The intracellular domain of the p75 neurotrophin receptor (p75ICD) can be released by γ-secretase in response to the previous activation of α-secretase by phorbol esters. However, ligand-dependent release of p75ICD has yet to be described. We show here that nerve growth factor can induce the release of p75ICD and facilitate its translocation to the nucleus in a γ-secretase-dependent manner. This effect was observed in RN22 schwannoma cells cultured under serum-free conditions, as well as in Schwann cells, and it was mimicked by other neurotrophins, such as brain-derived neurotrophic factor or neurotrophin-3. Unlike other known examples of regulated intramembrane proteolysis, ligand-dependent release of p75ICD did not need the previous activation of α-secretase. These results suggest that nuclear translocation of p75ICD may represent a novel neurotrophin-mediated signaling pathway.

Key words: α-secretase; γ-secretase; p75NTR; neurotrophin; nuclear translocation; regulated intramembrane proteolysis

Introduction

The neurotrophin family is composed of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4/5. These molecules act as ligands for the Trk neurotrophic receptor tyrosine kinases, as well as for the common p75 neurotrophic receptor (p75NTR), a member of the tumor necrosis factor (TNF) receptor family (López-Sánchez and Frade, 2002). In recent years, a number of signaling pathways have been described that mediate the response of ligand binding to p75NTR (López-Sánchez and Frade, 2002). More recently, the activation of α-secretase by phorbol esters was shown to provoke the release of the intracellular domain of p75NTR (p75ICD) in a number of cell lines, including RN22 schwannoma cells (RN22-SCs) (Jung et al., 2003; Kanning et al., 2003). However, evidence for ligand-mediated intramembrane cleavage of p75NTR has yet to be presented. In this article, we show that endogenously expressed p75ICD can be released and translocated to the nucleus in response to both NGF and other neurotrophins. Moreover, it seems that the release of p75ICD is independent of metalloproteinase-dependent shedding of the p75NTR ectodomain.

Materials and Methods

Materials. All of the chemical reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. The rabbit polyclonal antisera against p75ICD and against the extracellular domain of p75NTR (9651; diluted 1:800 for immunocytochemistry; diluted 1:2000 for Western blots) and the affinity-purified antiserum against the C terminus of SC-1 (used at 4 μg/ml) were kindly provided by Moses Chao (New York University, New York, NY). The anti-human p75ICD polyclonal antiserum (diluted 1:500; Promega, Madison, WI) also revealed nuclear staining in response to NGF (data not shown). The anti-neurotrophin receptor homolog 2 (NRH2) antiserum (kindly provided by Phil Barker, McGill University, Montreal, Québec, Canada) was diluted 1:800 (immunocytochemistry), diluted 1:5000 (immunodepletion), or diluted 1:10,000 (Western blotting). The amyloid precursor protein (APP)-specific monoclonal antibody 22C11 (Chemicon, Hayward, CA) was used at 5 μg/ml. The γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was used at 1 μM, the metalloproteinase inhibitor TNF-α protease inhibitor-2 (TAPI-2) was used at 20 μM, and the metalloproteinase inhibitor GM 6001 was used at 10 μM (all purchased from Calbiochem, La Jolla, CA). PMA was used at 100 ng/ml. Human recombinant NGF-β, BDNF, and NT3 were stored at 10 ng/μl in PBS/0.5% bovine serum albumin and were used at 100 ng/ml unless otherwise stated. TrkB receptor bodies (human TrkB/Fc chimera; R & D Systems, Abingdon, UK) were used at 1 μg/ml.

Reverse transcription-PCR. Amplification of cDNAs derived from RN22-SCs, postnatal day 2 (P2) rat brain, or P2 rat cortex was performed using standard procedures. The PCR primers used were as follows: GAPDH (base pairs 502–521, 1101–1120; GenBank accession number X02231), TrkB (base pairs 852–871, 1569–1588; GenBank accession number M55291), TrkC (base pairs 266–285, 766–785; GenBank accession number L03813), NGF (base pairs 655–674, 973–992; GenBank accession number M36589), BDNF (base pairs 564–583, 745–764; GenBank accession number M61178), and NT3 (base pairs 773–792, 1032–1051; GenBank accession number NM_031073). TrkB/TrkC primers are specific for both full-length and truncated forms.

Cell culture. RN22-SCs were maintained at 37°C in DMEM/10% FCS (Invitrogen, Carlsbad, CA). For most experiments, RN22-SCs (10,000 cells/cm²) were initially cultured for 24 h in DMEM, whereas RN22-SCs expressing human APP were cultured in DMEM/10% FCS. Schwann cells from P2 mouse sciatic nerves were isolated as described by Chan et al. (2000), and they were cultured containing 2 μM forskolin and bovine pituitary extract (BPE) diluted...
using the Student’s t test.

**Immunochemistry.** Cells were fixed with 4% paraformaldehyde (15 min). Immunostaining was performed following a protocol described previously (López-Sánchez et al., 2005). Nuclei were counterstained with 1 µg/ml propidium iodide or bisbenzimide.

**Cell fractionation.** All of the steps were performed at 4°C. Cells were lysed in cytoplasmic buffer (CB) (10 mM HEPES, pH 7.6, 60 mM KCl, 1 mM EDTA, 0.075% Triton X-100, 1 mM ethylenediaminetetraacetic acid [EDTA], 0.2 mM dithiothreitol, and 1× protease inhibitor mixture) and homogenized by passing them through a 22 ga needle. The nuclei were spun down at 325 × g for 4 min, and the supernatant was isolated (cytoplasmic fraction). The pellets were washed with CB without detergent and extracted in nuclear buffer (20 mM Tris-HCl, pH 8.0, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM L-azidophenylmethyl sulfonfyl fluoride, 10% glycerol, and 1× protease inhibitor mixture). NRH2 immunodepletion of nuclear fractions was performed in 50 mM Tris-HCl, pH 8.0, 168 mM NaCl, 0.6 mM MgCl2, 0.08 mM EDTA, 10% glycerol, and 0.1% Triton X-100. Removal of anti-NRH2 was performed with protein G-agarose beads diluted 1:25 (Santa Cruz Biotechnology, Santa Cruz, CA). Conditioned media (7.5 ml) were concentrated using Centricon YM-30 (Millipore, Bedford, MA). Total extracts were obtained by cell solubilization in Laemmli’s buffer.

Western blot. Total extracts (equivalent to 200,000 cells), nuclear and cytoplasmic extracts (15 µg of protein), or concentrated conditioned media (equivalent to 330 µl of original culture media) were separated by SDS-PAGE on 11% acrylamide gels under nonreducing conditions and transferred to Immuno-Blot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). APP-conditioned media (20 µl) were separated on 8% acrylamide gels. Membranes were blocked in 5% nonfat dried milk in PBS with 0.1% Tween 20 (PB Tw) for 1 h and incubated overnight at 4°C with the antiserum in blocking buffer. After being washed five times in PB Tw, the membranes were incubated with goat anti-rabbit HRP-conjugated antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:50,000 in blocking buffer (1 h) and washed as above. Protein bands were visualized using ECL (Amersham Biosciences, Piscataway, NJ). The films were scanned, and the relative optical density (ROD) of the bands was determined using the Analytical Imaging Station (AIS) 4.0 (Imaging Research, St. Catharines, Ontario, Canada). ROD values were normalized to the value of the protein bands from the control cultures.

**Cell counting.** Cells were examined by confocal microscopy to define the subcellular localization of the immunostaining using a Leica (Nussloch, Germany) TCF-4D confocal setup. Cell counting was performed with a Nikon (Melville, NY) E80i microscope with phase contrast and epifluorescence illumination. In other experiments, randomly selected fields containing cells counterstained with bisbenzimide were digitalized using a DXM1200F camera (Nikon), and the ROD for p75ICD immunostaining was measured with AIS 4.0. The average ROD value of the cytoplasm defined the threshold that was used under all of the experimental conditions to identify those cells with stronger nuclear labeling for p75ICD. This approach yielded results similar to those obtained by direct visual counting (data not shown). On average, 500 cells were analyzed per coverslip.

**Statistical analysis.** Quantitative data are shown as the mean ± SEM from n independent experiments. Statistical differences were analyzed using the Student’s t test.

**Results**

NGF induces nuclear translocation of p75ICD as revealed by immunocytochemistry. A–H, RN22-SCs cultured in the presence (A) or absence (B–H) of FCS were treated for 1 h with vehicle (B, F), NGF (C, G), DAPT, or both (D, H), and then were immunostained for p75ICD (A–D) or p75ECD (E–H). B, Weak nuclear p75ICD immunoreactivity was observed in most cells after removal of FCS. However, exposure to NGF increased the proportion of nuclei containing strong nuclear p75ICD staining (C, E), an effect that was inhibited by DAPT (D, F). C, Inset, Propidium iodide-labeled nucleus (red) with strong p75ICD immunoreactivity (green). F–H, p75ECD was never observed in the cell nucleus. J–L, Schwann cells cultured in the presence of FCS, forskolin, and BPE (J–L) were treated for 2 h with vehicle (J, NGF (K), DAPT, or both (L). The proportion of nuclei containing high levels of p75ICD increased in the presence of NGF (K, L), whereas exposure to DAPT prevented this effect of NGF (L, *p < 0.001. Scale bar: A–D, F–H, 14 µm; J–L, 10 µm. Cont, Control. Error bars represent SEM.

The SC line RN22 lacks the NGF-specific neurotrophic receptor TrkA but contains high levels of p75NTR (Gentry et al., 2000). The cellular distribution of p75ICD was determined in these cells by immunocytochemistry, and laser-scanning confocal microscopy confirmed that, in the presence of FCS, p75ICD did not accumulate in the nucleus (Fig. 1A). However, in the absence of serum, the intensity of p75ICD labeling in the majority of nuclei was equivalent to or less than that in the cytoplasm (Fig. 1B). In the presence of NGF, a considerable accumulation of p75ICD occurred in ~70% of nuclei, in contrast to control cells, in which only ~20% of the nuclei were strongly labeled for p75ICD (Fig. 1C,E). The increase in the proportion of nuclei strongly labeled for p75ICD was observed 10 min after the addition of NGF (Fig. 2A) and lasted for at least 24 h (data not shown). The half-maximal effect of NGF was attained after 2–3 min. The proportion of nuclei strongly labeled for p75ICD increased in a concentration-dependent manner, with ~5–10 ng/ml NGF producing a half-maximal effect (Fig. 2B). Exposure to DAPT prevented the increase in the proportion of nuclei that accumulated high levels of p75ICD in response to NGF (Fig. 1D, E). Hence, the ligand-dependent release of p75ICD seems to require γ-secretase activity. As expected, p75ICD immunolabeling was absent from the nucleus, regardless of the presence or absence of NGF or DAPT (Fig. 1F–H).

To confirm that the p75ICD does indeed accumulate in the nucleus in response to NGF, nuclear and cytoplasmic extracts...
were subjected to Western blotting with a p75ICD-specific antiserum. A 25 kDa band, characterized previously as p75ICD (Jung et al., 2003; Kanning et al., 2003), was enriched in nuclear extracts from RN22-SCs exposed to NGF for 2 h (n = 2) (Fig. 3A). In the presence of the γ-secretase inhibitor DAPT, the levels of this protein diminished to those of control cells. Additional protein bands of 28, 33, and 37 kDa were observed in these extracts, but they remained at a similar intensity under all of the conditions (Fig. 3A). These bands are likely to represent NRH2 moieties that cross-react with the anti-p75ICD antisem because they were also detected with the NRH2-specific antisem (n = 2) (Fig. 3A). Immunodepletion with this anti-NRH2 antisem allowed us to unambiguously identify the 25 kDa band corresponding to p75ICD in the nuclear extracts from RN22-SCs (Fig. 3A). Densitometric analysis demonstrated that this band was significantly increased in the NGF-treated cultures, an increase that was prevented by DAPT [control, 1; NGF, 9.63 ± 1.88 (p < 0.01); DAPT, 0.74 ± 0.25; DAPT/NGF, 1.57 ± 0.10; n = 3].

In the cytoplasmic fractions, the p75ICD-specific antisem only detected the full-length p75NTR protein (Fig. 3A). Interestingly, the intensity of the full-length protein was reduced in the fraction obtained from the NGF-treated cells, in accordance with the cleavage and nuclear translocation of p75ICD, and this decrease was blocked in the presence of DAPT [control, 1; NGF, 0.45 ± 0.01 (p < 0.001); DAPT, 1.24 ± 0.03; DAPT/NGF, 1.14 ± 0.02; n = 2]. No higher-mobility bands corresponding to the p75NTR C-terminal domain were observed in the cytoplasmic fraction, even in the presence of DAPT. Thus, the release of p75ICD in response to NGF does not seem to require the previous shedding of the ectodomain attributable to α-secretase activity. Alternatively, NGF might induce α-secretase cleavage and the release of the p75NTR extracellular domain, but immunoblotting may not be sufficiently sensitive to detect the C-terminal fragment in the presence of DAPT.

To discriminate between these two alternatives, total cell extracts were subjected to Western blotting with the p75ICD-specific antisem (Fig. 3B). This analysis confirmed that NGF induces the accumulation of a faint 25 kDa band, and this effect is blocked by DAPT. No high-mobility bands specific for the p75NTR C-terminal domain were observed in extracts from cells treated with NGF and DAPT, thus indicating that the release of p75ICD does not seem to require two cleavage steps.

To study further whether the activation of α-secretase is necessary for the release of p75ICD provoked by NGF, culture media conditioned by RN22-SCs were analyzed in Western blots using the anti-p75ICD antisem (Fig. 3C). A protein of 55 kDa was identified in medium conditioned by NGF-treated cells but not in medium from control cells. This protein was slightly larger than the 50 kDa p75NTR ectodomain released by α-secretase in response to PMA in the presence of serum (n = 2) (Weskamp et al., 2004) (Fig. 3C) and could represent the complete p75NTR ectodomain cleaved by γ-secretase. In agreement with this idea, the intensity of this band in the medium conditioned by cells treated with NGF was dramatically reduced by the presence of DAPT [NGF, 1; DAPT/NGF, 0.08 ± 0.06 (p < 0.05); n = 3] (Fig. 3C). These data strongly suggest that the interaction of NGF with p75NTR results in γ-secretase cleavage of p75NTR without the previous activation of α-secretase. Moreover, it seems that matrix metalloproteinases are not involved in the γ-secretase cleavage of p75NTR. In the presence of the TAPI-2, an inhibitor of matrix metalloproteinases that prevents PMA-dependent shedding of p75NTR (Jung et al., 2003; Kanning et al., 2003; Weskamp et al., 2004), the proportion of RN22-SC nuclei strongly labeled for p75ICD when visualized by immunocytochemistry still increased in response to NGF [control, 22.18 ± 6.09%; NGF, 69.42 ± 1.57% (p < 0.005); TAPI-2, 24.49 ± 3.09%; TAPI-2/NGF, 70.48 ± 1.96% (p < 0.001); n = 3]. Likewise, the release of the p75NTR ectodomain in response to NGF was not affected by the presence of TAPI-2 (NGF, 1; TAPI-2/NGF, 0.88 ± 0.12; n = 2; nonsignificant) (Fig. 3D). The activity of TAPI-2 under these conditions was confirmed by its ability to prevent APP shedding from RN22-SCs expressing human APP [control, 1; TAPI-2, 0.18 ± 0.03 (p < 0.005); n = 2] (Fig. 3D). The metalloproteinase inhibitor GM 6001 also failed to prevent the accumulation of p75ICD in the nuclei of the RN22-SCs in response to NGF [control, 28.90 ± 8.80%; NGF, 73.21 ± 6.71% (p < 0.005); GM 6001, 20.19 ± 5.16%; GM 6001/NGF, 85.24 ± 0.69% (p < 0.001); n = 4].

To determine whether p75ICD can also accumulate in the cytoplasm in response to NGF, the cytoplasmic fractions of RN22-SCs cultured with different combinations of NGF and DAPT were analyzed by Western blot using the p75ECD-specific antisem. These cytoplasmic fractions did not contain the 55 kDa p75ICD but rather the full-length p75NTR, which was less intense in the extracts obtained from NGF-treated cells than in control cells [control, 1; NGF, 0.34 ± 0.03 (p < 0.005); DAPT, 0.97 ± 0.15; DAPT/NGF, 1.17 ± 0.05; n = 2] (Fig. 3C). A 28 kDa protein band was also observed after all of the treatments, which may be attributable to nonspecific binding, or it may correspond to a degradation product of p75ICD. This band might account for the strong staining for p75ICD observed in the cytoplasm of NGF-treated cells (Fig. 1G).

NGF induces nuclear translocation of p75ICD in Schwann cells

We determined whether the nuclear accumulation of p75ICD in response to NGF might occur in nontransformed cells. In Schwann cells derived from P2 mice grown in DMEM supplemented with FCS, p75ICD did not accumulate in the nuclei (data not shown). However, in the presence of forskolin and BPE, ~5% of the nuclei contained p75ICD (Fig. 1I). Moreover, in the presence of NGF, the proportion of p75ICD-immunoreactive nuclei increased to ~25% (Fig. 1J, K). The effect of NGF on the nuclear accumulation of p75ICD was inhibited by the addition of DAPT (Fig. 1I, L). Hence, the activation of p75NTR by NGF seems to provoke regulated intramembrane proteolysis (RIP) and the nuclear translocation of p75ICD in primary cell cultures.

BDNF and NT3 also induce nuclear translocation of p75ICD

The possibility that other neurotrophins could induce nuclear accumulation of p75ICD was also evaluated in RN22-SCs. Be-
cause these cells do not express either TrkB or TrkC (Fig. 4F), any interference of these BDNF- and NT3-specific tyrosine kinase receptors with the signaling of p75NTR was avoided. Both BDNF and NT3 increased the proportion of cells showing strong nuclear p75ICD immunoreactivity in a manner similar to NGF (Fig. 4A–D). These data indicate that all of the neurotrophins have a similar capacity to induce p75ICD release and nuclear translocation.

We tested whether RN22-SCs cultured in the absence of serum express BDNF, NGF, or NT3. BDNF expression could be detected by reverse transcription (RT)-PCR in cDNAs derived from these cells (Fig. 4E). This suggests that the presence of endogenous BDNF in the culture medium during the 24 h period before the treatment with NGF could be responsible for the 20% of nuclei strongly stained with the anti-p75ICD antiserum under control conditions (Fig. 1E). Indeed, the addition of DAPT at the time of serum withdrawal strongly reduced the background levels of cells with strong nuclear p75ICD immunoreactivity [1.17 ± 0.19%, (p < 0.001); n = 4]. Blockage of endogenous BDNF with the addition of TrkB receptor bodies at the 4th day of culture was also reversed by DAPT.

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Discussion

In this study, we present evidence that the activation of p75NTR with NGF promoted the release of p75ICD via the activity of γ-secretase. We have demonstrated the increased immunoreactivity of p75ICD in the nuclei of RN22-SCs or Schwann cells in the presence of NGF was impaired by the γ-secretase inhibitor DAPT. Furthermore, the detection of a p75ICD-specific protein band in the nucleus and total cell extracts from RN22-SCs treated with NGF was also inhibited by DAPT. Finally, Western blot analysis indicated that the levels of full-length p75ICD were reduced in the cytoplasmic and total cell extracts of RN22-SCs treated with NGF, suggesting that p75ICD is released after neurotrophin binding. This effect was also reversed by DAPT.

p75ICD accumulation in the nucleus was dependent on the concentration of NGF and was completed in <10 min. This rapid activation of γ-secretase has also been seen in other RIP paradigms (Ray et al., 1999). Furthermore, the rapid response of p75NTR is not unique because the association of TRAF6 (TNF receptor-associated factor 6) with p75NTR occurs 1 min after NGF treatment and also reaches a maximum after 10 min (Kurpisz et al., 1999). In contrast, internalization of p75NTR in response to neurotrophins is much slower, taking ~45 min (Bronfman et al., 2003). This suggests that the release of p75NTR does not need a previous internalization step and that it can take place directly at the cell surface. It is unclear why neurotrophin-dependent RIP of p75NTR was only observed when RN22-SCs were cultured in serum-free medium. Schwann cells grown in the presence of serum, forskolin, and BPE showed a similar, although less pronounced, effect in response to NGF. It is possible that protein kinase A (PKA), an indirect target of forskolin, might sensitize Schwann cells to undergo RIP in response to NGF. Indeed, PKA has been shown to be necessary for TNF receptor-1 shedding (Douddevani et al., 1996).

The capacity of p75NTR to translocate its intracellular domain to the nucleus in response to ligand activation resembles the pathway used by Notch or ErbB-4 to transduce intracellular signals (Ebinu and Yankner, 2002). Unlike these receptors, p75NTR seems to undergo ligand-dependent RIP in the absence of γ-secretase-dependent ectodomain shedding. This conclusion was based first on the failure to detect the C-terminal p75NTR fragment derived from ectodomain shedding of the receptor in the cytoplasmic and total cell extracts of NGF-treated RN22-SCs cultured in the presence of DAPT. Moreover, the p75ICD released in response to NGF as a 55 kDa fragment is larger than the p75NTR ectodomain released by γ-secretase (Weskamp et al., 1999). These results indicate that all of the neurotrophins have a similar capacity to induce p75ICD release and nuclear translocation.
the absence of serum (data not shown), consistent with the possibility that SC1 stabilizes p75ICD in the nucleus of these cells.

The analysis of the nuclear fraction by Western blotting with the anti-p75ICD-specific antiserum identified the presence of 28, 33, and 37 kDa bands, whose intensity was not affected by the experimental manipulations. These bands were probably the result of a cross-reaction with NRH2 because they were detected by a well characterized anti-NRH2 antiserum (Paul et al., 2004). Moreover, they were absent in the NRH2-immunodepleted nuclear fractions analyzed with the anti-p75ICD antiserum. For unknown reasons, the NRH2-specific bands were not detected by the p75ICD antiserum in total cell extracts, as described previously by Murray et al. (2004).

References


