TrkA Receptor Activation by Nerve Growth Factor Induces Shedding of the p75 Neurotrophin Receptor Followed by Endosomal γ-Secretase-mediated Release of the p75 Intracellular Domain

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Soledad Urra‡1, Claudia A. Escudero‡1, Patricio Ramos‡1, Fernanda Lisbona‡, Edgardo Allende‡, Paulina Covarrubias‡, Jose I. Parraguez‡, Niccolo Zampieri‡, Moses V. Chao¶, Wuim Annaert‡, Wim Annaert‡, Jose I. Parraguez‡, Moses V. Chao¶, Wim Annaert‡, and Francisca C. Bronfman‡1,3

From the ‡Department of Physiology, Center for Cellular Regulation and Pathology Joaquin V. Luco, Faculty of Biological Sciences, Pontificia Universidad Catolica, Alameda 340, Santiago 8320000, Chile, the ‡Molecular Neurobiology Program, Skirball Institute for Biomolecular Medicine and Department of Cell Biology, New York University School of Medicine, New York, New York 10016, and the ¶Laboratory for Membrane Trafficking, Center for Human Genetics, Gasthuisberg KULeuven & VIB, B-3000 Leuven, Belgium

Neurotrophins are trophic factors that regulate important neuronal functions. They bind two unrelated receptors, the Trk family of receptor-tyrosine kinases and the p75 neurotrophin receptor (p75). p75 was recently identified as a new substrate for γ-secretase-mediated intramembrane proteolysis, generating a p75-derived intracellular domain (p75-ICD) with signaling capabilities. Using PC12 cells as a model, we studied how neurotrophins activate p75 processing and where these events occur in the cell. We demonstrate that activation of the TrkA receptor upon binding of nerve growth factor (NGF) regulates the metalloprotease-mediated shedding of p75 leaving a membrane-bound p75 C-terminal fragment (p75-CTF). Using subcellular fractionation to isolate a highly purified endosomal fraction, we demonstrate that p75-CTF ends up in endosomes where γ-secretase-mediated p75-CTF cleavage occurs, resulting in the release of a p75-ICD. Moreover, we show similar structural requirements for γ-secretase processing of p75 and amyloid precursor protein-derived CTFs. Thus, NGF-induced endocytosis regulates both signaling and proteolytic processing of p75.

Neurotrophins belong to a small family of neurotrophic factors that include nerve growth factor (NGF),¹ brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4 (NT4). They regulate different aspects of the developing and adult nervous system by binding to specific members of the Trk family of receptor-tyrosine kinases (TrkA, -B, and -C) or to the p75 neurotrophin receptor (p75). Their involvement includes neuronal migration, cell death, axonal elongation, myelinization, neuronal differentiation, and synaptic plasticity (1–3). p75 is a multifunctional type I transmembrane protein that is structurally related to the tumor necrosis factor receptor superfamily. It binds all neurotrophins, alone or in complex with Trk receptors, but also other ligands such as amyloid peptides and pro-neurotrophins (4, 5). The association of p75 with different receptors shapes the outcome of a signaling event. For example, binding of p75 to TrkA in the presence of NGF promotes neuronal survival, whereas interaction with the Nogo receptor (NgR) in the presence of its ligands, such as Nogo and myelin-associated glycoprotein (MAG), results in growth cone collapse and the inhibition of axonal regeneration (1, 4, 5). In addition, different downstream p75-associated signaling cascades are triggered through the interaction of its cytosolic portion with multiple adaptor proteins (6). Neurotrophin binding to p75 also induces accumulation of p75 in recycling endosomes where it associates with specific p75 downstream signaling effectors (7–9). Hence trafficking of p75 in the cell body or in axons (10, 11) may determine downstream signaling of p75 as previously shown for Trk receptors (12). The regulation of p75 signaling becomes even more complicated with the finding that p75 is subject to a dual processing starting with shedding of the ectodomain and followed by γ-secretase cleavage. This adds p75 to the growing list of substrates for regulated intramembrane proteolysis (RIP) that includes the (3-[(3-cholamidopropyl)-2-hydroxy-1-propanesulfonate); CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate); CHAPSO, (3-[(3-cholamidopropyl)-2-hydroxy-1-propanesulfonate); and CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate); C9E, compound E; CTF, C-terminal fragment; DAPT, (N-[N-(difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyler ester); HA, hemagglutinin; NTF, N-terminal fragment; p75, p75 neurotrophin receptor; p75-CTF, p75 membrane-bound C-terminal fragment; p75-ICD, p75-derived intracellular domain; PBS, phosphate-buffered saline; PMA, phorbol ester 12-myristate 13-acetate; RIP, regulated intramembrane proteolysis; HRP, horseradish peroxidase; P5, presenilin; PKC, protein kinase C; mAb, monoclonal antibody; pAb, polyclonal antibody; BDNF, brain-derived neurotrophic factor.

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‡ To whom correspondence may be addressed: Laboratory for Membrane Trafficking, Center for Human Genetics, Gasthuisberg KULeuven and VIB, B-3000 Leuven, Belgium. Tel.: 32-16-330520; Fax: 32-16-330522; E-mail: Wilm.Wim@med.kuleuven.be.

§ To whom correspondence may be addressed: Dept. of Physiology, Faculty of Biological Sciences, Pontificia Universidad Catolica, Alameda 340, Santiago 8320000, Chile. Tel.: 56-2-6602879; Fax: 56-2-6602824; E-mail: fbronfman@bio.puc.cl.

The abbreviations used are: NGF, nerve growth factor; ADAMS, a metalloprotease and disintegrin; APP, amyloid precursor protein; CHAPS, (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate); CHAPSO, (3-(3-cholamidopropyl)-2-hydroxy-1-propanesulfonate); C9E, compound E; CTF, C-terminal fragment; DAPT, (N-[N-(difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyler ester); HA, hemagglutinin; NTF, N-terminal fragment; p75, p75 neurotrophin receptor; p75-CTF, p75 membrane-bound C-terminal fragment; p75-ICD, p75-derived intracellular domain; PBS, phosphate-buffered saline; PMA, phorbol ester 12-myristate 13-acetate; RIP, regulated intramembrane proteolysis; HRP, horseradish peroxidase; P5, presenilin; PKC, protein kinase C; mAb, monoclonal antibody; pAb, polyclonal antibody; BDNF, brain-derived neurotrophic factor.
amyloid precursor protein (APP), Notch, Syndecan 3, ErbB4, and N-cadherins among others (13–22). Shedding of p75 can be induced by protein kinase C (PKC) activators such as the phorbol ester 12-myristate 13-acetate (PMA), reminiscent of the regulatory component of APP shedding by the ADAM (a metalloprotease and disintegrin) family of metalloproteases (23, 24). Indeed, in particular ADAM17 has been shown to be involved in phorbol ester-stimulated shedding of p75 in murine fibroblasts (33). This event produces a membrane-bound C-terminal fragment (p75-CTF) that is further processed by γ-secretase resulting in the release of the intracellular domain of p75 (p75-ICD). γ-Secretase is a multiprotein complex that consists of the catalytic presenilin 1 (PS1), nicastrin, Pen-2, and Aph-1. In the case of APP, γ-secretase cleavage releases both amyloid β-peptides and APP-ICD. Initial studies suggested a nuclear translocation and signaling role for APP-ICD (25), but recent reports have contradicted these findings (26). In contrast, for the Notch receptor, the ICD has the potential to regulate gene expression by forming transcriptionally active complexes (27–29). This may be true also for p75-ICD. Soluble p75-ICD regulates NFκB signaling (21) and has been localized in the nucleus of Schwann and 3T3 cells (overexpressing p75) in a ligand-dependent manner (30–33). γ-Secretase cleavage of p75 is required for MAG-induced inhibition of neurite outgrowth in cerebellar neurons (34) and down-regulation of growth cone collapse mediated by p75 and NgR in the presence of myelin ligands (35). Finally, the addition of pro-BDNF or BDNF to sympathetic neurons results in RIP of p75, which is necessary for nuclear translocation of NRIF (a p75 intracellular interactor) and for p75-induced neuronal apoptosis (36). These examples clearly demonstrate the growing importance of p75 processing for its downstream signaling.

Different lines of evidence suggest that early/recycling endosomes might be a major compartment where γ-secretase mediates the cleavage of APP and other substrates such as Notch (37–39). Indeed, the inhibition of γ-secretase activity results in the accumulation of APP-CTFs in recycling endosomes in addition to the cell surface (40). Considering that p75 can become internalized (8, 9) and the increasing evidence implicating p75 shedding and RIP in downstream signaling, we have investigated the regulation of p75 processing in PC12 cells. Here we show that p75 shedding is regulated through the activation of the TrkA receptor and that the resulting p75-CTFs end up in endosomes. Using a subcellular fractionation approach we furthermore demonstrate that γ-secretase processing occurs in early/recycling endosomes, resulting in the production of the corresponding p75-ICD fragment. Our data identify the endocytic pathway as a platform for processing of p75 and as a critical junction for regulation of neurotrophin signaling.

**EXPERIMENTAL PROCEDURES**

Recombinant BDNF, mouse NGF, and MC192 monoclonal antibody (mAb) against rat p75 ectodomain are from Alomone Labs (Jerusalem, Israel). Transferrin-Alexa555, donkey anti-mouse-Alexa488 and -555 are from Molecular Probes (Eugene, OR), and transferrin-HRP from Jackson ImmunoResearch (West Grove, PA). Rabbit polyclonal antibody (pAb) against the C terminus of p75 is from Upstate (Charlotteville, VA), rab11 and LAMP2 Abs are from Zymed Laboratories (San Francisco, CA), rab5 and the β1 subunit of the Na+/K+-ATPase Abs are from Santa Cruz Biotechnology (Santa Cruz, CA), and pAb against phospho-TrkA490 is from Cell Signaling Technology (Danvers, MA). mAb against β-tubulin, HA (hemagglutinin), GM130, and transferrin receptor are from Sigma-Aldrich, Roche Applied Sciences (Hertfordshire, UK), Abcam (Cambridge, UK), Zymed Laboratories, respectively. Lactacystin, compound E (CpE), DAPT, inhibitor X (L685,458), GM6001, and APP-CTF-derived fluorescent peptide are from Calbiochem. pAbs against PS1-NTF (B19.2) and PS1-CTF (B32.1) are described (42, 43) as well as mAb 9C3 against nicastrin (41). pAb SB129 against human PS1 was kindly provided by C. Van Broeckhoven (Antwerp, Belgium).

**Immunofluorescence—**PC12 and nnr5 cells were routinely cultured as described (8). To determine cell surface-associated p75, PC12 cells were starved in incubation buffer (IB; 25 mM Dulbecco’s modified Eagle’s medium/HEPES, pH 7.4 and 1 mg/ml bovine serum albumin) for 1 h at 37 °C, followed by PMA treatment (100 ng/ml) in IB for 1 h at 37 °C. Next, cells are washed with ice-cold phosphate-buffered saline (PBS) and incubated on ice with MC192 (3 μg/ml) in IB for 1 h. After fixation (3% PFA in PBS) and blocking (10% donkey serum in PBS, 30 min at room temperature) the primary antibody was visualized using donkey-anti-mouse-Alexa488, washed and mounted in Mowiol. In the case of immuno-endocytosis, PC12 cells were starved (1 h, 37 °C) and incubated with MC192 (3 μg/ml) for 1 h at 4 °C. After washing, cells were treated with PMA (100 ng/ml) or NGF (4 nm) for 1 h at 37 °C. Following fixation (3% paraformaldehyde in PBS) and blocking (10% donkey serum in PBS, 30 min at room temperature) cells were incubated with the first secondary antibody (donkey anti-mouse-Alexa647, 30 min in PBS + 5% donkey serum). Next, cells were permeabilized (PBS, 5% donkey serum, 0.2% saponin), incubated with secondary donkey anti-mouse-Alexa555 pAbs, washed, and finally mounted in Mowiol. To study the internalization of the N-terminal HA epitope-tagged p75-CTF construct (HA-p75-CTF), lacking the complete p75 ectodomain in PC12 cells, Lipofectamine 2000 (Invitrogen) was used, and cells were processed 24 h after transfection (8, 44). Internalization of HA-p75-CTF was studied by starving the cells (1 h, 37 °C) followed by incubation with anti-HA (3 μg/ml) for 1 h at 4 °C. After washing, cells were treated with NGF (4 nm; 1 h, 37 °C) in the presence of transferrin-Alexa555 (100 μg/ml). Following fixation and blocking as described above, cells were permeabilized (PBS, 5% donkey serum, 0.2% saponin) and incubated with secondary donkey-anti-mouseAlexa488 pAb, washed, and finally mounted in Mowiol.

**Microscopy—**Confocal images were collected on a Zeiss LSM Pascal 5 (including a triple laser module (Arg 458/488/514 nm, HeNe 543 nm, HeNe 633 nm, Carl Zeiss, Thornwood, NY) connected to an inverted microscope (Axiovert 2000) with a ×63 objective. Quantification of the fluorescence intensity was done as described in Ref. 8 and as shown in Fig. 1. Briefly, for each experimental point, 4–5 digital images were taken from two different coverslips (×63 and zoom ×2) in which 100 cells were imaged and quantified with Sigma Scan software.
Western Blot Analysis of p75 Fragments—PC12 cells were starved and pretreated with the different inhibitors (5 μM lactacystin, 10 μM GM6001, 0.2 μM compound E) for 1 h at 37 °C in IB followed by neurotrophin treatment (NGF or BDNF, 4 nM) for different times (15 min to 6 h) or PMA (100 ng/ml) for 2 h at 37 °C. After treatment, cells were lysed (20 μg Tris-HCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, protease inhibitors (Roche Applied Science), 250 mM sucrose, pH 7.6) and cleared extracts processed for Western blotting using a pAb against the C terminus of p75. For detection, HRP-conjugated secondary antibodies were used followed by ECL using West Pico (p75-FL) or West Femto (p75 fragments) kits (Pierce).

γ-Secretase Presence and Activity in Intracellular Membranes (P2)—A P2 pellet from PC12 cells was prepared essentially as described (8, 45). Briefly, 7–9 confluent 10-cm dishes were preincubated for 1 h at 37 °C with IB to deplete endogenous growth factors, followed by 2 h at 37 °C with NGF (4 nM). Next, cells were washed with ice-cold PBS and, after a final wash with sucrose/HEPES buffer (SHB; 320 mM sucrose, 20 mM HEPES, pH 7.2), scraped, and sedimented (800 × g, 15 min). The pellet was resuspended in 0.6 ml of SHB and homogenized with a ball-bearing homogenizer (cell cracker; EMBL, Germany) with 12-μm clearance. A postnuclear supernatant (1,000 × g, 10 min) was centrifuged over a cushion of 1% Ficoll in SHB (190,000 × g, 20 min). The resulting P2 pellet was resuspended in SHB (+ protease inhibitors) or solubilized in CHAPSO buffer (50 mM Tris-HCl, 2 mM EDTA, protease inhibitors, 0.25% CHAPSO; pH 6.8) and incubated for different times at 37 °C without or with specific γ-secretase inhibitors (0.2 μM CpE, 10 μM DAPT, or 0.5 μM inhibitor X). After incubation, membranes resuspended in SHB were solubilized with 0.2% Triton X-100 for 1 h on ice in addition to 5 min at 37 °C. Cleared supernatants were finally analyzed by Western blotting for p75. For co-immunoprecipitation experiments the P2 pellet was solubilized in CHAPSO buffer (250 mM sucrose, 50 mM HEPES, 2 mM EDTA, 2 mM EGTA, 125 mM NaCl, 2% CHAPS, pH 7.4). From the cleared lysate, 900 μg of protein was incubated overnight at 4 °C with 7 μg of anti-p75-ICD (Upstate Biotechnologies) and further incubated with 30 μl of TrueBlot anti-rabbit IgG IP beads (eBioscience) for 1 h at 4 °C. The beads were washed four times with buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 1% SDS in PBS. Bound proteins were eluted in sample buffer (10 min, 70 °C) and analyzed by Western blotting for PSI interaction.

γ-Secretase-mediated Peptide Cleavage Assay—γ-Secretase activity was assayed in vitro using an APP-CTF-derived intramolecularly quenched fluorescent peptide according to the manufacturer’s instructions (CALBIOCHEM) (46, 47). Briefly, 50 μg from CHAPSO-solubilized P2 membranes were incubated at 37 °C for different times in 150 μl of assay buffer containing 50 mM Tris-HCl, protease inhibitors, 2 mM EDTA, 0.25% CHAPSO, pH 6.8, and 8 μM fluorescent APP-CTF-derived peptide. After incubation, the cleared reaction mixture (16,100 × g, 15 min) was transferred to a 96-well plate. Fluorescence was measured using a PerkinElmer Luminescence spectrometer LS50B (excitation/emission at 350/440 nm). Specific γ-secretase activity was determined after subtracting the fluorescence obtained in the presence of DAPT (10 μM). Background fluorescence was calculated by incubating separately 50 μg of CHAPSO-solubilized P2 membranes and 8 μM APP-CTF-derived peptide with assay buffer for different times and mixing them just before fluorescence determination.

Cell Fractionation—Labeling of early/recycling endosomes with transferrin-HRP was done as described (8). To label late endosomes, cells were incubated for 90 min with NGF (4 nM) followed by a pulse (10 min) with HRP (0.5 mg/ml) and NGF (4 nM) and a chase (20 min) in the presence of NGF (4 nM) at 37 °C. Next, a P2 pellet was prepared, resuspended in 0.5 ml of SHB and loaded on a 4-ml linear Ficoll gradient (2–16%) over a 1-ml 16% Ficoll cushion. After velocity gradient centrifugation (SW50, 190,000 × g, 20 min), 0.5-ml fractions were manually collected from the top. The three transferrin-HRP or HRP peak fractions from gradient 1 were pooled, centrifuged again (SW50, 190,000 × g, 20 min) over a 4-ml linear Ficoll gradient prepared in SHB (3–16%), and collected in 0.5-ml fractions. HRP activity was measured as described previously (8, 48).

RESULTS

The neuroendocrine PC12 cell line is an established model for NGF signaling and expresses both p75 and TrkA receptors (49, 50). It is also used as a model system for synaptic membrane recycling because synaptic proteins recycle through PC12 endosomes (51, 52) and is therefore suited to study p75 processing and trafficking. We initiated our studies by analyzing the cell surface–associated full-length p75 (p75-FL) following PMA treatment using a p75 ectodomain-specific mAb. Quantitative confocal microscopy revealed a reduction of nearly 50% of cell surface-associated p75-FL (Fig. 1, A and B). Western blot analysis did not show a similar or substantial reduction of total p75-FL (Fig. 1, E and F). This could be explained by the fact that cell surface-associated p75 represents a part of the total cellular p75, and the levels of PMA-induced p75-CTFs are a minor fraction of the total p75-FL. Alternatively, PKC activation may induce p75 internalization, as has been shown for other receptors (52, 53). We tested this by measuring the cell surface and intracellular pool of p75 before and after permeabilization in control and PMA-treated cells. PMA induced a 5-fold increase in p75 internalization compared with basal levels (Fig. 1, C and D). However, when cells were challenged with NGF, the level of p75 internalization increased 3-fold compared with PMA treatment. This indicates that the trafficking of p75, in addition to being regulated in a ligand-dependent manner, is also regulated by PKC in a ligand-independent manner.

In PMA-treated PC12 cells, we also observed the appearance of p75-CTF and p75-ICD fragments (Fig. 1E). The latter could be stabilized by lactacystin or blocked by compound E (CpE), a highly specific γ-secretase inhibitor (54) (Fig. 1, E and F). GM6001, a general metalloprotease inhibitor, inhibited both p75-CTF and p75-ICD production, thus demonstrating that p75-CTF generation is a prerequisite for specific γ-secretase activity...
processing and production of p75-ICD (Fig. 1F). This contradicts previous findings reported in glial cells (30) but agrees with other studies performed in different cell types (21, 22, 44).

The PMA effect on p75 shedding is most likely because of the activation of ADAMs by PKC (55). We thought that similar to PMA, NGF might trigger such an effect by activating downstream signaling leading to the regulation of ADAMs activity. Indeed, like PMA, NGF induced p75-CTF and p75-ICD fragments in a metalloprotease- and γ-secretase-dependent manner (Fig. 1G). Following 2 h of NGF treatment, the levels of p75-ICD were similar to those of PMA-treated PC12 cells (Fig. 1H). In addition, overexpression of a dominant negative mutant of human PS1 (27) (Fig. 1I) resulted in a significant drop of p75-ICD levels after NGF treatment, further demonstrating that NGF induces p75 processing in a γ-secretase-dependent manner (Fig. 1G). Over time, NGF-induced p75-ICD production reached a maximum after 2 h and then decreased (Fig. 2A and B). It should be noted here that p75-ICD production could only be detected when lactacystin was present to prevent proteasomal degradation. This indicates that newly formed ICD fragments are unstable, which is characteristic for other RIP-generated ICDs like Notch and APP (27, 56).

Next we investigated whether the NGF effect on p75 processing is mediated by TrkA activation and/or direct p75 stimulation. We compared NGF with BDNF, a neurotrophin that specifically binds TrkB, which is not expressed in PC12 cells, and nnr5 cells, a clonal variant of PC12 deficient in TrkA expression. We performed in different cell types (21, 22, 44).

FIGURE 1. PMA and NGF induce internalization and shedding of the p75 neurotrophin receptor. A, cell surface localization of p75 in PC12 cells. PC12 cells were incubated with DMSO (DMSO) or PMA for 1 h at 37 °C, cooled to 4 °C, and subsequently incubated for 1 h with mAb MC192, recognizing the p75 ectodomain. After fixation cells were immunolabeled with donkey anti-mouse Alexa647 (green) washed and mounted in Mowiol. A clear decrease in immunoreactivity was noticed upon PMA treatment. Cell surface-associated immunofluorescence was equal to total immunofluorescence (white contour minus intracellular fluorescence (yellow circle) (inset). The graph shows the average ± S.E. of three independent experiments in which 100 cells were quantified. B, p75 internalization is induced by PMA and NGF. Serum-starved PC12 cells were incubated for 1 h at 4 °C with mAb MC192, and following a brief wash either fixed immediately or incubated with Me2SO (control) or PMA for 1 h at 37 °C. After fixing and blocking, cells were incubated first with donkey anti-mouse Alexa488 (red) for labeling cell surface-associated MC192 and then permeabilized and incubated with donkey anti-mouse Alexa555 (green) for labeling internalized MC192, and finally washed and mounted in Mowiol. D, quantification of p75 internalization induced by PMA and NGF. Internalized p75 was equal to total immunofluorescence (white contour) minus cell-associated fluorescence as quantified in B. The graph shows the average ± S.E. of 200 cells (of two independent experiments). E–H, characterization of p75 processing in PC12 whole cell lysate. Serum-starved PC12 cells were pretreated with the different inhibitors (5 μM lactacystin, 10 μM GM6001, 0.2 μM compound E (CpE)) for 1 h at 37 °C in incubation buffer followed by NGF (4 nm) or PMA (100 ng/ml) treatment for 2 h at 37 °C. After treatment, the samples were processed for Western blotting on nitrocellulose using the rabbit pAb against the C terminus of p75; FL, full-length p75; CTF, p75 membrane-bound C-terminal fragment; ICD, p75 intracellular domain. E and F, Western blot analysis of p75 processing after PMA and lactacystin (LAC) treatment (E), or in the presence of PMA, LAC, CpE, and GM6001 treatment (F). PMA induces whereas GM6001 represses p75-CTF and -ICD production. CpE only decreased p75-ICD production. Lactacystin was added to stabilize the newly produced p75-ICD. G and H, Western blot analysis (G) and quantification of p75-ICD production (ratio of p75-ICD to p75-FL) (H) induced by NGF compared with PMA (mean ± S.E., n = 5). I, Western blot analysis of human full-length PS1 (PS1-FL), before (non-transfected (NT)) or after transfection with wild-type human PS1 (WT) or a dominant negative mutant of human PS1 (DN). The SB129 pAb was raised against human PS1 but cross-reacts with rat N-terminal PS1 (PS1-N). After stripping, the nitrocellulose membrane was reprobed with a mAb against β-tubulin as a control for sample loading. J, Western blot analysis of p75-ICD production after NGF treatment (2 h) of PC12 cells transfected with human wild type (WT) or dominant negative (DN) PS1.
and when PC12 cells were incubated with NGF in the presence of K252a, an inhibitor of Trk tyrosine kinase activity (57), NGF-induced production of p75-CTF and p75-ICD was abrogated (Fig. 2C). Consistent with a specific effect of K252a on inhibition of tyrosine activity of TrkA, K252a had no effect on PMA-induced p75 processing (Fig. 2C).

Moreover, and using a phosphospecific antibody, we could demonstrate that NGF, but not PMA, significantly increased the levels of phosphorylated TrkA, which were again reduced by K252a (Fig. 2C). Phosphorylation of TrkA at tyrosine 490 links TrkA activity to the ERK1/2 pathway (58). Taken together, our data clearly demonstrate that NGF-mediated p75 processing is indirect and requires the activation of TrkA receptors in PC12.

The combined p75 internalization and appearance of proteolytic fragments after NGF stimulation suggests that a major part of p75 processing may occur within the cell. Indeed, γ-secretase processing of APP and Notch was suggested to occur in endosomal compartments (37, 59–62). We therefore further characterized the γ-secretase-mediated cleavage of p75 in the membrane fraction, which is mainly enriched with intracellular membranes (8, 45). P2 membranes derived from NGF-treated PC12 cells were resuspended in detergent-free buffer and incubated for different times (30 min up to 10 h) at 37 °C. In vitro production of p75-ICD was evident already after 30 min of incubation and accumulated in a time-dependent manner up to 10 h (Fig. 3, A and B). Similar kinetics of γ-secretase activity was found in a CHAPSO-solubilized P2 fraction mixed with an APP-CTF-derived fluorescent peptide harboring the γ-secretase cleavage site (Fig. 3B). It should be noted here that CHAPSO protects the integrity of the γ-secretase complex and hence activity (55). p75-ICD production could be inhibited by known γ-secretase inhibitors including DAPT, inhibitor X (L-685,458), and CpE (15, 21, 22, 36, 54, 63) in a concentration-dependent manner (Fig. 3, C and D). Moreover, and supporting a specific γ-secretase p75 cleavage, the N-terminal fragment of endogenous PS1 was co-immunoprecipitated with an antibody against the intracellular domain of p75 (Fig. 3E). Interestingly, increasing amounts of the APP-CTF-derived fluorescent peptide inhibited p75-ICD production indicating that it competed with p75-CTF for γ-secretase processing (Fig. 3F).

The above findings demonstrate that a fully active γ-secretase complex is involved in the processing of p75-CTF in PC12 intracellular membranes. To determine the intracellular localization of this processing, we used a modified protocol for the isolation and separation of early/recycling endosomes from late endosomes in PC12 cells (8, 64). Endogenous PS1 and mature nicastrin were found co-distributing with both markers of early/recycling (Rab5, transferrin receptor, and transferrin-HRP) and late endosomes (LAMP2, HRP) (Fig. 4A) suggesting that γ-secretase complexes are widely distributed in the endosomal system. On the other hand, p75-FL and p75-CTF were uniquely recovered in the early/recycling endosomal fractions (Fig. 4B). When cells were preincubated with NGF or PMA prior to subcellular fractionation a striking increase in the levels of p75-CTF were observed in early/recycling endosomes (Fig. 4, A and C). When we transfected PC12 cells with an N-terminal HA epitope-tagged p75-CTF, which lacks the p75 ectodomain (EC) and mimics a direct γ-secretase substrate, we observed that it is efficiently internalized and co-localizes with transferrin, confirming its association with early/recycling endosomes (Fig. 4D). These results suggest that p75-CTF enters the early/recycling endosomal compartments where it undergoes γ-secretase processing. This was confirmed by incubating at 37 °C (for 2 and 10 h) purified early/recycling endosomal fractions derived from PC12 cells treated with NGF. This treatment resulted in
the extensive generation of p75-ICD in a γ-secretase-dependent manner (Fig. 5A). The significant drop of p75-CTF but not p75-FL immunoreactivity in the absence of Cpe demonstrates that p75-ICD is generated directly from the pool of p75-CTF present in these endosomal fractions (Fig. 5A). The endosomal fractions used for this assay were practically free from plasma membrane and Golgi contamination (Fig. 5B).

DISCUSSION

In this study we report for the first time that TrkA receptor activation by NGF regulates p75 ectodomain shedding in PC12 cells generating a p75-CTF that is further processed by PS1-dependent γ-secretase in endosomes. Therefore our findings now couple regulated p75 processing to internalization and endosomal trafficking and provide a novel mechanism for regulating p75-mediated neurotrophin signaling.

Neurotrophin-induced p75 processing is still a matter of debate because of apparently conflicting data in different studies (21, 30, 31, 36). The fact that all studies on this topic so far were carried out in different cell types may partly account for these discrepancies. For instance, the overexpression of Trk receptors inhibits the processing of p75 in HEK293 cells (21). On the other hand, in glial cells, 3T3 cells (overexpressing p75) and sympathetic neurons, p75 processing is directly triggered by p75 stimulation (30, 31) but with different kinetics ranging from minutes in glial cells (30) to hours in sympathetic neurons (36). Our data clearly indicate that in PC12 cells initial binding of NGF first activates the TrkA receptor and then induces p75 ectodomain shedding and an increase in p75-CTF production. Interestingly, p75-CTF is known to form complexes with TrkA (22). Moreover the high affinity NGF binding site requires only the transmembrane and intracellular domains of p75 (equivalent to p75-CTF) suggesting that TrkA-induced shedding may increase NGF binding affinity (65). This mechanism might be particularly important when neurotrophin levels are low and higher affinity binding sites for NGF-facilitated cell response. The observed cell type-specific regulation of p75 shedding by neurotrophins might be related to the cell typespecific expression and distribution of metalloproteases; for instance those belonging to the ADAM family, and to the signal transduction system by which these metalloproteases are regulated. For example, the enzymatic activity of ADAM17, a prominent p75 sheddase (35, 66, 67), is regulated through phosphorylation by ERK1/2 kinases known to be activated by TrkA (68). Thus, TrkA activation may indirectly regulate ADAM17 activity. Indeed, addition of K252a to NGF-treated PC12 cells reduced TrkA phosphorylation at Tyr490, which mediates TrkA activation of the ERK1/2 pathway (58) (Fig. 2). Furthermore, this treatment led to a decrease in p75-CTF and p75-ICD levels, demonstrating that the TrkA activation initiates a downstream signaling cascade that regulates p75 processing by phosphorylating and activating an unknown ADAM.
p75 Endosomal γ-Secretase Processing

FIGURE 4. The p75-CTF is recovered in early/recycling, but not late endosomes in PC12 cells. A, separation of early/recycling and late endosomal fractions of NGF-treated PC12 cells using velocity Ficoll gradient centrifugation. Labeling of early/recycling endosomes was achieved by incubating serum-starved PC12 cells with NGF (4 nM) for 1 h followed by incubation with transferrin-HRP (8 μg/ml) and NGF for 1 h at 37 °C. To label late endosomes, serum-starved PC12 cells were incubated with NGF for 90 min followed by a 10-min pulse of HRP (0.5 mg/ml) and a 20-min chase in the presence of NGF at 37 °C. A P2 pellet of treated PC12 cells was resuspended in SHB and loaded in a linear Ficoll gradient (2–16% over a 1 ml 16% Ficoll cushion), and separation of early/recycling and late endosomal fractions was achieved by ultracentrifugation. Fractions representing the HRP activity peak were pooled and loaded over a second gradient (4 ml of 3–16% Ficoll) and centrifuged again.

B, Western blot analysis of the distribution of p75-FL and p75-CTF in early/recycling endosomes and late endosomes. Rabbit polyclonal antibodies specific for p75, transferrin receptor (Tfr), LAMP2, PS1-CTF, and nicastrin were used to analyze fractions highly enriched in endosomes. Lower panel, Western blot analysis shows a co-distribution of transferrin-HRP with the transferrin receptor (Tfr) and the small GTPase rab11 (both marking recycling endosomes). Plasma membranes (β1-subunit of the Na⁺/K⁺ ATPase) as well as Golgi (GM130) are virtually absent from these fractions.

So far, all type I transmembrane receptors that are subject to PS1-dependent γ-secretase cleavage, require a first step of ectodomain shedding (17, 18, 70, 71). Moreover, this first cleavage event appears to be highly regulated, supporting the overall view that the generation of CTFs is likely to be the major regulatory step in the generation of soluble ICDs. An open question is whether p75 shedding occurs at the cell surface prior to internalization. There are relatively few studies addressing the subcellular localization of ADAM5 activity (72). However, inhibition of ADAM17 at the cell surface reduces p75 shedding in neurons (35), suggesting that p75-CTF is generated at the cell surface prior to internalization.

Our study provides strong evidence that γ-secretase processing of p75 occurs within early/recycling endosomes. First, and using established γ-secretase inhibitors, we demonstrated endogenous p75-ICD production from intracellular membranes, with kinetics similar to those of APP-CTF processing. Second, a synthetic APP-CTF-derived peptide competed with p75-CTF for processing. Third, we observed a strong enrichment of p75-CTF in early/recycling endosomal fractions colo-
calized with PS1 and nicastrin, two components of the γ-secretase complex. Fourth, incubation of these endosomal membranes at 37 °C revealed γ-secretase-dependent production of p75-ICD. Although our results do not preclude γ-secretase activity in the plasma membrane (73), the kinetics of p75-ICD production in PC12 is consistent with the need for shedding, internalization, and trafficking of p75-CTF prior to final intramembrane proteolysis.

The increasing importance of the endosomal system for γ-secretase processing is supported by several recent findings. γ-Secretase activity regulates the protein clearance from the endocytic recycling compartment, where transferrin and APP-CTFs accumulate after inhibition of γ-secretase activity (40). In this respect it is interesting to notice that p75 is also internalized via clathrin-coated pits into early-recycling endosomes together with transferrin, suggesting that this compartment might be a meeting point for different γ-secretase substrates (8, 74). Using a fluorescent APP-CTF reporter substrate, Kaether et al. (73) provided additional evidence that intramembrane proteolysis occurs in endosomes next to the plasma membrane. On the other hand, a small amount of Aβ peptides were found in multivesicular bodies and exosomes, suggesting that at least some activity may also reside along the late endosomal sorting route (75). This is also consistent with the immunoreactivity of PS1 and nicastrin in late endosomes found in our studies (Fig. 4). The accessibility and relative affinities of the different substrates for γ-secretase-mediated processing in the plasma membrane and different endosome compartments will determine the ultimate cellular outcome after receptor activation.

Alternatively, dual p75 proteolysis by metalloproteases and γ-secretase may also serve to regulate the half-life of the p75 receptor. This is consistent with the idea that p75 and p75-CTF (8, 74) (see also Fig. 4) are poorly targeted to the lysosomal system after ligand binding, and that the p75-ICD fragment is rapidly degraded by the proteosome (21). Hence, instead of maintaining or initiating downstream signaling, processing may equally abrogate specific signaling cascades supporting the role of γ-secretase as the proteosome of the membrane (76).

Neurons are polarized cells, and signals generated in the synaptic terminal have to reach the cell body for transcriptional regulation. Whereas retrograde signaling via Trks is well characterized (77), little is known about p75 retrograde signaling, although it is localized at the synaptic terminal and is retrogradely transported in axons (78, 79). One possibility is that p75 endosomal signaling complexes are transported along the axon after ligand stimulation (5, 8, 79). Another possibility is that processing may occur at the synaptic terminal and p75 soluble signaling complexes generated by γ-secretase may subsequently associate with dynein for retrograde transport, as reported for other soluble signaling complexes in axons (80, 81). Alternatively, p75-CTF complexes may be retrogradely transported in endosomes prior to intramembrane proteolysis. This mechanism has the advantage that it would bring proteolysis and release of a soluble signaling complex to the vicinity of the nucleus. Our data, together with recent findings (8, 12, 78, 79), suggest that trafficking of neurotrophin receptors is a key event in the regulation of neurotrophin signaling in neurons.

The overexpression of p75 and its proteolytic-derived fragments have been shown to induce apoptosis in different systems, and p75 has been proposed as a cell death mediator in neurodegeneration (6). On the other hand several neurodegenerative diseases, including Alzheimer Disease (AD), Niemann Pick type C, and Huntington (82–84), present endocytic abnormalities. Therefore endosomal dysfunction might cause an imbalance in the proteolytic processing of p75 and other γ-secretase substrates resulting in abnormal intracellular signaling and disease. In this respect, p75 is highly expressed in cholinergic neurons that are more vulnerable to degeneration in AD (85, 86). Indeed, in transgenic mice models mimicking the cholinergic deficit observed in AD, endosomal abnormalities may underlie cholinergic neurodegeneration (87).

To summarize, we report for the first time direct endocytic cleavage of an endogenously expressed substrate in intact endosomal membranes, and we reveal endosomes as the major site of γ-secretase processing of p75. Taken together, our results suggest a model in which the activation of TrkA stimulates the activity of a metalloprotease resulting in p75 shedding, internalization, and final PS1-dependent γ-secretase processing of p75 in early/recycling endosomes. These findings identify the endocytic pathway as a platform for processing of p75 and as a critical junction for regulation of neurotrophin signaling.

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