showing that sortilin$_{mut}$ and p75^{NTR} 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 sortilin$_{mut}$-p75^{NTR} 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 p75^{NTR}, whereby the pro-domain of proNGF binds sortilin and the mature domain binds p75^{NTR} (5).

p75^{NTR} Binding Properties of the Extracellular and Transmembrane Domain of Sortilin—We next evaluated whether the extracellular and transmembrane domains of sortilin are required for it to bind to p75^{NTR}. To do this, co-immunoprecipitations were carried out using a truncated sortilin variant lacking the entire cytoplasmic domain (sortilin$_{tailless}$). HEK293 cells co-expressing sortilin$_{tailless}$ and p75^{NTR} were cross-linked in the absence or presence of 25 nM proNGF, and lysates were immunoprecipitated with anti-p75^{NTR} antibodies. Sortilin$_{tailless}$ clearly bound p75^{NTR}, demonstrating that residues within the extracellular domain and/or the transmembrane region account for complex formation in the absence of ligand (Fig. 2*A*). As expected, the interaction between sortilin$_{tailless}$ and p75^{NTR} was again further increased in the presence of proNGF (2.1-fold ± 0.3, $n = 4$, $p < 0.02$).

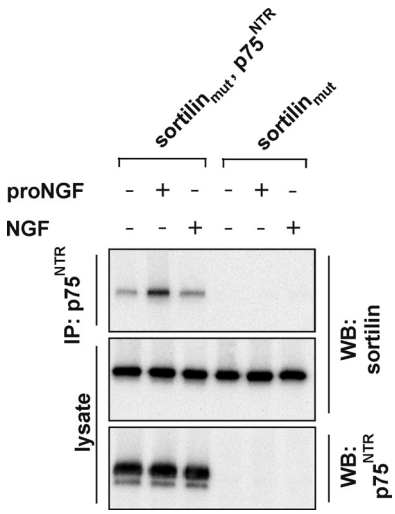


FIGURE 1. **Effect of pro- and mature NGF on the sortilin-p75^{NTR} interaction.** Representative ($n = 4$) Western blot (*WB*) of lysates of HEK293 cells stably expressing either sortilin$_{mut}$ or sortilin$_{mut}$ and p75^{NTR} incubated with 25 nM proNGF or NGF as indicated. The cells were cross-linked with dithiobis succinimidylpropionate, and the lysates were immunoprecipitated (*IP*) with anti-p75^{NTR} antibodies prior to SDS-PAGE and Western blotting. Sortilin is co-immunoprecipitated by p75^{NTR}, and the interaction is stabilized by proNGF but not NGF.

To confirm these findings in another cellular context, we developed a sensitive protocol for monitoring sortilin and p75^{NTR} complex formation using the β-galactosidase complementation assay (23). This assay is based on two weakly interacting subunits of β-galactosidase that upon dimerization reconstitute the active site measured by luminescence (23). We substituted the cytoplasmic domains of sortilin and HA-tagged p75^{NTR} with each of the two β-galactosidase subunits (Fig. 2*B*). 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 p75^{NTR} 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 p75^{NTR} (Fig. 2*E*). These results further show that proBDNF and proNT3 bind with very high affinity to sortilin and p75^{NTR}, similar to what we previously have reported for proNGF (5).

Sortilin and p75^{NTR} Interact through Their Extracellular Domains—To investigate whether p75^{NTR} interacts with the extracellular domain of sortilin, we constructed a sortilin-interleukin-2 receptor (IL_2R) chimeric receptor designated sortilin$_{ecd}$-$IL_2R_{tm,icd}$ that combined the extracellular domain of sortilin and the transmembrane region and intracellular domain of IL_2R (Fig. 3*A*). The binding properties of sortilin$_{ecd}$-$IL_2R_{tm,icd}$ with p75^{NTR} were then tested in transfected HEK293 cells by co-immunoprecipitation experiments. We found that sortilin$_{ecd}$-$IL_2R_{tm,icd}$ readily interacted with p75^{NTR} and that proNGF increased this interaction 1.9-fold ± 0.1, $n = 5$, $p < 0.002$ (Fig. 3*B*). These findings were further substantiated by surface plasmon resonance analysis using the commercially available fusion protein of the extracellular domain of p75^{NTR} linked to

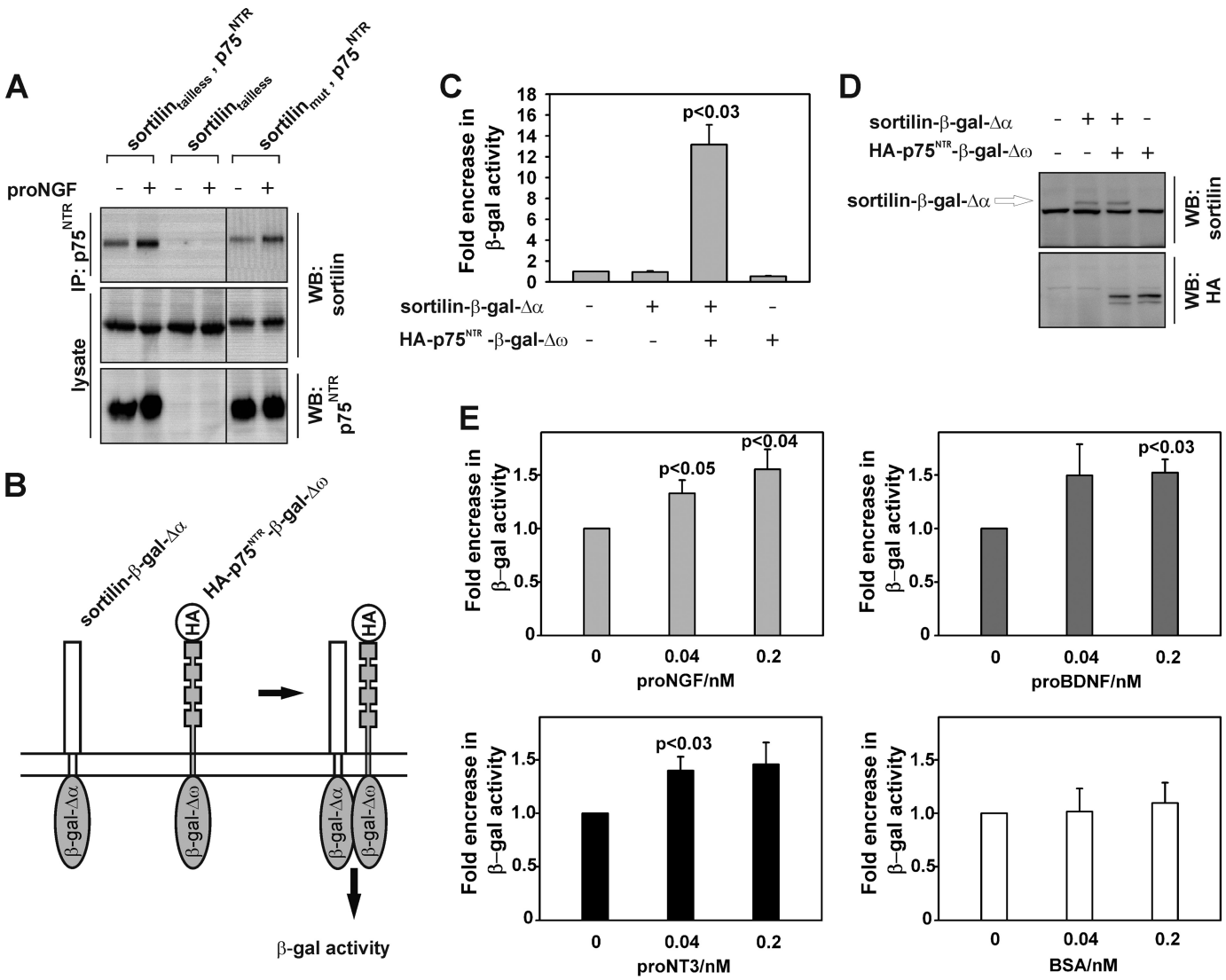


FIGURE 2. **p75^{NTR} binding epitopes within the extracellular and transmembrane domains of sortilin.** *A*, representative ($n = 4$) Western blot (*WB*) of immunoprecipitates (*IP*) and lysates of p75^{NTR} and sortilin$_{tailless}$, lacking its intracellular domain, compared with sortilin$_{mut}$. Stable cultures of HEK293 cells expressing either sortilin$_{tailless}$ or sortilin$_{tailless}$ and p75^{NTR} were incubated with or without proNGF and cross-linked with dithiobis succinimidylpropionate. Precipitates and crude lysates were then resolved by SDS-PAGE and Western blotted as indicated. *B*, schematic representation of expression constructs of sortilin and HA-p75^{NTR} lacking their cytoplasmic domains and fused to one of two weakly interacting subunits of β-galactosidase that were used in the β-galactosidase complementation assay. Upon dimerization of receptor chimeras, the β-galactosidase active site and thus activity is reconstituted. *C*, measurement of the luminescence of β-galactosidase in HEK293 cells transfected with HA-p75^{NTR}- and sortilin-β-galactosidase fusion expression constructs. Co-transfected cells produced significantly higher luminescence than single- and nontransfected cells ($p < 0.03$). The data represent the means ± S.E. of three experiments. *D*, representative Western blot ($n = 4$) of crude lysates from transfectants used in *C*. *E*, luminescence of the β-galactosidase substrate added to cells transfected with HA-p75^{NTR}- and sortilin-β-galactosidase expression constructs and incubated for 30 min with increasing concentrations of proNGF, proBDNF, or proNT3. In contrast to BSA, at 40 pM all three proNTs increased complex formation (means ± S.E. of three to six experiments).

the constant region of human IgG (p75$^{NTR}{}_{ecd}$-Fc). When p75$^{NTR}{}_{ecd}$-Fc was injected onto a Biacore sensor chip containing immobilized soluble sortilin (sol-sortilin), binding was clearly evident (Fig. 3*C*). 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. 3*C*), confirming that the binding between sortilin and p75^{NTR} relies on residues within these extracellular domains.

We next addressed whether p75^{NTR} engages the ligand-binding site in the 10-bladed β-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$^{NTR}{}_{ecd}$-Fc to a minor extent, suggesting that p75^{NTR} predominantly binds sortilin outside the neurotensin binding area (Fig. 3*D*).

We next determined whether p75^{NTR} also interacts with the sortilin intracellular domain. To address this, we made an addi-

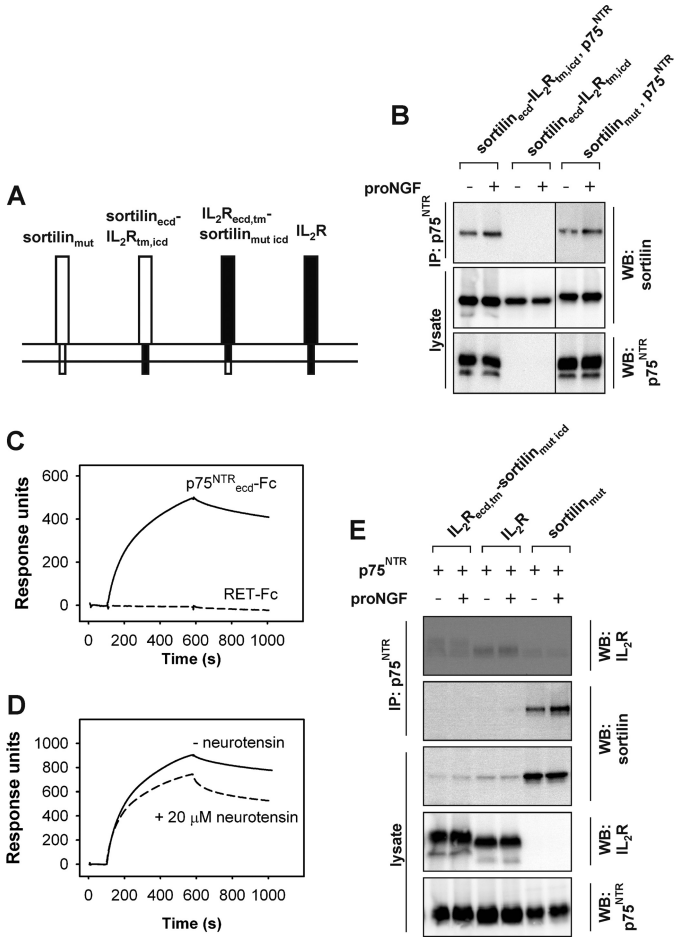


FIGURE 3. **Residues within the extracellular domain of sortilin account for binding to p75^{NTR}.** *A*, schematic representation of chimeric sortilin variants used to map the domain required to interact with p75^{NTR}. *B*, representative ($n = 5$) Western blots (*WB*) of lysates and immunoprecipitates (*IP*) of p75^{NTR} and sortilin$_{ecd}$-IL$_2$R$_{tm,icd}$ and sortilin$_{mut}$, with and without proNGF treatment. Cross-linked proteins were immunoprecipitated with anti-p75^{NTR} antibodies and precipitates were Western blotted with anti-sortilin antibodies. *C*, representative sensorgram ($n = 3$) obtained from surface plasmon resonance analysis of 20 nM p75^{NTR}$_{ecd}$-Fc (*solid line*) and 20 nM RET-Fc (*broken line*) injected onto a Biacore sensor chip containing immobilized sol-sortilin. *D*, representative sensorgram ($n = 3$) from surface plasmon resonance analysis of 20 nM p75^{NTR}$_{ecd}$-Fc poured onto a Biacore sensor chip with immobilized sol-sortilin in the absence (*solid line*) or presence of 20 μM neurotensin (*broken line*). The response for neurotensin alone has been subtracted. *E*, representative ($n = 4$) Western blots of lysates and immunoprecipitates of p75^{NTR} and sortilin variants with or without treatment with proNGF.

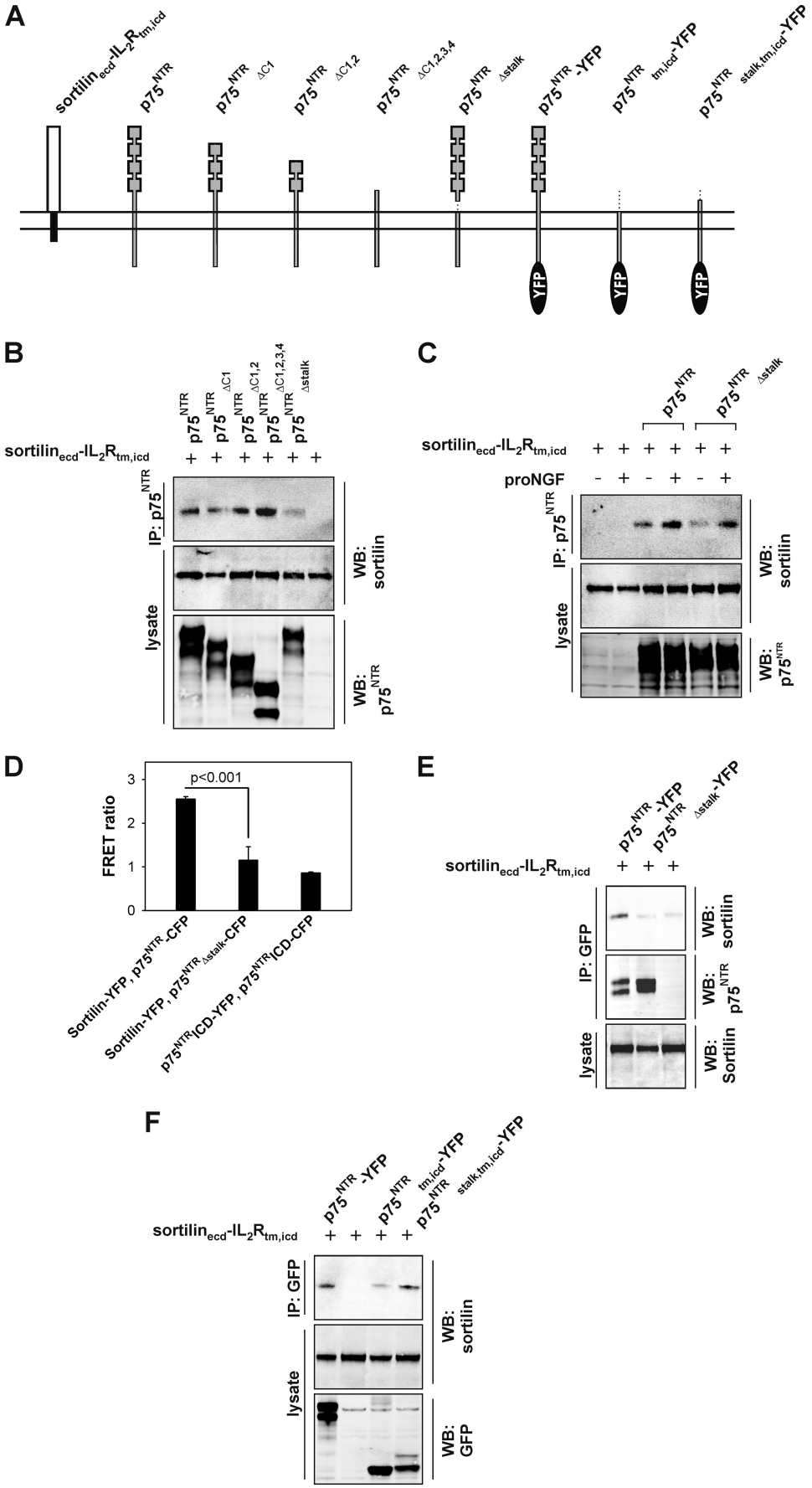
tional sortilin-IL$_2$R-based chimeric receptor by substituting the intracellular domain of IL$_2$R with that from sortilin$_{mut}$, thereby generating IL$_2$R$_{ecd,tm}$-sortilin$_{mut,icd}$, (Fig. 3*A*). The chimera-encoding plasmid was then transfected into HEK293 cells together with p75^{NTR}, and binding of the sortilin intracellular domain was assessed by co-immunoprecipitation experiments. Binding of full-length IL$_2$R to p75^{NTR} acted as the control. We were not able to co-immunoprecipitate IL$_2$R$_{ecd,tm}$-sortilin$_{mut\ icd}$ or IL$_2$R, demonstrating that the intracellular domain of sortilin does not interact with p75^{NTR} (Fig. 3*E*).

Defining a Sortilin-binding Region of the p75^{NTR} Extracellular Domain—To map the interaction site(s) of the extracellular domain at which p75^{NTR} binds sortilin, co-immunoprecipitation experiments using various truncated p75^{NTR} variants and sortilin were carried out. p75^{NTR} variants include N-terminally truncated forms lacking one or more ligand binding repeats and a p75^{NTR} variant lacking 23 amino acids (Thr^{228}–Asp^{250}) of the extracellular juxtamembrane stalk region (p75^{NTR}Δ$_{stalk}$) (Fig. 4*A*). Following co-immunoprecipitation, we found that all truncated p75^{NTR} variants bound equally well to sortilin$_{ecd}$-IL$_2$R$_{tm,icd}$ (Fig. 4*B*) except for p75^{NTR}Δ$_{stalk}$, which had greatly reduced binding to sortilin$_{ecd}$-IL$_2$R$_{tm,icd}$. However, in the presence of 25 nM proNGF, p75^{NTR}Δ$_{stalk}$ was co-immunoprecipitated with sortilin$_{ecd}$-IL$_2$R$_{tm,icd}$ (Fig. 4*C*). Because p75^{NTR}Δ$_{stalk}$ 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 p75^{NTR}Δ$_{stalk}$. Rather, these data suggest that the stalk region of p75^{NTR} is responsible for its constitutive interaction with sortilin.

To substantiate these results, we subjected p75^{NTR}Δ$_{stalk}$ and sortilin to FRET analysis. To do this, full-length p75^{NTR} and p75^{NTR}Δ$_{stalk}$ were C-terminally fused with CFP to generate p75^{NTR}-CFP and p75^{NTR}Δ$_{stalk}$-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 p75^{NTR} is not mediated through the intracellular domain (29), FRET originating from cells co-transfected with the ICD of p75^{NTR} fused to either CFP or YFP (*i.e.*, p75^{NTR}$_{ICD}$-CFP and p75^{NTR}$_{ICD}$-YFP) served as a negative control. The extent of FRET between p75^{NTR}-CFP and sortilin-YFP was increased 3-fold relative to background; however, the extent of FRET between p75^{NTR}Δ$_{stalk}$-CFP and sortilin-YFP was significantly lower, being equivalent to that of p75^{NTR}$_{ICD}$-CFP and p75^{NTR}$_{ICD}$-YFP (Fig. 4*D*). We also assessed the binding properties of YFP-tagged p75^{NTR} receptor constructs by co-immunoprecipitation using sortilin$_{ecd}$-IL$_2$R$_{tm,icd}$ as a binding partner. Similar to FRET analysis, p75^{NTR}Δ$_{stalk}$-YFP had a reduced binding to sortilin$_{ecd}$-IL$_2$R$_{tm,icd}$ compared with p75^{NTR}-YFP (Fig. 4*E*). However, it is noteworthy that a deletion of the stalk region of p75^{NTR} abrogates binding to sortilin as measured by FRET, indicating that the extracellular domains alone mediate the binding between sortilin and p75^{NTR}.

Next, we sought to determine whether the 23 amino acids in the stalk region of p75^{NTR} alone are sufficient for binding to the sortilin extracellular domain. Co-immunoprecipitation experiments were therefore performed using a truncated variant of p75^{NTR}-YFP designated p75^{NTR}$_{stalk,tm,icd}$-YFP containing only the stalk, transmembrane, and intracellular domains of p75^{NTR}. Because sortilin$_{ecd}$-IL$_2$R$_{tm,icd}$ was used as a binding partner, only the 23 amino acids of the p75^{NTR} stalk region overlap with sortilin (Fig. 4*A*). We found that the presence of the stalk region fully rescued binding of the p75^{NTR} extracellular domain with sortilin$_{ecd}$-IL$_2$R$_{tm,icd}$ (Fig. 4*F*). This strongly suggests that the residues Thr^{228}–Asp^{250} within the C-terminal stalk region of p75^{NTR} constitute a core-binding surface of the p75^{NTR} extracellular domain for sortilin.

Sortilin Intracellular Domain Regulates RIP of p75^{NTR} and proNGF-dependent Apoptosis—Given that the interaction between sortilin and p75^{NTR} maps to the extracellular domain, we sought to determine whether this domain is sufficient to affect p75^{NTR} function. Depending on the molecular context,



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

To confirm these findings in another cellular context, we assessed the effect of sortilin on p75^{NTR} ICD release using the Gal4/UAS system (31). This assay is based on binding of the nuclear protein Gal4 to the UAS element, which controls the nuclear transcription of luciferase. Because Gal4 is fused to the C-terminal end of p75^{NTR}, only shedding and release of p75^{NTR} ICD-Gal4 and subsequent translocation to the nucleus induces transcription of luciferase, which can be measured by a chemiluminescent substrate (Fig. 5*C*). To validate our assay, HEK293 cells were stably co-transfected with p75^{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^{NTR} (Fig. 5*D*). Furthermore, the effect of PMA was fully inhibited in the presence of an α-secretase inhibitor (TAPI-2), showing that RIP of p75^{NTR} is required for up-regulation of luciferase transcription and activity (Fig. 5*D*). To test the effect of sortilin variants, p75^{NTR}-Gal4 and UAS-luciferase stably transfected HEK293 cells were transiently transfected with wild type sortilin, sortilin$_{mut}$, or sortilin$_{tailless}$ 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. 5, *E* and *F*). Again, sortilin$_{mut}$ and sortilin$_{tailless}$ did not significantly induce p75^{NTR} ICD-Gal4-mediated transcription above that of the control plasmid.

We next assessed the accumulation of endogenous p75^{NTR} ICD in rat RN22 Schwannoma cells, which naturally express high levels of p75^{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$_{mut}$, and sortilin$_{tailless}$ and measured the accumulation of p75^{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^{NTR} ICD formation, whereas sortilin$_{mut}$ induced a less pronounced nonsignificant accumulation of p75^{NTR} ICD (Fig. 6, *A* and *B*). Sortilin$_{tailless}$ did not affect p75^{NTR} ICD accumulation, which was again comparable with that observed in untransfected RN22 cells.

Because sortilin apparently regulates RIP of p75^{NTR} and given that RIP has been associated with neuronal apoptosis (16), we next determined the ability of wild type sortilin, sortilin$_{mut}$, and sortilin$_{tailless}$ 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. 6*C*). Cells expressing sortilin$_{mut}$ exhibited an intermediate rate of death, whereas cells transfected with sortilin$_{tailless}$ were largely unaffected. This strongly suggests that the intracellular domain of sortilin regulates proNGF-dependent cell death through p75^{NTR} in RN22 cells.

To test the effect of sortilin on p75^{NTR} RIP under physiological conditions, we measured accumulation of p75^{NTR} ICD within primary cultures of SCG neurons isolated from sortilin knock-out mice. SCG neurons endogenously express high amounts of sortilin and p75^{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^{NTR} RIP compared with SCG neurons isolated from wild type littermates (Fig. 6*D*). These findings, together with the results obtained from HEK293 and RN22 cells, collectively suggest that sortilin promotes RIP of p75^{NTR}, resulting in increased release of p75^{NTR} ICD and ultimately in apoptosis.

DISCUSSION

Here we have mapped the binding site between sortilin and p75^{NTR} and found that the interaction relies on residues within their extracellular domains. Although we found no evidence

FIGURE 4. **The stalk region of p75^{NTR} binds sortilin.** *A*, schematic representation of truncated and deletion p75^{NTR} receptor variants used to map the domain of p75^{NTR} required to interact with sortilin. *B*, representative ($n = 4$) Western blots (*WB*) of immunoprecipitates (*IP*) and lysates of HEK293 cells that were transiently co-transfected with sortilin$_{ecd}$-Il$_2$R$_{tm,icd}$ and p75^{NTR} receptor variants. The most C-terminal 23 amino acids of the p75^{NTR} stalk region interact with sortilin. *C*, representative ($n = 3$) Western blots of immunoprecipitates and lysates from HEK293 transiently expressing sortilin$_{ecd}$-Il$_2$R$_{tm,icd}$ and p75^{NTR} receptor variants as indicated. p75$^{NTR}\Delta_{stalk}$ binds exogenous proNGF (25 nM) and thereby interacts with sortilin$_{ecd}$-Il$_2$R$_{tm,icd}$. *D*, FRET analysis of sortilin and p75^{NTR} interactions. HEK293 cells were transiently co-transfected with the indicated constructs and analyzed 48 h post-transfection. Full-length p75^{NTR} and sortilin induced FRET above background, whereas p75$^{NTR}\Delta_{stalk}$ did not, indicating that the stalk region of p75^{NTR} is required for the interaction with sortilin. The data represent the means ± S.E. of three experiments. *E*, representative ($n = 3$) Western blot of lysates and immunoprecipitates of sortilin$_{ecd}$-IL$_2$R$_{tm,icd}$ and YFP-tagged p75^{NTR} variants demonstrating that p75$^{NTR}\Delta_{stalk}$ does not significantly interact with sortilin$_{ecd}$-IL$_2$R$_{tm,icd}$. Constructs were transiently transfected into HEK293 cells, and p75^{NTR}-YFP receptor constructs were immunoprecipitated with anti-GFP antibodies. *F*, representative ($n = 3$) Western blots of lysates and immunoprecipitates of sortilin$_{ecd}$-Il$_2$R$_{tm,icd}$ and YFP-tagged p75^{NTR} variants demonstrating that the 23 amino acids of the p75^{NTR} stalk region are sufficient to rescue extracellular binding of two otherwise nonoverlapping sortilin and p75^{NTR} constructs.

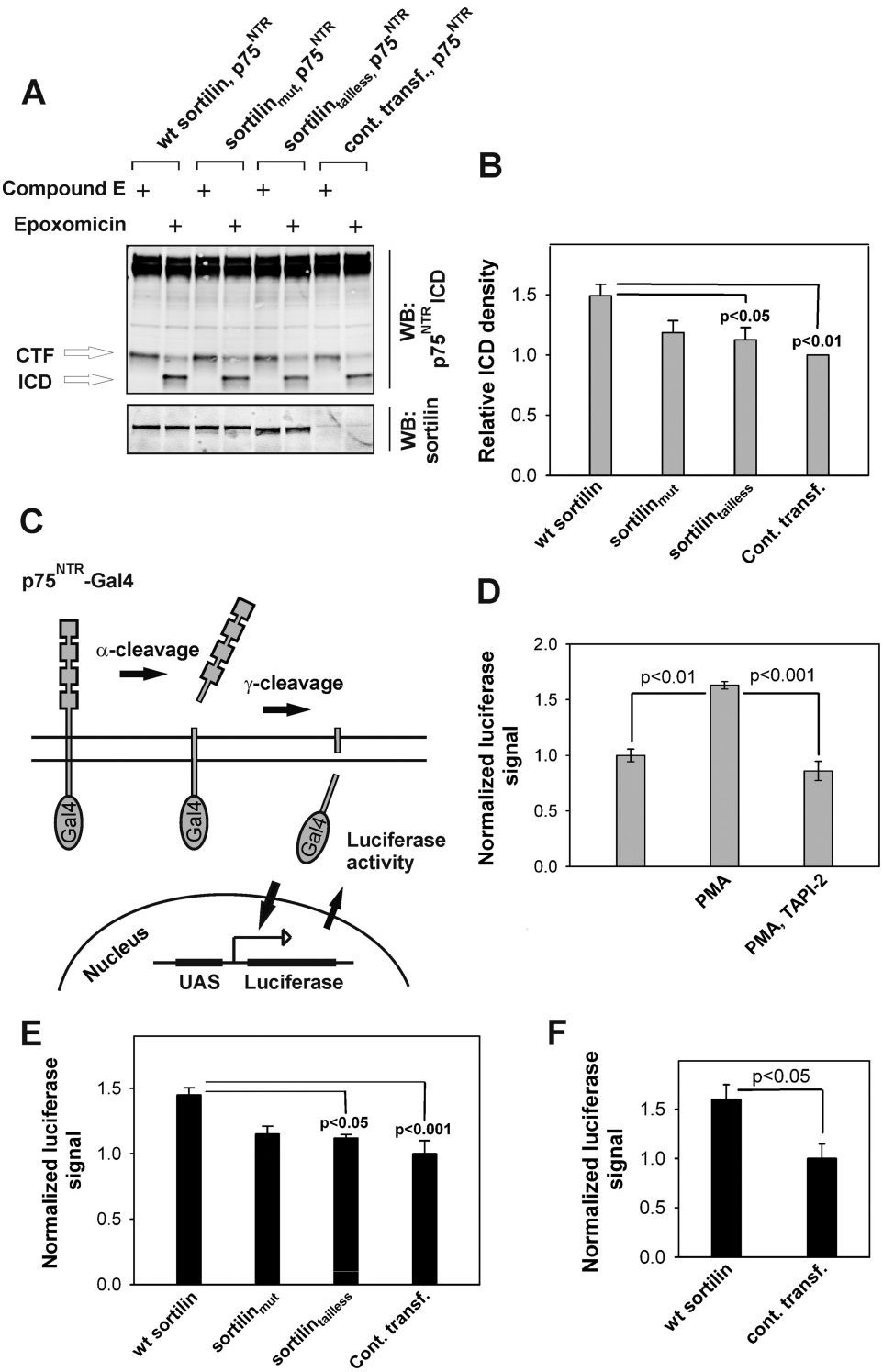


FIGURE 5. **The intracellular domain of sortilin regulates RIP of p75^{NTR}.** *A*, representative ($n = 6$) Western blot (*WB*) of HEK293 cell lysates, which were transiently co-transfected with p75^{NTR} and sortilin constructs as indicated and treated for 3 h with 200 nM PMA, 200 nM γ-secretase inhibitor compound E, or 1 μM proteasome inhibitor epoxomicin as indicated. *B*, densitometric quantification of the amount of p75^{NTR} ICD in each condition in *A* relative to the amount of full-length p75^{NTR}. 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^{NTR}-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^{NTR}-ICD-Gal4-mediated luciferase activity (the data represent the means ± S.E. of three experiments). *E*, luciferase luminescence mediated by p75^{NTR} ICD-Gal4 generated in HEK293 cells stably expressing p75^{NTR}-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^{NTR}-Gal4 and luciferase and transiently transfected with wild type sortilin or control DNA. *Cont. transf.*, control transfected; *CTF*, C-terminal fragment.

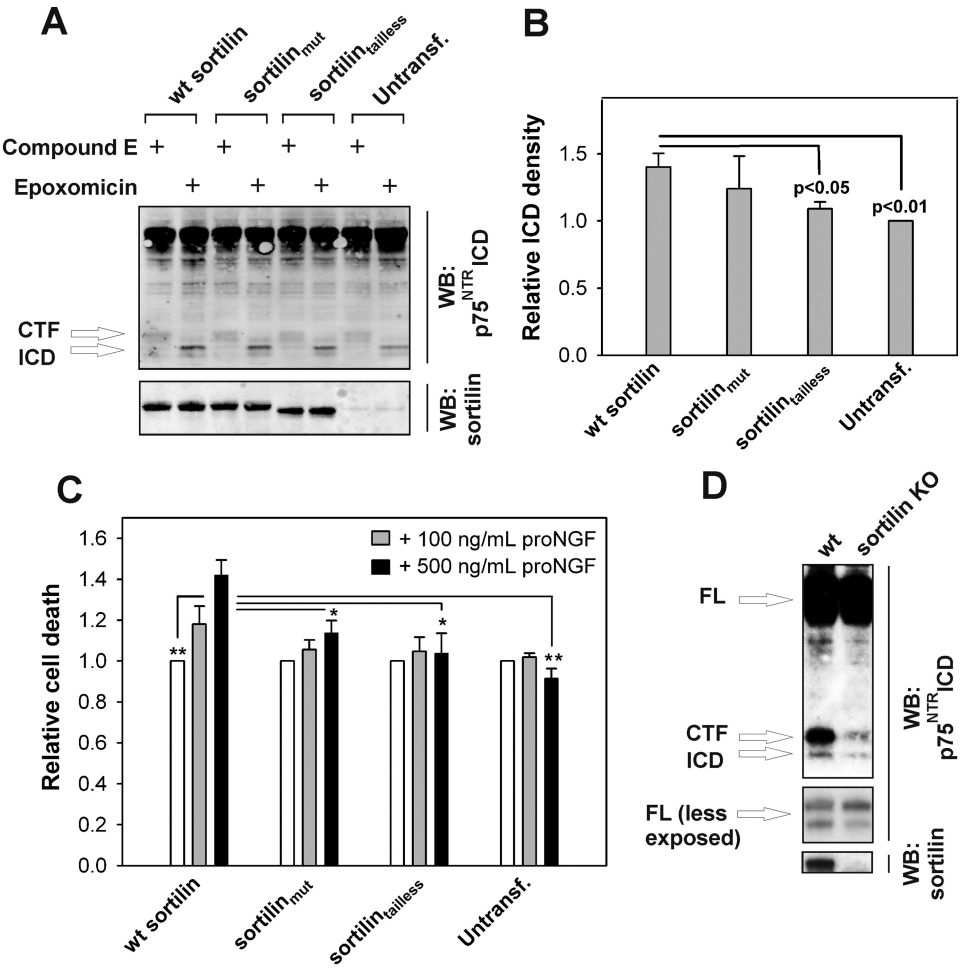


FIGURE 6. **Sortilin intracellular domain promotes RIP of p75^{NTR} and proNGF-dependent cell death in RN22 cells.** *A*, representative ($n = 6$) Western blots (*WB*) of lysates from RN22 cells that were stably transfected with sortilin constructs as shown and treated for 6 h with 200 nM PMA, 200 nM compound E, or 1 μM epoxomicin as indicated. *B*, densitometric quantification of the amount of p75^{NTR} ICD in each condition in *A* relative to the amount of full-length p75^{NTR}. 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.

that the sortilin intracellular domain interacts with p75^{NTR}, we found that it does regulate RIP of p75^{NTR} and p75^{NTR}-dependent cell death signaling.

The present results confirm previous findings that co-expression of sortilin and p75^{NTR} generates a high affinity receptor complex for proNTs, which increases p75^{NTR} 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 p75^{NTR}, similar to what has been reported for proNGF (5). Mapping of the interaction site between sortilin and p75^{NTR} revealed that the extracellular domain of sortilin alone is sufficient to mediate an interaction with full-length p75^{NTR}. 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 p75^{NTR}. 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 p75^{NTR}, we conclude that the ligand-binding tunnel of the 10-bladed β-propeller domain of sortilin is unlikely to contain the p75^{NTR} interaction site and that the 10CC domain is more likely to mediate this interaction.

Using a variety of truncated p75^{NTR} receptor constructs, we determined that the extracellular domain of p75^{NTR} is alone sufficient to mediate the co-association with sortilin in the absence of ligand. Deletion of the cysteine-rich ligand-binding domains of p75^{NTR} had no effect on sortilin binding; however, deletion of 23 amino acids within the membrane-proximal region of the p75^{NTR} stalk domain significantly reduced the ability of p75^{NTR} 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 p75^{NTR} and sortilin, indicating that this is the major sortilin-binding site of p75^{NTR}. These findings further support our notion that the 10CC domain of sortilin is likely to contain the p75^{NTR}-binding site because the 10CC domain is

expected to be located relatively close to the plasma membrane surface (12).

By use of Western blotting and an assay that measures p75^{NTR} ICD-dependent transcription of luciferase, we analyzed the effect of sortilin on p75^{NTR} RIP. We found that sortilin increases RIP of p75^{NTR}, leading to significantly increased levels of p75^{NTR} ICD. Moreover, the increased RIP of p75^{NTR} was accompanied by an increase in cell death signaling mediated by p75^{NTR}. Our finding that sortilin expression facilitates the generation of the p75^{NTR} ICD is consistent with our data and published reports that proNTs facilitate both an interaction between p75^{NTR} and sortilin and the generation of a p75^{NTR} ICD-dependent cell death signal (5, 7, 8, 16). Importantly, the *YXXØ* and dileucine trafficking motifs of sortilin were required for sortilin to increase the rate of p75^{NTR} RIP and to promote cell death in the presence of proNGF in RN22 cells. Interestingly, the dileucine motif together with the *YXXØ* 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. Our findings that sortilin$_{mut}$ and sortilin$_{tailless}$ engage in a complex with p75^{NTR} but fail to stimulate RIP of p75^{NTR} and RN22 cell death indicate that sortilin-dependent apoptosis is the result of two events, which require high affinity binding of proNTs, as well as endocytosis of sortilin. Because generation of p75^{NTR} ICD occurs predominately in endosomes where γ-secretase activity is concentrated (32, 33), it is tempting to speculate that sortilin facilitates internalization of p75^{NTR} or fragments thereof destined for endosomes. However, additional studies would be required to elucidate the mechanism of sortilin trafficking in relation to RIP of p75^{NTR} and neuronal cell death.

In summary, we have mapped the binding site between sortilin and p75^{NTR} to the extracellular juxtamembrane stalk region of p75^{NTR}. The interaction between sortilin and p75^{NTR} is further strengthened by the pro-apoptotic proNT ligands, which promote generation of the p75^{NTR} ICD (16). Furthermore, sortilin-dependent release of the p75^{NTR} ICD correlates with proNT-dependent apoptosis facilitated by sortilin. We therefore conclude that sortilin mediates cell death not only by interacting with p75^{NTR} as a co-receptor for proNTs but also through generation of p75^{NTR} cleavage fragments and release of p75^{NTR} ICD.

Acknowledgments—We thank Prof. Helen M. Blau (Institute for Regenerative Medicine and Stem Cell Biology, Stanford University School of Medicine) for providing us with constructs encoding mutated subunits of β-galactosidase and Prof. Moses V. Chao (Skirball Institute of Biomolecular Medicine, New York University School of Medicine) for the anti-p75^{NTR} ICD antibody. Prof. Elisabeth Schwarz (Institute for Biotechnology, Martin-Luther-Universität, Halle-Wittenberg) generously supplied us with proNTs. Anne Marie Bundsgaard, Anja Aagaard, and Benedicte Vestergaard (Department of Biomedicine, University of Aarhus) are thanked for excellent technical assistance. Rowan Tweedale (Queensland Brain Institute, The University of Queensland) is acknowledged for critical reading of the manuscript.

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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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J. Biol. Chem. 2012, 287:43798-43809.
doi: 10.1074/jbc.M112.374710 originally published online October 26, 2012
