Brain-derived neurotrophic factor (BDNF) regulates neuronal differentiation, synaptic plasticity, and morphology, and modest changes in BDNF levels result in complex behavioral phenotypes. BDNF levels and intracellular localization in neurons are regulated by multiple mechanisms, including use of distinct promoters, mRNA and protein transport, and regulated cleavage of proBDNF to mature BDNF. Sortilin is an intracellular chaperone that binds to the prodomain of BDNF to traffic it to the regulated secretory pathway. However, sortilin binds to numerous ligands and plays a major role in mannose 6-phosphate receptor-independent transport of lysosomal hydrolases utilizing motifs in the intracellular domain that mediate trafficking from the Golgi and late endosomes. Sortilin is modified by ectodomain shedding, although the biological implications of this are not known. Here we demonstrate that ADAM10 is the preferred protease to cleave sortilin in the extracellular stalk region, to release the ligand binding sortilin ectodomain from the transmembrane and cytoplasmic domains. We identify sortilin shedding at the cell surface and in an intracellular compartment. Both sortilin and BDNF are trafficked to and degraded by the lysosome in neurons, and this is dependent upon the sortilin cytoplasmic tail. Indeed, expression of the sortilin ectodomain, which corresponds to the domain released upon the sortilin cytoplasmic tail, reduces BDNF release from the regulated secretory pathway. This is not dependent upon the cytoplasmic tail (5). These findings characterize the regulation of sortilin shedding and identify a novel mechanism by which sortilin ectodomain shedding acts as a regulatory switch for delivery of BDNF to the secretory pathway or to the lysosome, thus modulating the bioavailability of endogenous BDNF.

Brain-derived neurotrophic factor (BDNF) is dynamically regulated in the central nervous system and is a critical factor in development and plasticity (1). Whereas mature BDNF promotes survival and long term potentiation, the proform induces long term depression and apoptosis (2, 3). This diversity of function suggests that BDNF is subject to sophisticated regulatory mechanisms, with studies showing dynamic regulation at the level of transcription, translation, enzymatic cleavage, and trafficking of mRNA and protein (4). In addition, numerous chaperone proteins regulate subcellular localization of BDNF mRNA and protein in neurons including sortilin, carboxypeptidase, and translin (5–7).

Sortilin, a member of the Vps10p domain containing family of proteins that includes sorLA and sorCS1–3, is highly expressed in the cortex and hippocampus (8–10), where it can function as a cell surface receptor for proneurotrophins, neutrotensin, and lipoprotein lipase (11–13). The majority of sortilin, however, is expressed within intracellular compartments, where it chaperones diverse ligands, including proBDNF and acid hydrolases (5, 13, 14). The sortilin cytoplasmic tail is highly homologous to mannose 6-phosphate receptor and is required for the intracellular trafficking of cargo proteins via interactions with distinct adaptor molecules (8, 15). In addition to mediating lysosomal targeting of specific acid hydrolases, the sortilin cytoplasmic tail also directs trafficking of BDNF to the secretory pathway in neurons, where it can be released in response to depolarization to modulate cell survival and synaptic plasticity (16–19). Prior studies have demonstrated that reduced sortilin levels or expression of a mutant sortilin lacking the cytoplasmic tail reduces BDNF release from the regulated secretory pathway. Furthermore, expression of sortilin lacking the cytoplasmic domain results in increased constitutive release of BDNF, evidence that BDNF trafficking by sortilin is dependent upon the cytoplasmic tail (5).

Ectodomain proteolysis of sortilin leads to separation of the ligand binding domain from the trafficking motifs encoded by the intracellular domain; however, little is known about the mechanism and biological consequences of this process (20). Here we identify the extracellular and intracellular products of sortilin proteolysis and determine that the transmembrane metalloprotease ADAM10, but not the related ADAM17, is required for sortilin shedding. We demonstrate that the cleavage recognition site is located within the extracellular juxtamembrane stalk and that shedding occurs at the cell surface.
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and in an intracellular compartment. In addition, we report a novel mechanism whereby endogenous BDNF levels in neurons are decreased via sortilin dependent lysosomal degradation. Trafficking of BDNF to the lysosome requires the sortilin cytoplasmic tail, providing evidence that the levels of endogenous BDNF are modulated by sortilin ectodomain shedding. These studies provide insight into pathological states such as inflammation, cancer, and Alzheimer disease in which ADAM proteases are dysregulated and BDNF levels are altered (21–25).

EXPERIMENTAL PROCEDURES

Reagents—Phorbol 12-myristate 13-acetate (PMA), TAPI-2, and iomycin were purchased from Calbiochem. Leupeptin hemisulfate and Dnase were from Sigma. E64 was from Biomol (Farmingdale, NY). The rabbit anti-p75NTR(9993) (26) antibodies and the rabbit anti-sortilin ectodomain antibody (anti-NTR3) was purchased from BD Biosciences, and the biotinylated goat anti-mouse sortilin antibody and TrkB-Fc were from R&D Systems (Minneapolis, MN). Mouse anti- H.A.11 was from Covance (Princeton, NJ) and rabbit anti-HA was from Sigma. Rabbit anti-Myc was from Bethyl Laboratories (Montgomery, TX). Anti-BDNF was from Santa Cruz (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse secondary antibodies were purchased from Calbiochem and visualized using the enhanced chemiluminescence (ECL) detection system purchased from GE Healthcare. The BDNF Emax ELISA kit and peptide N-glycosidase F were purchased from Promega (Madison, WI). The QuikChange II XL site-directed mutagenesis kit was from Stratagene (Cedar Creek, TX). Custom primers were generated by Invitrogen. Taq polymerase was purchased from Denville (Denville, NJ). DNTPs were purchased from Invitrogen. The GeneClean II kit was purchased from Qbiogene/MP Biomedicals (SOLON, OH).

Plasmids—Human sortilin cDNA was subcloned into pcDNA3.1 hygro expression vector using Nhel and Xhol sites, and an HA epitope tag was added to the 3’ end by PCR. Human p75NTR was subcloned into pcDNA3. The sortilin(mut) variant impaired in endocytosis (alanine substituted for Tyr-14, Leu-17, Leu-51, and Leu-52 in the cytoplasmic tail) was described in Nielsen et al. (27). The Δstalk sortilin-HA cDNA was generated by deletion of amino acids 741–755 of human sortilin. To do so, a unique XbaI restriction enzyme site was introduced by site-directed mutagenesis of positions 2220 with the addition of an XbaI site directly upstream of the stalk domain. The Δstalk cDNA was generated by PCR to amplify the luminal domain of sortilin and cloned into pcDNA3.1 hygro vector using Nhel and Xhol sites. N-terminal Myc-tagged sortilin was a generous gift from the laboratory of Frances Lee.

Cell Culture and Transfection—Human embryonic kidney (HEK) 293T cells (ATCC), COS-1 cells (ATCC), and immortalized mouse embryonic fibroblasts deficient in ADAM10 or ADAM17 (28) (29) were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1% penicillin/streptomycin. Lipofectamine 2000 (Invitrogen) was used to transfect DNA into cell lines. Dissociated E16-E19 rat corticocerebellar granule neurons (5) or P7-P8 rat cerebellar granule (30) were transfected by electroporation using the rat Amaxa kit. Briefly, 4–6 x 10^6 dissociated neurons were transfected with 3 µg of pcDNA according to the manufacturer’s specifications (Amaxis Cologne, Germany). Cells were plated at a density of 3 x 10^6 cells per well of a 6-well plate and maintained in Neurobasal medium containing B27, 0.5 mM or 2 mM glutamine, and 25 mM KCl. Experiments were performed 4–5 days after plating.

Deglycosylation—100 µl of media or 50 µg of lysate from HEK293T cells or rat cortical neurons transfected with sortilin-HA or 50 µg of whole cell extracts from rat brain and mouse cortices and hippocampi was denatured by boiling at 100 °C and incubated with N-glycanase or endoglycosidase H for 2 h at 37 °C according to the manufacturer’s guidelines (Prozyme).

Surface Biotinylation—HEK293T cells were transiently transfected with pcDNA containing wild-type sortilin-HA or Δstalk sortilin-HA. Cells were washed twice in PBS containing Ca^2+ and Mg^2+ 4 °C and incubated with 0.5 mg/ml Sulfo-NHS-LC-Biotin (Sigma) in binding buffer (0.1 mM triethanolamine, pH 7.4, 2 mM CaCl_2, 150 mM NaCl) at 4 °C for 30 min with gentle rocking. The reaction was quenched for 20 min at 4 °C in PBS containing 50 mM Tris, pH 8. To precipitate labeled proteins, 50 or 400 µg of whole cell lysate was incubated with streptavidin-conjugated beads (Pierce) overnight at 4 °C.

Shedding Assays—Cells were plated in 6-well tissue culture plates and incubated in DMEM without serum for 30 or 40 min to measure base-line levels of shedding. To assess ectodomain shedding, cells were washed 2 times in serum-free DMEM and treated with 100 ng/ml PMA (40 min) or 2.5 µM iomycin (30 min) in the presence or absence of 25 µM TNF-α protease inhibitor (TAPI-2, N-(R)-[2-hydroxymuconaryl]-4-methylpentanoyl-1- t-butyl-alanyl-1-alanine, 2-aminoethyl amide). Immediately after treatment, cell media was harvested and centrifuged for 5 min at 1000 rpm to remove cellular debris. Cells were lysed in buffer containing 10 mM Tris, pH 7.0, 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and the protease inhibitors aprotinin, leupeptin, and phenylmethyl-sulfonyl fluoride (PMSF). Proteins in media and lysate were resolved by SDS-PAGE followed by immunoblotting with antibodies to the sortilin ectodomain (anti-NTR3) or anti-HA.11.

Neuronal Depolarization—E18 rat cortical neurons were transfected with 1 µg of N-terminal Myc-tagged sortilin or control vector. Cells were quiesced in media lacking additional KCl from DIV4 to DIV6. On DIV6, cells were incubated in media containing 56 mM KCl for 90 min. Proteins in the media were resolved by SDS-PAGE followed by immunoblotting with anti-Myc.

Immunoprecipitation—Cell supernatant was harvested in the presence of aprotinin, leupeptin, and PMSF and centrifuged for 5 min at 1000 rpm to remove cellular debris. For immuno-
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precipitation, 3.5 ml of media or 0.5 mg of protein from whole cell lysate was incubated with rabbit anti-sortilin (1:500), biotinylated goat anti-sortilin (1:100), or rabbit anti-HA (1:100) at 4 °C overnight followed by incubation with protein A-Sepharose or streptavidin-conjugated beads. Immunoprecipitates were subjected to SDS-PAGE (7.5 or 12.5% polyacrylamide) and immunoblotting with mouse anti-NTR3 or mouse anti-HA.11.

Immunoblotting—Cultured cells were lysed in buffer containing 10 mM Tris, pH 7.0, 100 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40. Total protein concentration was determined by the Bradford assay using the Bio-Rad Protein Assay Dye and bovine serum albumin (Sigma) as the standard. Equal amounts of protein were by SDS-PAGE (7.5% or 12.5% polyacrylamide) followed by immunoblotting with primary antibodies and visualization using HRP-conjugated secondary antibodies.

Immunocytochemical Staining and Fluorescence Microscopy—Cells were plated on poly-D-lysine-coated 8 chamber slides (NUNC) at a density of 5 × 10^5 cells per well. Cells were fixed in 3% paraformaldehyde and permeabilized with 0.1% Triton in PBS containing 5% BSA for 30 min. Rabbit anti-HA (1:100) was applied in 5% BSA and incubated overnight at 4 °C. Subtype-specific fluorescent secondary antibodies and DAPI nuclear stain were applied in PBS. Fluorescence images were acquired using a Retiga Exi digital camera (Qimaging, BC, Canada) mounted on an Olympus (Center Valley, PA) BX61 microscope. For controls, the same concentration of subtype-specific normal IgG was applied.

BDNF ELISA—BDNF levels in 30 μg of whole cell lysates were measured by ELISA using the BDNF Emax Immunoassay kit (Promega) using recombinant BDNF as the standard. Standards and samples were analyzed in duplicate, and each group contains three independent samples.

Lysosomal Inhibition—Triplicate cultures of rat-dissociated cortico-hippocampal neurons were treated for 22–24 h with 50 μM leupeptin hemisulfate and 5 mM E64 in Neurobasal/B27 medium on DIV5. BDNF capture experiments were performed as described in Yang et al. (22). Briefly, 0.7 μg/ml TrkB-Fc (R&D) was added to the culture medium in the presence or absence of leupeptin and E64 from DIV5–7. Media were harvested, centrifuged for 5 min at 1000 rpm to remove cellular debris, and incubated with protein A-Sepharose for 90 min. Precipitates were subjected to SDS-PAGE (15% polyacrylamide) and immunoblotting with rabbit anti-BDNF.

Metabolic Labeling—Dissociated cortico-hippocampal neurons were transfected with wild-type sortilin-HA, Δstalk sortilin-HA, or pcDNA3.1 empty vector using Amaxa electroporation and plated at a density of 3 × 10^6 cells per 60-mm dish. Five days after transfection, cells were washed twice and starved for 30 min in cysteine-methionine-free DMEM containing 5% dialyzed fetal bovine serum. The cells were pulse-labeled with cysteine-methionine-free DMEM containing 250 μCi/ml ^35S for 1 h at 37 °C. The cells were washed and chased in DMEM containing 5 mM methionine and 5 mM cysteine for 1, 2, 4, or 6 h at 37 °C. Cell lysates were washed, and lysates were harvested in radioimmune precipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1.25% deoxycholic acid, 1 mM EDTA) and centrifuged at 16,000 × g for 15 min. 200 mg of total protein were incubated with 4 μg of rabbit anti-HA antibody (Sigma) overnight followed by precipitation with protein A-Sepharose for 90 min. Proteins were eluted with SDS-PAGE sample buffer and resolved by reducing 7.5% SDS-PAGE gel. The gel was fixed in 30% methanol, 10% acetic acid, stained with EN3HANCE solution (PerkinElmer Life Sciences) for 1 h, dried, and analyzed by autoradiography.

Preparation of Human Blood Components—Blood was drawn from a human volunteer using a 21-gauge butterfly needle. To obtain the serum, ~5 ml was added to a glass tube and incubated at 37 °C for 1 h to promote coagulation. The serum fraction was collected after centrifugation at 800 rpm for 15 min, and supernatant was collected. To obtain the plasma and platelet fractions, 30 ml of blood was collected into a glass tube containing acid citrate-dextrose (38 mM citric acid, 75 mM sodium citrate, 135 mM glucose) as an anticoagulant and incubated on ice for 30 min. To obtain the platelet-rich plasma, whole blood was centrifuged at 1000 rpm for 10 min with no brake at room temperature followed by a second spin at 600 rpm for 10 min with no brake. A 100-μl aliquot of the platelet-rich plasma was washed for analysis. To obtain the platelet-poor plasma fraction, the platelet-rich plasma was acidified in CIT solution (0.105 sodium citrate to prevent coagulation) and centrifuged at 2500 rpm for 10 min with no brake at 5 °C. A 100-μl aliquot of the poor plasma fraction was removed for analysis. The pellet of platelets was washed twice in 7 ml of Tris/CIT solution with centrifugation at 1500 × g for 10 min with no brake at 4 °C and lysed in TNE lysis buffer (10 mM Tris, pH 7.0, 100 mM NaCl, 1 mM EDTA, 1% Nonidet P-40).

RESULTS

Constitutive Proteolysis of Sortilin—Sortilin encodes a large luminal/ectodomain with a predicted molecular mass of 95 kDa and an intracellular domain of 18 kDa, and the sortilin ectodomain is detected in the media of sortilin-overexpressing cells (20, 31). To determine whether shedding of the ectodomain of sortilin occurs in vivo, we evaluated human blood components. We observe that human platelets express full-length sortilin (110 kDa), whereas an immunopositive 95-kDa sortilin product was detected in both human plasma and serum using an ectodomain specific antibody, providing evidence that sortilin shedding is observed in humans (Fig. 1a). Sortilin is expressed by central neurons, including pyramidal cells of hippocampal regions CA1–3 (9, 10). To determine whether sortilin ectodomain shedding occurs in neurons, hippocampal neurons were cultured from E18 rat brains using conditions to deplete cultures of glia. Using a sortilin ectodomain antibody, a 95-kDa sortilin immunoreactive species was found to accumulate in the media of DIV5 neurons over time (Fig. 1b). Immunoblotting lysates of DIV5 hippocampal neurons detects a 110-kDa full-length sortilin species, indicating that the 95-kDa sortilin detected in the media is likely a truncated soluble form (Fig. 1b). These studies suggest that shedding of endogenous sortilin occurs in hippocampal neurons.

To better characterize sortilin cleavage products, a human sortilin construct encoding a C-terminal HA epitope tag (sortilin-HA, Fig. 1c) was expressed in HEK293 cells. The shed 95-kDa ectodomain was observed in cell media (Fig. 1d, left
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![Diagram](https://example.com/diagram.png)

FIGURE 1. Identification of sortilin cleavage products. a, immunoblotting of human platelets with an antibody against the sortilin ectodomain detects a 110- and 95-kDa species, whereas human plasma and serum contain a single 95-kDa species. b, anti-sortilin immunoblotting of cultured rat hippocampal neuron lysate detects endogenous full-length 110-kDa sortilin, whereas immunoblotting of media shows the accumulation of 95-kDa soluble sortilin over time. c, shown is a schematic of human sortilin protein engineered to express a C-terminal HA epitope tag. The extracellular domain (ECD) contains the ligand binding region. A 9-amino acid HA epitope tag is adjacent to the intracellular domain (ICD). TM, transmembrane. d, immunoblotting of media and lysate from HEK293T cells expressing sortilin-HA or endogenous sortilin with an antibody against the ectodomain detects the full-length 110-kDa sortilin (A) in lysate and the shed ectodomain (B) in the media after 24 h (left and center panels). A longer film exposure was utilized to enhance visualization of the endogenous shed ectodomain in the media of pcDNA control-transfected cells. Immunoblotting with anti-HA detects intact 110-kDa sortilin (A) and an 18-kDa C-terminal fragment (C) in the lysate, but not in the media, of cells expressing sortilin-HA (right panel) is shown. L, lysate; M, media.

Sortilin Shedding Depends on ADAM10—The ADAM (A Disintegrin And Metalloprotease) proteases are a family of transmembrane metalloproteases that promote ectodomain shedding of numerous transmembrane receptors, including p75NTR, an ADAM17/TACE substrate that interacts with sortilin to form a high affinity site for proneurotrophins (Refs. 12 and 32; for reviews on ADAMs, see Refs. 33 and 34). ADAMs 10 and 17 are the major proteases for most shed proteins (35–37) and exhibit widespread expression in brain regions where sortilin is expressed (38, 39). Therefore, we focused on identifying potential roles for ADAM10 or ADAM17 in sortilin shedding.

To determine whether ADAM10 or ADAM17/TACE is the major sortilin sheddase, we analyzed proteolysis in the presence of reagents that specifically activate either enzyme. PMA specifically activates ADAM17, but not ADAM10, in short term experiments (<1 h) (36, 40, 41). Although the precise mechanism by which this occurs is unknown, it requires PKC activation and is independent of the ADAM17 cytoplasmic domain (42). Consistent with a previous study (43), treatment of COS-1 cells with PMA (100 ng/ml) did not increase the level of the shed sortilin ectodomain in media or production of the cell-associated 18-kDa C-terminal fragment (Fig. 2a, lane 2; b, lower panel, lane 2) but did enhance production of the intracellular domain of p75NTR, a known ADAM17 substrate (Ref. 32 and Fig. 2c, lower panel, Lane 2). These results suggest that a different ADAM family member is the major sortilin sheddase.

To assess the ability of ADAM10 to cleave sortilin, cells expressing sortilin-HA were treated with ionomycin, a calcium ionophore known to strongly activate ADAM10 in a calmodulin-dependent manner (40). Ionomycin treatment led to a significant increase (27 ± 7-fold; p < 0.01) in the level of the shed ectodomain in the cell media as well as the 18-kDa intracellular fragment detected in the cell lysate (Fig. 2, a, lane 7, and b, lower panel, lane 5). To confirm that the effect of ionomycin was metalloproteinase-dependent, cells were treated with ionomycin in the presence of the known peptide hydroxamate ADAM inhibitor, TNF-α, protease Inhibitor 2, or TAPI-2 (44). Ionomycin-induced ectodomain shedding was significantly inhibited by TAPI-2 (7 ± 4-fold reduction; p < 0.01), implicating an ADAM as the putative sortilin sheddase (Fig. 2, a, lane 8, and b, lower panel, lane 6). The ionomycin-induced production of the p75NTR C-terminal fragment was also prevented by treatment with TAPI-2 (Fig. 2c, lower panel, lanes 5 and 6).

The fact that ionomycin stimulated sortilin shedding led us to hypothesize that depolarization-dependent elevations in intracellular calcium may also increase sortilin shedding. Rat cortical neurons expressing full-length sortilin with an N-terminal Myc tag were treated with 56 mM KCl for 90 min. Western blotting of media from KCL-treated cells showed an
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FIGURE 2. The sortilin sheddase is a member of the ADAM family proteases. a–c, COS-1 cells were transfected with C-terminal HA-tagged human sortilin or c, human p75NTR, a and b, ionomycin, and not PMA, enhances production of the N- and C-terminal sortilin cleavage products, and this effect is inhibited by TAPI-2. Cells were treated with PMA (100 ng/ml, 40 min) or ionomycin (Iono, 2.5 μM, 30 min) in the presence or absence of TAPI-2 (25 μM) followed by immunoblotting (IB) of media with anti-sortilin (a) and cell lysate with anti-HA (b). FL, full length, c, cells were treated as in a and b followed by immunoblotting of cell lysate with an antibody against the p75NTR intracellular domain. d, ionomycin induces matrix metalloproteinase-dependent shedding of endogenous sortilin from neurons. Cultured P7 rat cerebellar granule neurons were treated as in a–c. Media was immunoprecipitated (IP) with a polyclonal antibody against the sortilin extracellular domain followed by Western blotting with a monoclonal antibody against the extracellular domain.

increase in the shed sortilin ectodomain in the media relative to the amount of sortilin constitutively shed from the same cells in 48 h (supplemental Fig. 1).

To confirm that ADAM10 is the preferred protease in neurons expressing endogenous sortilin, cultured rat cerebellar granule neurons were stimulated with PMA or ionomycin. Sortilin shedding into the media was detected after treatment with ionomycin but not PMA (Fig. 2d, lanes 2 and 4). The effect of ionomycin-induced sortilin ectodomain shedding was again blocked by TAPI-2 (Fig. 2d, lane 5). Although both ADAM17 and ADAM10 are reportedly expressed in the cerebellum (45, 46), these findings indicate that ADAM10 is the major sortilin sheddase in cultured granule neurons.

To directly compare the contribution of ADAM17 and ADAM10 to sortilin shedding, ectodomain cleavage was assessed in immortalized mouse embryonic fibroblasts lacking either ADAM10 or ADAM17 (29, 35) and expressing sortilin-HA. Both constitutive and ionomycin-induced sortilin proteolysis were intact in ADAM17-deficient cells as assessed by detection of the sortilin ectodomain in the media and generation of the 18-kDa C-terminal fragment in the cell extract (Fig. 3a, lane 5, and b, first and third lanes). Furthermore, ectopic expression of ADAM17 in ADAM17-deficient MEFs (A17Resc) did not promote sortilin proteolysis above base-line levels (Fig. 3, a, lanes 2, 4, and 6, and b, fourth through the sixth lanes), ruling out a significant role for ADAM17 in constitutive and ionomycin-induced sortilin proteolysis.

In contrast, using ADAM10-deficient MEF cells, both constitutive and ionomycin-induced production of the soluble ectodomain (Fig. 3c, Lanes 1, 3, and 5) and 18-kDa sortilin CTF (Fig. 3d, first, third, and fifth lanes) were abolished. Expression of wild-type ADAM10 (A10Resc) effectively rescued sortilin proteolysis in ADAM10−/− MEFs, and this effect was further enhanced by treatment with ionomycin (Fig. 3, c, lanes 2, 4, and 6, and d, second, fourth, and sixth lanes). Taken together, these data demonstrate that ADAM10, but not ADAM17, is required for constitutive and ionomycin-stimulated sortilin cleavage.

Deletion of the Sortilin Juxtamembrane Stalk Inhibits Shedding—Prior structure:function studies demonstrate that ADAM proteases do not recognize canonical sequences but cleave substrates on the basis of distance from the membrane and on secondary structure (47–50). The sortilin ectodomain contains a 15-amino acid unstructured stalk directly adjacent to the highly structured cysteine-rich region identified as the site of proneurotrophin binding (51). The sortilin secondary structure (52) as well as the molecular mass of shed sortilin predicts that proteolysis likely occurs within this region. Therefore, we generated a deletion mutant lacking the 15-amino acid juxtamembrane stalk (amino acids 741–755; Δstalk sortilin-HA; Fig. 4a).

To ensure that deletion of the stalk does not alter localization, we performed surface biotinylation of HEK293T cells expressing WT or Δstalk sortilin-HA. Although Δstalk sortilin-HA expression was reduced relative to WT sortilin-HA, immunoblotting of avidin-precipitated cell lysates showed that a similar proportion of the Δstalk sortilin-HA localizes to the plasma membrane compared with WT sortilin-HA (Fig. 4b). In a second approach, lysates of cells expressing WT sortilin-HA or Δstalk sortilin-HA were treated with endoglycosidase H. Immunoblotting with anti-HA revealed similar endoglycosidase H-resistant pools of WT and mutant sortilin, indicating that the mutant is trafficked through the medial Golgi compa-
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FIGURE 3. ADAM10, and not ADAM17, is required for sortilin shedding. ADAM17<sup>−/−</sup> (a and b) or ADAM10<sup>−/−</sup> (c and d) MEF cells were transfected with sortilin-HA alone or cotransfected with sortilin-HA plus ADAM17 (a and b) or ADAM10 (c and d). Base-line levels of shedding in each condition were assessed by incubation in serum-free media (Pretx). Cells were treated with vehicle control, PMA (100 ng/ml; 40 min), or ionomycin (Iono, 2.5 μM; 30 min). a and c, immunoblotting (IB) of media with anti-sortilin is shown. b and d, immunoblotting of lysate with anti-HA demonstrates that sortilin proteolysis is intact in the absence of ADAM17 (a and b). Sortilin shedding is abolished in ADAM10<sup>−/−</sup> cells and rescued by coexpression of ADAM10 (c and d).

FIGURE 4. Deletion of the juxtamembrane stalk does not alter sortilin localization. a, shown is a schematic of the wild-type and Δstalk sortilin-HA constructs. The dotted line represents the 15 amino acids deleted in the Δstalk sortilin-HA mutant. An HA epitope tag is attached at the C terminus. b, HEK293T cells transfected with WT or Δstalk sortilin-HA were labeled with biotin at 4 °C. 50 μg of whole cell lysates were incubated with streptavidin beads followed by anti-HA Western blotting (IB) to detect sortilin-HA at the cell surface (top) and in 25 μg of whole cell extract (bottom). Approximately 10% of total WT sortilin-HA and total Δstalk sortilin-HA localizes to the plasma membrane as measured by densitometry. c, deglycosylation of Δstalk sortilin-HA is shown. Whole cell lysates from HEK293T cells transfected with WT sortilin-HA or Δstalk sortilin-HA were treated with peptide <sup>N</sup>-glycosidase F (PNGaseF) or endoglycosidase H (EndoH) followed by Western blotting with anti-HA. d, anti-HA immunofluorescent (IF) staining of the human HT1080 cell line and dissociated P7 rat cerebellar granule neurons transfected with WT or Δstalk sortilin-HA is shown.

rable with WT sortilin-HA (Fig. 4c). In addition, we performed anti-HA immunostaining of HT1080 cells or E18 rat hippocampal neurons expressing either WT or Δstalk sortilin-HA and confirmed that both the WT and Δstalk mutant display characteristic perinuclear and somatodendritic expression patterns as previously reported (Fig. 4d).
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To determine whether sortilin shedding occurs at the stalk, we examined the susceptibility of Δstalk sortilin-HA to shedsases. Because Δstalk sortilin-HA is expressed at lower levels than WT sortilin-HA, cells were transfected with a 2-fold concentration of Δstalk sortilin-HA relative to WT sortilin-HA to obtain approximately equal expression. Detection of the shed ectodomain in media of HEK293T cells expressing Δstalk sortilin-HA or WT sortilin-HA demonstrated that ionomycin-induced proteolysis of Δstalk sortilin-HA was markedly reduced (Fig. 5a). This was also observed in hippocampal neurons, where constitutive production of the 18-kDa sortilin CTF was detected in cells expressing WT sortilin-HA but not Δstalk sortilin-HA (Fig. 5b). Taken together, these results indicate that the ADAM cleavage site for sortilin is within the 15-amino acid juxtamembrane stalk, we examined the susceptibility of Δstalk sortilin-HA to shedsases. Because Δstalk sortilin-HA is expressed at lower levels than WT sortilin-HA, cells were transfected with a 2-fold concentration of Δstalk sortilin-HA relative to WT sortilin-HA to obtain approximately equal expression. Detection of the shed ectodomain in media of HEK293T cells expressing Δstalk sortilin-HA or WT sortilin-HA demonstrated that ionomycin-induced proteolysis of Δstalk sortilin-HA was markedly reduced (Fig. 5a). This was also observed in hippocampal neurons, where constitutive production of the 18-kDa sortilin CTF was detected in cells expressing WT sortilin-HA but not Δstalk sortilin-HA (Fig. 5b). Taken together, these results indicate that the ADAM cleavage site for sortilin is within the 15-amino acid juxtamembrane extracellular domain.

One goal of the generation of a sheddase-resistant mutant sortilin was to evaluate the biological actions of shedding in neurons. Although deletion of the sortilin juxtamembrane stalk did not significantly alter subcellular localization, we consistently observed reduced Δstalk sortilin-HA expression levels relative to wild-type sortilin-HA when expressed in cortical neurons (supplemental Fig. 2a). Pulse-chase analysis indicates that inhibition of shedding by removal of the juxtamembrane stalk leads to accelerated sortilin turnover (supplemental Fig. 2b), thus precluding further use of this mutant construct in addressing the functional role of shedding.

**Localization of Sortilin Shedding**—ADAM10 is localized primarily to intracellular compartments and is active within the secretory pathway (53), but is also present at the cell surface where it induces functional shedding of plasma membrane proteins such as amyloid precursor protein, L1 adhesion molecule, and EphB2 (54–56). Because sortilin functions both as a cell surface receptor and an intracellular chaperone, the effects of sortilin ectodomain shedding may depend upon the compartment in which it occurs. To identify where sortilin is cleaved, we utilized a sortilin mutant that is impaired in endocytosis by disruption of the C-terminal LL and YSVL trafficking motifs (27) (Y14A, L17A, L51A, and L52A in the cytoplasmic tail; Fig. 6a). This mutant localizes predominantly to the plasma membrane and is endocytosis-deficient (27). Expression of internalization-deficient sortilin in ADAM10−/− MEFs demonstrated that like wild-type sortilin, this mutant fails to undergo constitutive and ionomycin-induced shedding, and this effect is rescued by coexpression of wild-type ADAM10 (Fig. 6b). These findings suggest that sortilin cleavage by ADAM10 could occur during transit from the trans-Golgi network to the plasma membrane or on the cell surface but that internalization to an endosomal compartment is not required. Furthermore, the absence of shedding of this internalization mutant in ADAM10−/− cells indicates that enhancing expression in the plasma membrane does not render sortilin susceptible to shedding by other ADAM family members.

To test whether ectodomain cleavage can occur at the plasma membrane, we performed surface biotinylation of rat hippocampal neurons expressing sortilin-HA. Western blotting of the biotinylated membrane proteins demonstrates constitutive and ionomycin-induced production of the HA-tagged 18-kDa C-terminal fragment remaining on the plasma membrane after ectodomain shedding, suggesting that cleavage can occur at the cell surface (Fig. 6c).

Because only a minor fraction of sortilin (<10%) is expressed in the plasma membrane (13), we hypothesized that shedding may also occur intracellularly. To test this, lysates of HEK293T cells expressing endogenous sortilin or transfected with wild-type sortilin-HA were examined to determine if the shed ectodomain was detectable. In addition, we used peptide N-glycosidase F to remove carbohydrates, and proteins were resolved with urea containing SDS-PAGE to enhance resolution. In the presence of sugar modifications, a single sortilin species was detected at 110 kDa by anti-sortilin immunoblotting (Fig. 6d, top panel, A). Removal of N-linked sugar groups allowed for resolution of a 92- and 83-kDa species in deglycosylated samples corresponding to predicted molecular weights of propeptide containing and mature furin-processed sortilin, respectively (Fig. 6d, top panel, B and B’). In addition, antibodies specific for the sortilin ectodomain detected a doublet of ~75 kDa that we hypothesize corresponds to the pro- and furin-processed sortilin ectodomain (C and C’). Anti-HA immunoblotting of deglycosylated samples detects only the 92- and 83-kDa bands, but not the 75-kDa species, further evidence that the 75-kDa doublet represents the shed ectodomain (Fig. 6d). In contrast to WT sortilin-HA, the 75-kDa sortilin was not detected in cells expressing the uncleavable Δstalk sortilin-HA, indicating that this species is a product of sortilin shedding (Fig. 6d, top panel). Taken together, these observations suggest that sortilin ectodomain shedding occurs both at the cell surface and in an intracellular compartment.

**BDNF Levels Are Modulated by Lysosomal Proteases**—The established role of sortilin in delivery of hydrolases to the lysosome coupled with the observation that a proportion of sortilin is normally degraded by lysosomal proteases (57) led us to hypothesize that BDNF may also be targeted to the lysosome by sortilin. Indeed, lysosomal inhibition led to a 26.7 ± 3.8% (p < 0.01) increase in endogenous BDNF levels in cell lysates of cortical neurons expressing endogenous sortilin, and this effect...
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FIGURE 6. Localization of sortilin shedding. Sortilin shedding occurs in the plasma membrane and in an intracellular compartment. a, shown is a schematic of internalization-deficient sortilin illustrating mutations that localize sortilin to the plasma membrane and impair endocytosis. ECD, extracellular domain; ICD, intracellular domain. b, ADAM10−/− MEF cells were transfected with internalization-deficient sortilin alone or cotransfected with ADAM10 (AD10imm). Base-line levels of shedding were assessed by incubation in serum-free DMEM (Pretx) followed by treatment with ionomycin (IM, 2.5 μM, 30 min) followed by anti-sortilin immunoblotting (IB). Anti-sortilin immunoblotting of whole cell extract confirms levels of expression (right panel). c, rat hippocampal neurons were transfected with sortilin-HA and surface biotinylated. Lysates were incubated with streptavidin, and the eluted precipitates were subjected to Western blotting with anti-HA to detect full-length sortilin and the sortilin CTF. Anti-HA immunoblotting of whole cell extracts is shown in the right hand panel. Veh., vehicle. d, N-glycanase (PNGaseF)-treated whole cell extracts of HEK293T cells expressing endogenous, WT sortilin-HA, or Δstalk sortilin-HA were resolved using urea SDS-PAGE. Immunoblotting with anti-sortilin ectodomain antibody (top panel) detects full-length glycosylated sortilin (A). Removal of sugar groups permits resolution of the propeptide-containing and furin processed full-length deglycosylated sortilin (B and C). A doublet at ~75-kDa (C and C') is detectable with anti-sortilin immunoblotting of WT sortilin-HA expressing but not Δstalk sortilin-HA-expressing cells. Immunoblotting of cell extracts with anti-HA (bottom panel) detects the full-length sortilin species (A and B) and not the 75-kDa species (C).

was enhanced by expression of WT sortilin-HA (32 ± 6.4%, p < 0.01, Fig. 7a). Concomitantly, lysosome inhibition resulted in an ~25% increase in levels of endogenous sortilin and WT sortilin-HA (Fig. 7b). These results suggest that BDNF is synthesized in excess in cortical neurons and that sortilin regulates intracellular BDNF levels by targeting a proportion (~25%) to the lysosome.

Sortilin Sheding Modulates Lysosomal Targeting of BDNF—Targeting of acid hydrolases to the lysosome by sortilin is dependent upon the sortilin cytoplasmic tail (58), suggesting that shedding of the sortilin ectodomain during intracellular trafficking may inhibit lysosomal degradation of cargo, including BDNF. To this end we expressed in neurons a soluble form of the sortilin ectodomain to mimic the effects of shed sortilin. This soluble sortilin binds proBDNF with similar affinity to full-length sortilin (3) and is constitutively released from cells. Indeed, we observed a higher ratio of soluble sortilin in the media relative to the lysate of cells expressing WT sortilin-HA (Fig. 7b). To test whether the soluble sortilin ectodomain alters the targeting of endogenous BDNF to the lysosome, cells expressing WT sortilin-HA or soluble sortilin ectodomain were treated with lysosomal inhibitors. Lysosomal inhibition of cortical neurons expressing the soluble sortilin ectodomain led to a nonsignificant increase in cellular BDNF levels of 10.3 ± 3.8%, a significant reduction in BDNF degradation relative to control-transfected cell or cells expressing WT sortilin-HA (26.7 ± 3.8 and 32 ± 6.4%, respectively, p < 0.01, Fig. 7a).

Previous studies have shown that expression of the soluble sortilin ectodomain results in an increase in constitutive release of BDNF relative to cells expressing full-length sortilin (5). Because we did not observe an increase in base-line levels of BDNF in the whole cell lysate (Fig. 7a), we postulated that the BDNF that is protected from degradation by soluble sortilin is rapidly released into the media. To detect endogenous BDNF in the media, cortical neurons were cultured in the presence of TrkB-Fc receptor bodies for 48 h to prevent internalization of BDNF by TrkB (22). Anti-BDNF immunoblotting of media precipitated with protein A beads demonstrates an increase in constitutive release of mature BDNF from cells expressing soluble sortilin compared with cells expressing WT sortilin-HA (Fig. 7c). Lysosomal inhibition increased the amount of BDNF detected in the media of
cells expressing WT sortilin-HA by 1.7-fold relative to untreated cells but did not increase BDNF release from soluble sortilin-expressing cells (Fig. 7c).

Taken together, these findings indicate that sortilin modulates the intracellular levels of neuronal BDNF by targeting it to the lysosome where it is degraded. Furthermore, overexpression of the sortilin ectodomain, which mimics increased intracellular sortilin shedding, serves to increase endogenous neuronal BDNF by rescuing it from degradation by lysosomal proteases, allowing for an increase in constitutive secretion.

**DISCUSSION**

The identification of sortilin as both a cell surface receptor for proneurotrophins and an intracellular chaperone for BDNF has enhanced our understanding of neurotrophin biology, but little is known about the mechanisms by which sortilin mediates these distinct functions. The current studies were conducted to gain insight into these processes by elucidating the mechanism and function of sortilin ectodomain shedding. We present evidence that ADAM10 is the major sortilin sheddase and that the extracellular juxtamembrane stalk domain contains the cleavage recognition site. In addition, we demonstrate that shedding occurs both at the cell surface and in an intracellular compartment, suggesting multiple functions. We also describe for the first time a mechanism by which endogenous levels of BDNF in neurons are modulated by sortilin-dependent lysosomal degradation and demonstrate a role for sortilin shedding in the regulation of this process.

**ADAM10 Is Required for Sortilin Shedding—Ectodomain shedding regulates numerous transmembrane proteins. Previous studies reported conflicting findings for a role for ADAM17 in sortilin shedding (20, 43), but here, utilizing gain and loss of function approaches, we determined that ADAM10, but not ADAM17, is the major sortilin protease in neurons and fibroblasts. However, a previous study demonstrating that sortilin shedding is activated by PKC in a tumor cell line suggests that sortilin may be cleaved by ADAM17 in cell types other than those utilized in our experiments (20). As such, the ability of an ADAM to preferentially cleave a substrate depends on the local environment. ADAM10 and -17 expression are spatially and temporally regulated in the brain (39), with ADAM10 widely expressed in neurons, whereas ADAM17 is more restricted to glia and brain endothelium in the adult (38, 39). Thus, we cannot rule out a role for ADAM17 in cleavage of sortilin in other cell types or under conditions in which ADAMs are aberrantly expressed such as in cancer, inflammation, or neurodegenerative disease (21, 59).

Interestingly, loss of function studies identified ADAM17 as the major sheddase for the sortilin co-receptor p75NTR (32). Our observed ionomycin-induced proteolysis of p75NTR is likely due to activation of ADAM17, as both ADAM10 and ADAM17 respond to stimulation of cells with ionomycin (41). The cleavage of p75NTR and sortilin by different ADAM family members may allow for independent regulation of downstream functions that are dependent on shedding. p75NTR proteolysis is induced by proneurotrophin binding and leads to enhanced apoptosis; however, pharmacological studies do not exclude a role for an additional ADAM family member in this process (60).

The Sortilin Juxtamembrane Stalk Domain Is Required for Shedding—Based on its proximity to the plasma membrane and lack of structure (52), we hypothesized that the 15-amino acid extracellular juxtamembrane stalk domain contained the
ADAM cleavage recognition site (49). Indeed, deletion of this region dramatically reduced constitutive and stimulated shedding; however, it also led to the accelerated turnover of sortilin, rendering a sheddase-resistant loss of function approach untenable in identifying functional consequences of sortilin shedding. The inability to express the sortilin lacking the juxtamembrane stalk at levels comparable with wild-type sortilin may reflect enhanced turnover of sortilin that is not able to be shed, as has been shown for TNF-α (61). Alternatively, deletion of the stalk domain may lead to increased clearance of sortilin due to improper folding or insertion in the membrane; however, detection of the mutant was not enhanced by proteasomal inhibition (data not shown).

Localization of Shedding—Our studies demonstrate that sortilin shedding occurs both at the cell surface and in an intracellular compartment in neurons and non-neuronal cells. Although residing primarily in intracellular compartments, sortilin localization is dynamic, and modulation by NRH2 enhances surface levels of sortilin and proneurotrophin responsiveness (57). A functional role for sortilin relocalization in vivo is demonstrated by increased sortilin-p75NTR colocalization at the cell surface of retinal ganglion neurons during the developmental stage in which they undergo apoptosis (62). Our findings argue that ADAM10 is the major sheddase for sortilin both at the cell surface and in intracellular compartments; however, the subcellular localization in which cleavage occurs could dictate the downstream consequences of shedding. For example, ectodomain shedding at the cell surface could modulate pro-neurotrophin-dependent apoptosis, whereas shedding in an intracellular compartment may affect localization of cargo molecules due to separation from trafficking motifs in the sortilin cytoplasmic tail.

A Role for Sortilin Shedding in BDNF Trafficking—Our findings that sortilin is shed into the media of cultured neurons as well as during intracellular trafficking suggest a functional role in regulating the sorting of neuronal BDNF. BDNF is packaged into dense core vesicles and released in response to depolarization in a sortilin-dependent manner (5); as such, we posited that sortilin shedding may affect BDNF trafficking. As the sortilin cytoplasmic tail contains canonical motifs that dictate both trafficking between the trans-Golgi network and late endosome and internalization from the plasma membrane (15, 63), the separation of the ligand binding ectodomain from the intracellular sorting motifs via the actions of ADAM10 may alter trafficking of sortilin cargo proteins such as BDNF. Indeed, we found that ectopic expression of the soluble sortilin ectodomain, which is capable of binding to BDNF but lacks the sorting motifs required for trafficking to the late endosome, rescued the effect of lysosomal inhibition on BDNF levels. This suggests that a significant portion of BDNF is normally degraded by the lysosome, estimated here to represent more than 25% of synthesized levels. In addition, our findings indicate that the shed sortilin ectodomain largely redirects this pool of BDNF, destined for lysosomal degradation, to the constitutive secretory pathway. Our findings support and significantly extend those of Chen et al. (5), in which the soluble sortilin ectodomain enhanced constitutive release of BDNF, and suggest that this may be in part due to an increase in total BDNF levels as well as decreased targeting to the lysosome and to regulated secretory vesicles.

Taken together, our data support a model in which BDNF is normally produced in excess and delivered to the lysosome by a sortilin-dependent process for degradation. Our studies suggest that increased neuronal BDNF bioavailability may be rapidly achieved by ADAM10-mediated production of the soluble sortilin ectodomain. These results add yet another mechanism to the diverse array of processes by which BDNF is regulated (4). At the mRNA level, BDNF is regulated by alternative splicing of both 5’ and 3’ UTRs to produce multiple transcripts that differ in their regulatory regions while encoding identical protein products (64, 65). This allows for context-dependent BDNF expression and local translation of mRNA (65, 66). Furthermore, chaperone proteins regulate localization of BDNF mRNA and protein in neurons and include sortilin, carboxypeptidase E, and translin (5–7). Recent studies of a BDNF V66M single nucleotide polymorphism that displays altered protein binding to sortilin and mRNA binding to translin highlights the pathophysiological consequences of altered BDNF release. Expression of the BDNF Met allele results in a 10% reduction in activity-dependent secretion relative to the BDNF Val allele (5). This seemingly subtle difference correlates with behavioral or psychiatric susceptibility in human carriers of the Met allele including memory deficits and depression and schizophrenia (67, 68). Thus, our observation that lysosomal inhibition results in a greater than 25% increase in cellular BDNF suggests that perturbation of this system could drastically affect BDNF-dependent processes.

Sortilin Ectodomain Shedding and Disease—The characterization of a new mechanism by which BDNF levels are regulated may aid in the development of therapeutics for diseases in which BDNF levels are altered. For example, BDNF levels are decreased in Alzheimer diseased brains, and this may contribute to the observed cell death, synaptic dysfunction, and memory loss (25). A recent analysis showed that gene delivery of BDNF rescues degenerating neurons in Alzheimer disease models and in the aging brain of rodents and primates (69). Given the difficulties in delivering BDNF in the human brain, modulation of sortilin ectodomain shedding may provide an alternate strategy for the treatment of neurodegenerative disease and CNS injury via regulation of BDNF levels and secretion. However, if sortilin shedding is to be therapeutically targeted, consideration must be given to the effects of proteolysis on other sortilin functions, such as sorting of lysosomal hydrolases and glucose transporter isofrom 4 trafficking (70, 71).

In summary, we present evidence that sortilin shedding is an endogenous process in vivo and that ADAM10 is the major sortilin sheddase in neurons. In addition, we identified the juxtamembrane stalk as the primary site of sortilin shedding. Intriguingly, we have characterized a novel mechanism by which sortilin ectodomain shedding regulates BDNF levels in neurons via modulation of lysosomal targeting. These findings enhance our understanding of the complex mechanisms that regulate BDNF. Further study is warranted to determine the role of sortilin shedding during development and plasticity when BDNF levels are tightly regulated as well as in disease states in which BDNF levels are altered.
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References


