THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 25, Issue of June 22, pp. 23097–23104, 2001

Printed in U.S.A.

The p75 Neurotrophin Receptor Activates Akt (Protein Kinase B) through a Phosphatidylinositol 3-Kinase-dependent Pathway*

Received for publication, December 20, 2000, and in revised form, April 3, 2001
Published, JBC Papers in Press, April 18, 2001, DOI 10.1074/jbc.M011520200

Philippe P. Roux‡, Asha L. Bhakar§, Timothy E. Kennedy¶, and Philip A. Barker‖

From the Centre for Neuronal Survival, Montreal Neurological Institute, and the Department of Neurology and Neurosurgery, McGill University, Montréal, Québec H3A 2B4, Canada

The Akt kinase plays a crucial role in supporting Trk-dependent cell survival, whereas the p75 neurotrophin receptor (p75NTR) facilitates cellular apoptosis. The precise mechanism that p75NTR uses to promote cell death is not certain, but one possibility is that p75NTR-dependent ceramide accumulation inhibits phosphatidylinositol 3-kinase-mediated Akt activation. To test this hypothesis, we developed a system for examining p75NTR-dependent apoptosis and determined the effect of p75NTR on Akt activation. Surprisingly, p75NTR increased, rather than decreased, Akt phosphorylation in a variety of cell types, including human Niemann-Pick fibroblasts, which lack acidic sphingomyelinase activity. The p75NTR expression level required to elicit Akt phosphorylation was much lower than that required to activate the JNK pathway or to mediate apoptosis. We show that p75NTR-dependent Akt phosphorylation was independent of TrkA signaling, required active phosphatidylinositol 3-kinase, and was associated with increased tyrosine phosphorylation of p85 and Shc and with reduced cytosolic tyrosine phosphatase activity. Finally, we show that p75NTR expression increased survival in cells exposed to staurosporine or subjected to serum withdrawal. These findings indicate that p75NTR facilitates cell survival through novel signaling cascades that result in Akt activation.

The neurotrophins are a family of growth factors involved in the survival, development, and death of specific populations of neurons and non-neuronal cells. Nerve growth factor (NGF), the prototypic neurotrophin, is the best characterized member of this family, which in mammals, also includes brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5 (1). The signal transduction systems that mediate the diverse biological functions of the neurotrophins are initiated by two categories of cell-surface receptors: the Trk receptors and the p75 neurotrophin receptor (p75NTR).

One of the main survival pathways for neuronal cell survival is mediated by phosphatidylinositol 3-kinase (PI3K) and involves activation of the Akt serine/threonine kinase (2). Increased phosphatidylinositol 3,4,5-trisphosphate (PIP_3) production results primarily from relocalization of PI3K from the cytosol to a juxtamembrane location that provides access to PIP substrates. This redistribution of PI3K requires the association of the SH2 domain within the p85 regulatory subunit of PI3K with phosphorylated tyrosines present on activated cell-surface receptors or on receptor-associated adaptor proteins (reviewed in Ref. 3). Accumulation of PIP_3 and its phospholipid phosphatase product, phosphatidylinositol 3,4-bisphosphate, in the plasma membrane creates docking sites for the pleckstrin homology domains of phosphoinositide-dependent kinase-1 and Akt. Phosphorylation of Akt on threonine 308 by phosphoinositide-dependent kinase-1 followed by autophosphorylation on serine 473 activates Akt (4, 5) and allows the enzyme to facilitate survival by phosphorylation of downstream substrates that may include Bad, Caspase-9, Forkhead family members, IκB kinase, and glycogen synthase kinase-3 (6–12).

p75NTR binds all neurotrophins with similar affinity and is a member of the tumor necrosis factor receptor (TNFR) superfamily (13, 14). Current data suggest that the main physiological functions of p75NTR are to regulate Trk receptor activation and signaling (15–19) and to activate Trk-independent signal transduction cascades involving sphingomyelinase (20–22), nuclear factor-κB (23–25), and JNK (26–28). Several findings indicate that NGF binding to p75NTR can initiate a cell death cascade in some cell types. For example, NGF treatment of embryonic retinal cells or postnatal oligodendrocytes that express p75NTR, but not TrkA, increases cellular apoptosis (29–31). The precise signaling pathway(s) used by p75NTR to activate cell autonomous death cascades remain unclear, but may involve activation of caspase-1, -2, and -3 (32) as well as cyclin-dependent kinases (33). A number of cytosolic proteins that interact directly with the p75NTR intracellular domain have been identified, including TRAF proteins (25, 34), caveolin (35), SC-1 (36), NRIF (37), FAP-1 (38), NADE (39), RhoA (40), and NRAGE (41), but linking each of these to precise p75NTR signaling cascades remains a major challenge.

Activation of cell death cascades can result from suppression of signaling pathways that normally support survival. In some systems, sphingomyelinase activation results in a ceramide-dependent decrease in the generation of PIP_3 and a subsequent reduction in Akt activity (42, 43); and in others, ceramide

* This work was supported in part by operating grants from the Canadian Institutes of Health Research (to P. A. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by the Neuroscience Foundation of Canada and by a Canadian Institutes of Health Research studentship.

§ Supported by a Canadian Institutes of Health Research studentship.

¶ Canadian Institutes of Health Research Scholar.

‖ Killam Foundation Scholar and Canadian Institutes of Health Research Scholar. To whom correspondence should be addressed: Center for Neuronal Survival, Montreal Neurological Inst., McGill University, 3801 University Ave., Montréal, Québec H3A 2B4, Canada. Tel.: 514-398-3064; Fax: 514-398-1319; E-mail: mdpb@musica.mcgill.ca.

[1] The abbreviations used are: NGF, nerve growth factor; p75NTR, p75 neurotrophin receptor; PI3K, phosphatidylinositol 3-kinase; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; TNFR, tumor necrosis factor receptor; JNK, c-Jun N-terminal kinase; PTPase, protein-tyrosine phosphatase; BCS, bovine calf serum; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; m.o.i., multiplicity of infection; CREB, cAMP-responsive element-binding protein; MKK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; FAP-1, Fas-associated phosphatase-1; TRAF, tumor necrosis factor receptor-associated factor.

reduces Akt activity through specific dephosphorylation of serine 473 (44). Since p75NTR activates sphingomyelinase in a neurotrophin-dependent manner, we have determined if p75NTR activation can suppress Akt and thereby facilitate apoptosis. Our results show that p75NTR does indeed regulate Akt; but contrary to our expectations, we found that p75NTR increases Akt activation through a Trk-independent pathway that requires PI3K and show that p75NTR expression suppresses apoptosis. Although high levels of p75NTR will mediate cell death, the p75NTR expression level required to elicit Akt phosphorylation is much lower than that required to activate the JNK pathway or to mediate apoptosis. The effect of p75NTR on Akt correlates with increased tyrosine phosphorylation of the p85 regulatory subunit of PI3K and of Shc adaptor proteins, suggesting that PTPase inhibition may play a role in this effect. Consistent with this, p75NTR expression results in reduced cytosolic tyrosine phosphatase activity. These data indicate that a physiological role of p75NTR is to enhance cell survival through an Akt-dependent pathway.

EXPERIMENTAL PROCEDURES

Materials—NGF was purchased from Collaborative Research (Bedford, MA); cell culture reagents were from BioWhittaker, Inc. (Walkersville, MD); and all other reagents were from Sigma, ICN Biochemicals (Costa Mesa, CA), or Calbiochem unless otherwise indicated.

Preparation of Recombinant Adenoviruses—pAd-CMV5-F1 containing full-length rat p75NTR (45), the p75NTR intracellular domain (46), or the p75NTR intracellular domain modified to contain an N-terminal myristoylation tag derived from Hck, an Src-related kinase (47), was cotransfected with replication-defective adenoviral DNA (Quantum Biotechnologies, Laval, Québec, Canada) into 293A cells. Crude viruses derived from viral plaques were used to infect 293A cells. p75NTR expression was confirmed by immunoblot analysis, and positive plaques were repurified twice by limiting dilution. Recombinant adenoviruses were amplified in 293A cells, purified on sucrose gradients, and titered by plaque assay in 293A cells. Control recombinant adenoviruses expressing β-galactosidase (LacZ) or green fluorescent protein were generated using the same viral backbone and purification techniques as for the p75NTR viruses.

Cell Culture—The rat pheochromocytoma cell lines PC12 and PC12nnr5 were maintained in 7.5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium with 5% bovine calf serum (BCS), 5% horse serum, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin. Normal and Niemann-Pick human fibroblasts (obtained from the NIGMS/Human Genetic Mutant Cell Repository, Camden, NJ), MG87-3T3, HeLa, COS-7, and A875 cells were maintained in Dulbecco's modified Eagle's medium containing 10% BCS. Doxycycline-inducible p75NTR-expressing MG87-3T3 fibroblasts (TIMp75-3) were produced and maintained as described previously (24).

Immunoblotting—Immunoblotting for total and phosphorylated proteins was performed using rabbit polyclonal antibodies from New England Biolabs, Inc. (Beverly, MA) or from Upstate Biotechnology, Inc. (Lake Placid, NY). p75NTR immunoreactivity was detected using p75NTR-B1, a rabbit polyclonal antibody directed against a glutathione *S*-transferase fusion protein containing amino acids 276–425 of the intracellular domain of rat p75NTR (Babco, Berkeley, CA) (46, 48). Protein content from cell lysates was normalized using the BCA assay (Pierce), and 10–25 μg of protein was solubilized in Laemmli sample buffer (49), separated by SDS-polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose. Blocking and secondary antibody incubations of immunoblots were performed in Tris-buffered saline/Tween (10 mM Tris (pH 7.4), 150 mM NaCl, and 0.2% Tween 20) supplemented with 5% (w/v) dried skim milk powder. Primary antibody incubations were performed in Tris-buffered saline/Tween supplemented with 5% bovine serum albumin. For 4G10, 2% (w/v) bovine serum albumin was used for the blocking step. Horseradish peroxidase-conjugated donkey anti-rabbit IgG, horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), or horseradish peroxidase-conjugated protein A was used at a dilution of 1:5000. Immunoreactive bands were detected using enhanced chemiluminescence (PerkinElmer Life Sciences) according to the manufacturer's instructions.

Transfection and Subcellular Fractionation—COS-7 cells were transfected with a control plasmid or with plasmid encoding the p75NTR intracellular domain or the p75NTR intracellular domain modified to contain a myristoylation tag. Forty-eight hours after transfection, cells were scraped from plates in cold phosphate-buffered saline (PBS), centrifuged for 5 min at 2000 × *g*, and then resuspended in 15 ml of HES buffer (20 mM HEPES (pH 7.4), 1 mM EDTA, 255 mM sucrose, 10 μg/ml leupeptin, 25 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Cells were homogenized in a glass-on-Teflon homogenizer with 10 strokes at 1200 rpm and then triturated twice using a 25-gauge needle. An aliquot was set aside as the initial lysate. The lysate was centrifuged at 19,000 × *g* for 20 min; the resulting pellet was designated membrane, and the supernatant was designated cytosol. All fractions were resuspended in HES buffer containing 1% Nonidet P-40 and analyzed for protein concentration, and equivalent amounts were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Survival Assays—Analysis of cell survival was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which was added at a final concentration of 1 mg/ml for 4 h following a 48-h infection. The reaction was ended by the addition of 1 volume of solubilization buffer (20% SDS, 10% dimethylformamide, and 20% acetic acid). After overnight solubilization, specific and nonspecific absorbances were read at 570 and 630 nm, respectively. Each condition was tested six times, and results were analyzed for statistical significance by multiple analysis of variance.

Immunoprecipitation—Twenty-four hours after infection, cells were washed in cold Tris-buffered saline and lysed in Nonidet P-40 lysis buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM sodium orthovanadate). Immunoprecipitation was performed at 4 °C using polyclonal anti-pan-Trk 203 antibody (gift of David Kaplan, Montreal Neurological Institute, Montreal, Canada), polyclonal anti-Shc antibody (gift of Jane McGlade, University of Toronto, Toronto, Ontario, Canada), or polyclonal anti-p85 antibody (Upstate Biotechnology, Inc.). Complexes were precipitated using 45 μl of protein A-Sepharose (Amersham Pharmacia Biotech), which was added for 90 min at 4 °C and then subjected to multiple washes. For wheat germ agglutinin (Amersham Pharmacia Biotech) precipitation, beads were added to Nonidet P-40-extracted protein samples for 2 h at 4 °C, followed by centrifugation and multiple washes. Samples were lysed in Laemmli sample buffer and analyzed by immunoblotting as described above.

Apoptotic Assays—Apoptotic cell death was quantified using annexin V binding and FACS analyses. Briefly, cells were harvested using PBS with 2 mM EDTA and washed twice in PBS supplemented with 2% BCS. After the last wash, the cells were resuspended in 0.1 ml of PBS containing 1 μg/ml fluorescein isothiocyanate-conjugated annexin V (Becton Dickinson, Mountain View, CA) and incubated for 15 min in the dark at room temperature. PBS (0.3 ml) was added to each tube, and cells were analyzed on a FACScan flow cytometer (Becton Dickinson-Pharmingen). For FACS analysis of cells with sub-G_1 DNA content, cells were harvested and resuspended in 50% ethanol and PBS and left on ice for 15 min. An underlayer of 1 volume of cold BCS was added, and cells were spun at 250 × *g* for 5 min. Cells in the resulting pellet were resuspended in blocking buffer consisting of PBS containing 2% bovine serum albumin and 2% BCS and then incubated for 30 min on ice. Antibody p75NTR-B1 was added at a dilution of 1:500, and the incubation was continued for an additional 30 min. Cells were washed three times in blocking buffer and then incubated for 30 min in blocking buffer supplemented with a 1:500 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.). Cells were washed three times and resuspended in 0.1 ml of PBS with 0.1 mg/ml RNase A for 15 min at room temperature. PBS (0.3 ml) with 25 μg/ml propidium iodide was added to the cells, incubated for 15 min, and then analyzed on a FACScan. Each condition was tested in triplicate, and results were analyzed for statistical significance by multiple analysis of variance.

Protein-tyrosine Phosphatase Assays—Twenty-four hours after infection, PC12nnr5 cells were washed twice in cold HEPES-buffered saline to remove free phosphate and then harvested in 1 ml of suspension buffer (50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 0.25 M sucrose, 1 mM dithiothreitol, 10 μg/ml leupeptin, 25 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride), sonicated, and spun at 1000 × *g* for 5 min to remove intact mitochondria and nuclei. Cytosolic and membrane fractions were separated by spinning at 100,000 × *g* for 60 min at 4 °C. The membrane pellet was resuspended for 30 min in 1 ml of suspension buffer supplemented with 1% Triton X-100 and then spun at 1000 × *g* for 5 min to remove insoluble contaminants. Endogenous phosphate was removed from membrane and cytosolic fractions by buffer exchange

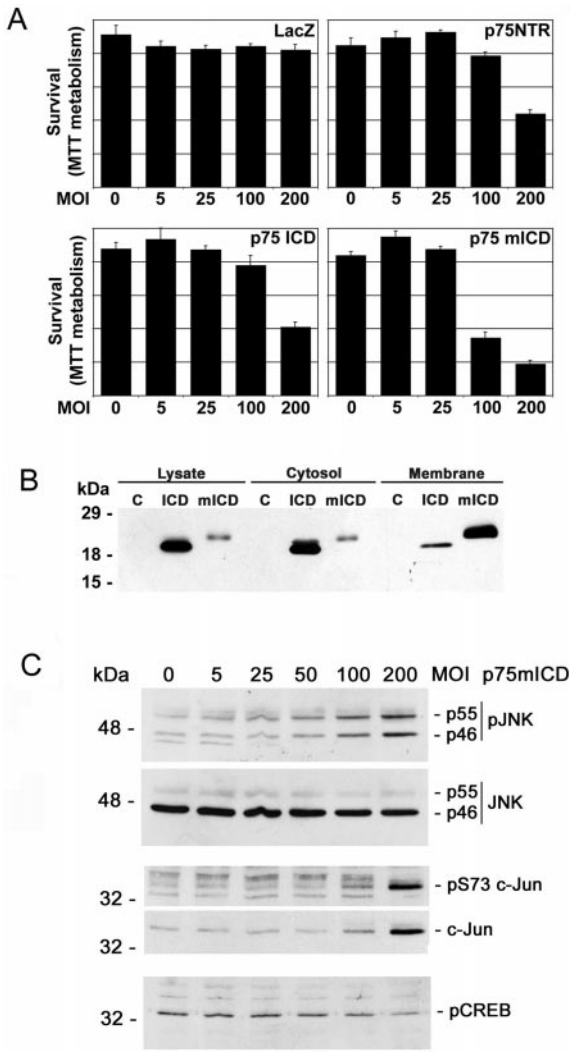

FIG. 1. **Overexpression of p75NTR in PC12nnr5 cells induces cell death.** *A*, PC12nnr5 cells were infected with recombinant adenoviruses expressing LacZ, full-length p75NTR, the intracellular domain of p75NTR (p75ICD), or the myristoylated p75NTR intracellular domain (p75mICD) at increasing multiplicities of infection and, after 48 h, were assayed for survival using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (*MTT*) assay as described under "Experimental Procedures." *B*, p75mICD is enriched in cell membranes, but p75ICD is cytosolic. COS-7 cells were transiently transfected with plasmids encoding either p75ICD (*ICD*) or p75mICD (*mICD*) and, after 48 h, fractionated as described under "Experimental Procedures." *C* indicates control. *C*, PC12nnr5 cells that were infected and incubated under the same conditions as described for *A* were lysed and analyzed for c-Jun (phospho-Ser^{73} (*pS73*)), JNK (phospho-Thr^{183}/Tyr^{185}), and CREB (phospho-Ser^{133}) phosphorylation by immunoblotting. Results shown in *A* are represented as the means ± S.E. of three independent experiments, and the experiment in *C* was repeated twice with similar results.

in Centricon-10 columns (Amicon, Inc., Beverly, MA). Protein assays were performed on the resulting fractions, and the tyrosine-phosphorylated substrate END(pY)INASL was added to 25 μg of protein to a final concentration of 100 μM. The reaction was stopped after 30 min at room temperature by the addition of 1 volume of stopping solution (0.02% malachite green, 0.5% ammonium molybdate tetrahydrate, and 0.1% polyvinyl alcohol). Absorbance at 630 nm was read after a 60-min incubation. As a control, sodium orthovanadate was added to a parallel set of samples to ensure the presence of specific tyrosine phosphatase activity. Background absorbance for parallel samples that did not receive the substrate was subtracted as contaminating free phosphate. Each condition was tested in triplicate, and results were analyzed for statistical significance by multiple analysis of variance.

RESULTS

Recombinant Adenoviruses Encoding p75NTR Activate JNK and Induce Cell Death of PC12nnr5 Cells—The actions of p75NTR are complex and not well understood. This is due in part to the lack of cellular systems in which p75NTR-dependent signaling events can be reliably observed. Previous work from our group (46) and others (50) has shown that overexpression of the intracellular domain of TNFR superfamily members will constitutively activate downstream signaling pathways. To reliably activate p75NTR signaling cascades in a variety of cellular circumstances, recombinant adenoviruses encoding full-length p75NTR, the intracellular domain of p75NTR (p75ICD), or a myristoylated form of the intracellular domain of p75NTR that is targeted to the plasma membrane (p75mICD) (Fig. 1*B*) were produced. Recombinant adenoviruses encoding LacZ or green fluorescent protein were used as controls for all studies. To validate signaling properties of these p75NTR recombinant viruses, we initially tested their effects on the survival of PC12nnr5 cells (which express endogenous p75NTR, but not TrkA). Fig. 1*A* shows that LacZ virus had no effect on cell survival, whereas viruses encoding p75NTR or p75ICD were cytotoxic at a multiplicity of infection (m.o.i.) of 200. Overexpression of p75mICD induced cytotoxicity at lower multiplicities of infection than that of p75NTR or p75ICD, suggesting that membrane localization of the intracellular domain may be important for p75NTR-dependent apoptosis. p75NTR-dependent cell death was not an artifact of protein overexpression or viral infection since equivalent quantities of LacZ adenovirus had no effect on cell survival. Several reports have shown that p75NTR activation can lead to JNK activation (26–28); and consistent with this, we found that expression of p75mICD (Fig. 1*C*), p75ICD, and p75NTR increased phosphorylation of JNK on threonine 183 and tyrosine 185 and increased c-Jun phosphorylation on serine 73. Equivalent levels of LacZ virus did not alter JNK or c-Jun phosphorylation (data not shown). The level of c-Jun protein was also elevated at high p75NTR expression levels, likely due to autoregulation of c-*jun* transcription (51) during the relatively long infection period (48 h). To demonstrate the specificity of the JNK activation initiated by p75NTR, we analyzed phosphorylation of the transcription factor CREB, which lies downstream of protein kinase A, and assessed the phosphorylation status of MKK3/6, which activates p38 MAPK. Neither CREB (Fig. 1*C*) nor MKK3/6 (data not shown) phosphorylation was altered by p75NTR expression . These results show that p75NTR signaling results in specific activation of the JNK pathway and promotes cell death and therefore demonstrate that the p75NTR recombinant adenoviruses show the expected constitutive signaling properties.

p75NTR Activates Akt—Recent reports have shown that ceramide inhibits PI3K activity, reduces cellular PIP_3 levels, and thereby inhibits Akt activity (42, 43). p75NTR activates sphingomyelinase upon neurotrophin binding (20, 21), and we hypothesized that p75NTR could facilitate apoptosis by attenuating PIP_3 production and reducing Akt activity. To determine if p75NTR alters Akt activity, PC12nnr5 cells were infected with recombinant virus expressing each of the three p75NTR isoforms or LacZ and analyzed for Akt activation using phospho-specific antibodies directed against Akt serine 473 (Ser^{473}), an Akt autophosphorylation site that correlates with Akt kinase activity (5). Surprisingly, expression of full-length p75NTR, p75ICD (Fig. 2), or p75mICD (see Figs. 3*B* and 6*B*) resulted in significant increases in the phosphorylation of Akt on Ser^{473}, whereas control adenovirus expressing LacZ had no effect on Akt Ser^{473} phosphorylation. As noted above, relatively high levels of p75NTR expression were required to observe JNK activation, c-Jun phosphorylation, and apoptosis, but much lower levels of p75NTR expression were sufficient to induce Akt phosphorylation. p75NTR-mediated reduction of Akt phosphorylation was never observed, even in cells exposed to high

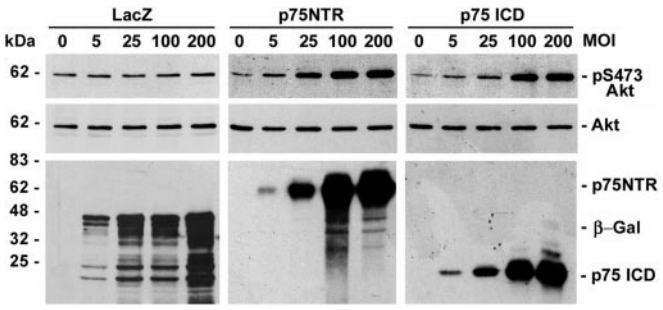

FIG. 2. **Expression of p75NTR in PC12nnr5 cells increases Akt Ser^{473} phosphorylation.** PC12nnr5 cells were infected with recombinant adenovirus expressing LacZ, full-length p75NTR, or the intracellular domain of p75NTR (p75ICD) at different multiplicities of infection. Cells were harvested 24 h after infection and analyzed for Akt levels, Akt serine 473 phosphorylation, LacZ expression, and p75NTR expression by immunoblotting. Experiments were repeated three times with similar results. *pS473*, phospho-Ser^{473}; *β-Gal*, β-galactosidase.

titers of p75NTR recombinant adenovirus. The effects of ligand binding to p75NTR on Akt activation in the presence and absence of virus were also tested; treatment of control and infected cells with NGF, brain-derived neurotrophic factor, or neurotrophin-3 at various concentrations and time courses had no effect on the p75NTR adenovirus-induced phosphorylation of Akt in PC12nnr5 cells (data not shown). Therefore, these data indicate that p75NTR expression activates Akt in a ligand-independent manner.

p75NTR-induced Akt Phosphorylation Is Trk-independent and Does Not Require Acidic Sphingomyelinase—The TrkA receptor is a potent activator of Akt, and our previous results (15) and those of others (16, 52) have shown that p75NTR can increase the response of TrkA to limiting NGF concentrations. It was therefore possible that the p75NTR-dependent Akt phosphorylation observed is secondary to activation of low levels of TrkA that may be present in PC12nnr5 cells. To address this, PC12nnr5 cells were first examined to determine if they express NGF-responsive TrkA. Fig. 3*A* shows that activated TrkA was not detected in PC12nnr5 cells under conditions in which TrkA was readily detected in normal PC12 cells. We then tested K252a, a specific TrkA inhibitor, for its ability to block p75NTR-mediated Akt phosphorylation using recombinant viruses encoding p75ICD and p75mICD, which are incapable of binding ligand. LacZ, p75ICD, and p75mICD adenoviruses were used at m.o.i. = 25 in these experiments since this infection level activated Akt (Fig. 2), but did not induce cell death (Fig. 1). Fig. 3*B* shows that K252a completely blocked NGF-mediated Akt phosphorylation in PC12 cells, but had no effect on p75ICD- and p75mICD-induced Akt phosphorylation in either PC12nnr5 or PC12 cells. Therefore, p75NTR-mediated activation of Akt occurs independently of TrkA signaling.

PC12nnr5 cells express endogenous p75NTR, and it is conceivable that p75NTR overexpression may mediate an increase in Akt phosphorylation by disrupting signaling from endogenous p75NTR. To address this, the effects of p75NTR on Akt phosphorylation were determined in a variety of cell types that do not express p75NTR or TrkA. p75NTR expression in MG87-3T3 fibroblasts (m.o.i. = 100) (Fig. 3*C*) or HeLa cells (data not shown) resulted in increased Akt phosphorylation, similar to that observed in PC12nnr5 cells. These results indicate that the effect of p75NTR on Akt phosphorylation does not involve disruption of endogenous p75NTR signaling.

Recombinant adenoviruses have significant effects on cellular physiology, and we therefore sought additional means to confirm that p75NTR expression increases Akt activation. TIMp75-3 is an MG87-derived cell line in which expression of p75NTR is tightly regulated through the addition of doxycycline (24) and therefore could be used to confirm that p75NTR overexpression results in Akt phosphorylation. Cells incubated with 2.5 μg/ml doxycycline for 24 h showed the expected increase in p75NTR expression, and this correlated with a rise in Akt Ser^{473} phosphorylation (Fig. 3*D*). Therefore, two independent means of p75NTR expression result in Akt activation in different cell types. The relatively modest increase in Akt phosphorylation observed likely reflects the fact that only a subpopulation of TIMp75-3 cells show robust doxycycline-induced p75NTR expression (see Fig. 5*B*).

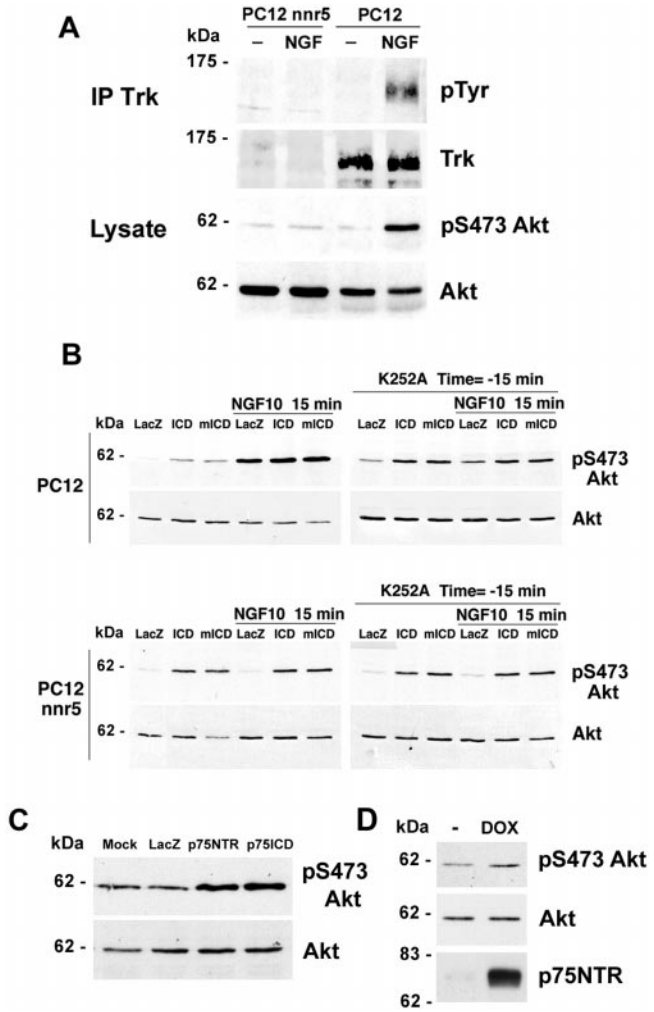

FIG. 3. **p75NTR-induced Akt phosphorylation is Trk-independent.** *A*, PC12 or PC12nnr5 cells were treated for 15 min with 10 ng/ml NGF and lysed in Nonidet P-40 lysis buffer as described under "Experimental Procedures." Trk receptors were immunoprecipitated (*IP*) using the anti-pan-Trk 203 antibody. Protein lysates were analyzed by immunoblotting for levels of total and phosphorylated Akt, phosphotyrosine (*pTyr*) content, and TrkA levels within immunocomplexes. *B*, PC12 or PC12nnr5 cells were infected with LacZ, p75ICD (*ICD*), or p75mICD (*mICD*) adenovirus at m.o.i. = 25 for 24 h and then treated with the Trk inhibitor K252a (200 nM) for 30 min, followed by 50 ng/ml NGF for 15 min before harvest. Comparison between the two cell lines shows that the Trk inhibitor K252a effectively inhibited Akt phosphorylation induced by NGF in PC12 cells, but had no effect on p75NTR-induced activation of Akt in PC12 or PC12nnr5 cells. *C*, MG87-3T3 cells were infected with recombinant adenovirus encoding p75NTR or p75ICD at m.o.i. = 100, harvested 24 h later, and analyzed for Akt levels and Akt Ser^{473} (*pS473*) phosphorylation. *D*, TIMp75-3 cells were treated with 2.5 μg/ml doxycycline (*DOX*) for 24 h; harvested; and analyzed for Akt levels, Akt Ser^{473} phosphorylation, and p75NTR expression. Experiments shown in *A* and *B* were repeated twice, and those in *C* and *D* were repeated three times, all with similar results.

Neurotrophin binding to p75NTR increases sphingomyelinase activity, and one possible explanation for the effect of

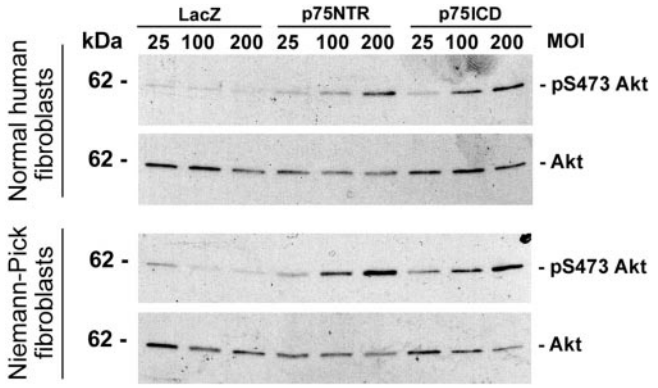

FIG. 4. **p75NTR-mediated Akt phosphorylation does not require acidic sphingomyelinase activity.** Overexpression of p75NTR or p75ICD in control human (*upper panel*) or Niemann-Pick (*lower panel*) fibroblasts induced Akt phosphorylation. High viral multiplicities of infection (m.o.i. = 100–200) were due to the low infectability of these lines (data not shown). Results shown were repeated twice with similar results. *pS473*, phospho-Ser^{473}.

p75NTR on Akt is that unliganded p75NTR functionally inactivates cellular sphingomyelinase, thereby reducing cellular ceramide levels and causing a consequent increase in PI3K activity, PIP_3 levels, and Akt phosphorylation. To address this, we compared the effects of p75NTR overexpression on Akt activation in normal and Niemann-Pick fibroblasts. Niemann-Pick fibroblasts are deficient in acidic sphingomyelinase, which is the only form of sphingomyelinase activated by p75NTR in PC12 cells.[2] Fig. 4 shows that p75NTR and p75ICD induced Akt phosphorylation in primary human fibroblasts derived from control and Niemann-Pick patients, suggesting that inactivation of acidic sphingomyelinase activation is not involved in this p75NTR response.

Expression of p75NTR Increases Cell Survival—These data are consistent with the hypothesis that p75NTR produces biphasic autonomous responses. High levels of p75NTR signaling result in JNK activation, c-Jun phosphorylation, and cell death, and lower levels of p75NTR signaling induce alternative pathways that include Akt activation and survival. To examine this, PC12nnr5 cells were infected with LacZ, p75NTR, or p75ICD adenovirus (all at m.o.i. = 25) and exposed to 0.5 μM staurosporine for 18 h, and levels of cellular apoptosis were determined by assessing annexin V binding by FACS analysis. Fig. 5*A* shows that infection of PC12nnr5 cells with p75NTR and p75ICD viruses significantly reduced the incidence of apoptosis compared with Me_2SO-treated cells or cells infected with control LacZ virus.

To extend these results to other cellular models, TIMp75-3 cells were analyzed for p75NTR-mediated survival properties. TIMp75-3 cells were produced from the MG87 cell line, which undergoes rapid cell death in the absence of serum-derived growth factors. TIMp75-3 cells were treated with 2.5 μg of doxycycline for 36 h (to induce p75NTR expression) and then deprived of serum for 18 h. FACS analysis was performed to assess p75NTR expression and apoptosis. Fig. 5*B* clearly shows that the TIMp75-3 population that expressed p75NTR was much less susceptible to cell death induced by serum deprivation, indicating that p75NTR promotes survival under these circumstances.

Activation of Akt by p75NTR Requires Active PI3K—To begin to determine the mechanisms used by p75NTR to activate Akt, the effect of PI3K inhibitors on p75NTR-induced Akt phosphorylation was examined. LY294002 and wortmannin were first tested for their ability to block Akt phosphorylation induced by NGF in PC12 cells. Fig. 6*A* shows that, as expected, both

[2] R. T. Dobrowsky, personal communication.

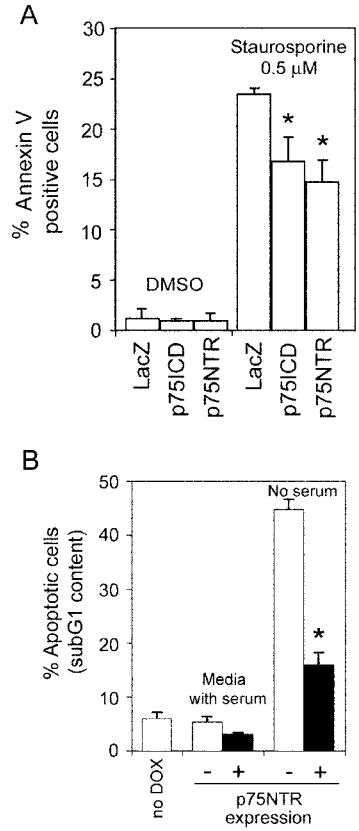

FIG. 5. **Expression of p75NTR facilitates cell survival.** *A*, PC12nnr5 cells were infected with recombinant adenovirus encoding LacZ, p75ICD, or p75NTR at m.o.i. = 25; incubated for 24 h; and then treated with 0.5 μM staurosporine or vehicle (dimethyl sulfoxide (*DMSO*)) for 18 h and analyzed for annexin V binding. A minimum of 20,000 cells were analyzed for each condition shown. *B*, TIMp75-3 cells were treated with 2.5 μg/ml doxycycline (*DOX*) for 36 h and serum-deprived for 18 h. Flow cytometry was carried out to determine p75NTR expression levels (using antibody p75NTR-B1) and to determine the proportion of cells with sub-G_1 DNA content using propidium iodide staining. A minimum of 30,000 cells were analyzed by flow cytometry for each condition. Statistically significant differences determined by multiple analysis of variance are indicated with *asterisks* in *A* and *B* (*, $p < 0.05$; **, $p < 0.001$). Data in *A* and *B* represent the means ± S.E. of three independent experiments.

inhibitors efficiently reduced NGF-induced Akt activation. Both inhibitors also completely blocked Akt phosphorylation induced by the p75mICD (Fig. 6*B*) or p75ICD or p75NTR (data not shown) recombinant adenovirus in PC12nnr5 cells and in A875 human melanoma cells. Therefore, PI3K activity is required for p75NTR-mediated activation of Akt.

p75NTR Expression Increases Tyrosine Phosphorylation of p85 and Shc—Activated receptor tyrosine kinases increase PIP_3 production largely by allowing PI3K proximity to the plasma membrane through SH2 domain-mediated interactions of the p85 regulatory subunit with receptor or with receptor-associated adaptor proteins such as Shc. To determine if Shc could contribute to the p75NTR-mediated increase in Akt phosphorylation, the tyrosine phosphorylation level of immunoprecipitated Shc was analyzed in cells expressing p75ICD or control adenovirus. Fig. 7*A* shows that phosphorylation of all three Shc isoforms (p66, p52, and p46) was increased relative to controls and that this effect was potentiated when tyrosine phosphatase activity (PTPase) was inhibited by treating the cells with sodium orthovanadate for 1 h prior to harvesting. A 160-kDa protein that co-immunoprecipitated with Shc also showed increased tyrosine phosphorylation in response to p75NTR overexpression; this protein is likely SHIP, a 160-kDa

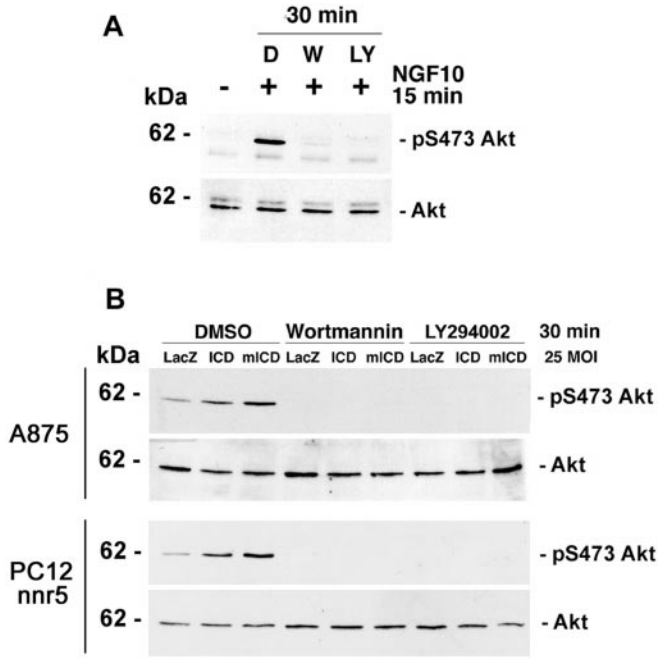

FIG. 6. **PI3K activity is required for p75NTR-induced phosphorylation of Akt.** *A*, PC12 cells were treated with 50 ng/ml NGF and 100 nM wortmannin (*W*), 20 μM LY294002 (*LY*), or equivalent quantity of DMSO (*D*) for 15 min prior to lysis and analyzed by immunoblotting for Akt levels and for Akt Ser^{473} (pS473) phosphorylation. *B*, A875 human melanoma cells and PC12nnr5 cells were infected with adenovirus encoding for LacZ, p75ICD (*ICD*), or p75mICD (*mICD*) at m.o.i. = 25 for 24 h and then treated for 30 min with wortmannin (100 nM) or LY294002 (20 μM) prior to lysis and analysis by immunoblotting for Akt levels and Akt Ser^{473} phosphorylation. Results shown were repeated three times with similar results.

lipid phosphatase that binds activated Shc with high affinity (53). Experiments were also performed to determine if the p85 subunit of PI3K shows a similar p75NTR-dependent increase in tyrosine phosphorylation. Fig. 7*B* shows that full-length p75NTR, but not green fluorescent protein, strongly increased tyrosine phosphorylation of p85. These results indicate that p75NTR activates a PI3K/Akt pathway by increasing tyrosine phosphorylation of adaptor proteins that relocalize PI3K to the plasma membrane. To determine if the phosphotyrosine content of any cell-surface proteins is altered by p75NTR expression, PC12nnr5 cells were infected with p75ICD recombinant adenovirus, and cell-surface glycoproteins were precipitated using Sepharose-conjugated wheat germ agglutinin and assayed for phosphotyrosine content by 4G10 immunoblotting. Fig. 7*C* shows that p75NTR expression specifically increased tyrosine phosphorylation of a 120-kDa protein, particularly in cells pretreated with sodium orthovanadate.

p75NTR Decreases Protein-tyrosine Phosphatase Activity—Our results show that p75NTR expression increased tyrosine phosphorylation of several proteins, particularly in the presence of orthovanadate, a nonspecific competitive PTPase antagonist. p75NTR has no intrinsic enzymatic activity, and its effect on phosphorylation must be due to regulatory interactions with proteins capable of increasing kinase activity, decreasing PTPase activity, or some combination of the two. A physical interaction between p75NTR and the FAP PTPase has recently been reported (38), raising the possibility that cellular tyrosine phosphatase activity could be modulated by p75NTR. To test this, PC12nnr5 cells were infected with full-length p75NTR, p75ICD, or LacZ adenovirus at m.o.i. = 25, subjected to subcellular fractionation, and analyzed for cytosolic and membrane-bound PTPase activities. Fig. 8 shows that expression of p75NTR or p75ICD resulted in a significant decrease in cytosolic PTPase activity, whereas membrane-associated PTPase activity was unchanged by p75NTR expression. These results indicate that p75NTR is capable of inhibiting cytosolic PTPase(s) and suggest that increases in the phosphotyrosine content mediated by p75NTR may be secondary to alterations in PTPase activity.

DISCUSSION

We have demonstrated that p75NTR expression leads to Trk-independent, PI3K-dependent Akt phosphorylation. High levels of p75NTR expression increase JNK and c-Jun phosphorylation and promote apoptosis, yet lower p75NTR expression levels are associated with activation of Akt and suppression of apoptosis induced by distinct stressors. p75NTR expression levels that potentiate Akt phosphorylation and survival increase the phosphotyrosine content of several cellular proteins, including p85 and Shc, suggesting that p75NTR affects the activity of tyrosine kinases or PTPases. Consistent with this, we demonstrate that p75NTR expression is associated with a decrease in cytosolic PTPase activity.

Many studies have demonstrated that p75NTR can facilitate apoptosis. Our earlier work (46) has shown that overexpression of the p75NTR intracellular domain within neurons of transgenic mice results in dramatic loss of peripheral and central neurons, and Barde and co-workers (30, 55) has shown that embryonic retinal cells that express p75NTR undergo cell death that can be prevented by the application of antibodies against either NGF or the p75NTR extracellular domain. Genetically altered mice rendered null at p75NTR or NGF loci show deficits in developmental apoptosis within the retina and spinal cord (31), and p75NTR can facilitate apoptosis of cultured rat oligodendrocytes (29) and sympathetic neurons (27). The precise pathways that p75NTR activates to induce apoptosis are unclear, but JNK, caspase activation, and increased p53 levels have been observed in some systems (26–28, 32). To reliably activate p75NTR signaling cascades, we created recombinant adenoviruses that encode either full-length p75NTR or the p75NTR intracellular domain. As expected from our earlier work in transgenic mice (46), adenovirus-mediated overexpression of p75ICD resulted in cellular apoptosis that was associated with increased JNK activity and c-Jun phosphorylation. Expression of p75NTR or myristoylated p75ICD gave similar results, with p75mICD proving a particularly potent apoptotic inducer. These reagents will be useful for studies designed to identify specific signaling events in the p75NTR apoptotic cascade.

Neurotrophin binding to p75NTR results in the activation of sphingomyelinase and the production of ceramide (20–22, 29, 56). Ceramide generated by p75NTR activation may inhibit Trk receptor activation (57), activate JNK (29, 58, 59), and affect neuronal differentiation (22). In some systems, sphingomyelinase activation results in a ceramide-dependent decrease in PIP_3 production and a subsequent reduction in Akt activity (42, 43), and our initial hypothesis was that p75NTR-dependent ceramide accumulation would suppress PI3K activity and thereby reduce Akt activation. However, overexpression of p75NTR resulted in ligand-independent activation of Akt in multiple cell types. Indeed, Akt was activated even at p75NTR expression levels that facilitate apoptosis, indicating that when the apoptotic pathway is activated, it can override the pro-survival effect of Akt.

The activation of Akt by p75NTR requires active PI3K and correlates with increases in the phosphotyrosine content of several proteins, including the adaptor protein Shc, the p85 regulatory subunit of PI3K, and a 120-kDa cell-surface protein. The increased phosphotyrosine content of these proteins correlates with reduced cytosolic PTPase activity in the presence of

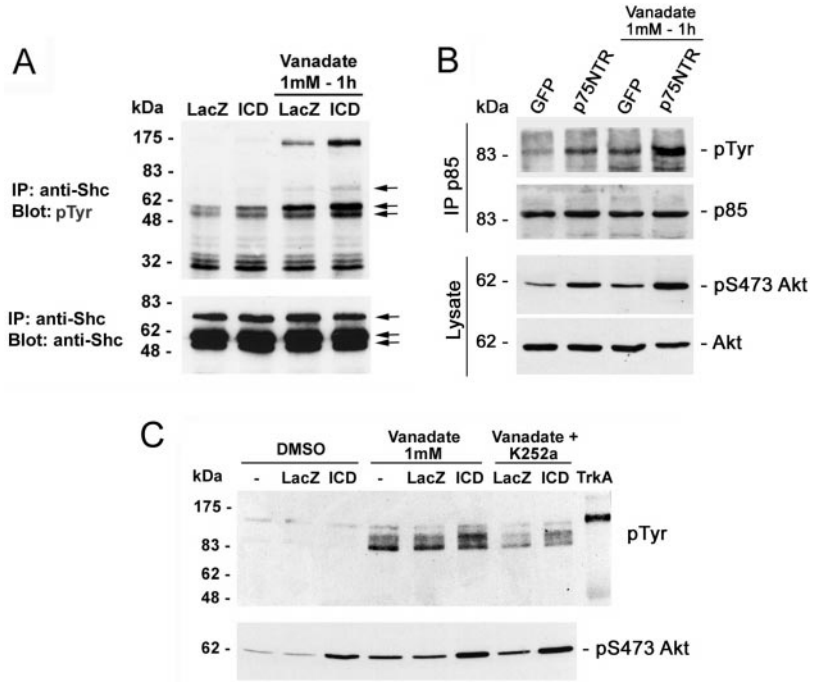

FIG. 7. **Tyrosine phosphorylation of Shc and p85 is increased by p75NTR expression.** *A*, PC12nnr5 cells were infected with LacZ, p75NTR, or p75ICD (*ICD*) adenovirus for 24 h and, where indicated, incubated in 1 mM sodium orthovanadate for 1 h and harvested. Shc was immunoprecipitated (*IP*), and immunoblots were performed to determine Shc levels and Shc phosphotyrosine (*pTyr*) content. *B*, the PI3K p85 subunit was immunoprecipitated, and immunoblots were performed to determine p85 tyrosine phosphorylation and protein levels. *GFP*, green fluorescent protein; *pS473*, phospho-Ser^{473}. *C*, PC12nnr5 cells were infected with LacZ or p75ICD adenovirus for 24 h and, where indicated, incubated in 1 mM sodium orthovanadate. Cells were lysed, and glycoproteins were precipitated using wheat germ agglutinin conjugated to Sepharose. Phosphotyrosine content of the precipitates was analyzed by 4G10 immunoblotting. As a control, the *last lane* shows activated TrkA to emphasize the difference in SDS-polyacrylamide gel electrophoretic migration of the two proteins. The phosphorylation state of Akt in the total protein lysates is shown in the *lower panel*. These experiments were repeated four times with similar results. *DMSO*, dimethyl sulfoxide.

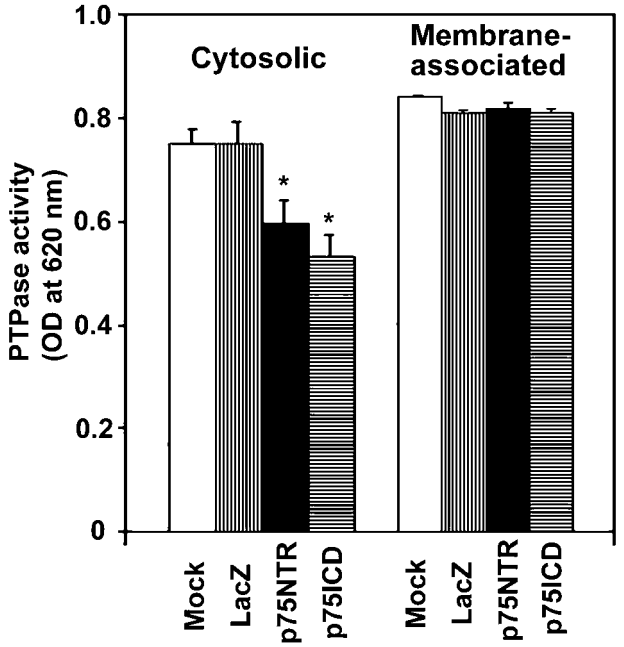

FIG. 8. **p75NTR expression decreases cytosolic protein-tyrosine phosphatase activity.** PC12nnr5 cells were infected with LacZ, p75NTR, or p75ICD adenovirus for 24 h and then harvested in high salt buffer. Cytosolic and membrane compartments were separated by centrifugation and analyzed for phosphatase activity as described under "Experimental Procedures." Conditions that were statistically different from controls ($p < 0.001$) are indicated by *asterisks*. Data shown represent the means ± S.E. of three independent experiments.

p75NTR. p75NTR-mediated inhibition of a cytosolic PTPase may therefore be a proximal event in the signaling cascade that may allow a Shc·PI3K complex to associate with the plasmalemma and increase PIP_3 production. Additional studies will be required to identify specific phosphatase(s) inhibited by p75NTR, but one candidate is FAP, which physically interacts with p75NTR when overexpressed in 293T cells (38). FAP also binds human Fas (60), and Fas-mediated apoptosis can be suppressed by FAP (61, 62) through an unknown mechanism. An alternative mechanism that may account for p75NTR-mediated Akt activation involves a link between TRAF proteins and Src kinases. The TRANCE receptor is a member of the TNFR superfamily that activates survival pathways in osteoclasts in part by activating Akt, and a recent study has found that a complex of Src kinase and TRAF-6 is required for this effect (63). p75NTR interacts with several members of the TRAF family, including TRAF-6 (25, 34), raising the possibility that this signaling path may also contribute to p75NTR-mediated Akt activation. Future experiments specifically examining FAP and Src signaling will be required to reveal the relative contributions of each of these pathways to p75NTR-mediated Akt activation.

The signaling mechanisms employed by p75NTR are not well understood, and the relationship of neurotrophin binding to p75NTR action remains unclear. All neurotrophins activate sphingomyelinase when bound to p75NTR (20, 21), but only NGF is capable of inducing apoptosis and nuclear factor-κB activation in most systems (23, 29, 30). Paradoxically, some studies suggest that the receptor signals apoptosis when free of ligand and that this function is suppressed by ligand binding to p75NTR (64, 65). For our studies, we produced a group of recombinant adenovirus that would constitutively activate p75NTR signaling and thereby allow us to identify p75NTR cascades irrespective of ligand binding. All of the p75NTR constructs employed specifically activate the JNK pathway and mediate apoptosis when expressed at high levels, indicating that they are capable of activating p75NTR signaling pathways. The high expression levels required to in-

duce apoptosis presumably reflect forced formation of a receptor signaling complex normally obtained in the presence of appropriate ligand (46). It is noteworthy that proximity of the intracellular domain to the plasma membrane appears important for activation of apoptosis by p75NTR since the p75mICD fragment elicits stronger apoptotic signaling than either p75NTR or p75ICD.

Cytotoxic effects are observed when p75NTR is highly overexpressed, but much lower expression levels of full-length p75NTR and the intracellular domain mutants elicit Akt phosphorylation and enhance survival. The canonical view of p75NTR action is that receptor signaling is activated by ligand binding, but recent studies on other TNFR superfamily members suggest an alternative paradigm for p75NTR signaling. Lenardo and co-workers (66, 67) have recently shown that some TNFR superfamily members must pre-assemble into cell-surface oligomers before binding ligand. Many investigators have observed oligomeric p75NTR in the absence of ligand in a variety of preparations (for an early example, see Ref. 54), consistent with the possibility that p75NTR may also pre-assemble into oligomers. With Fas and TNFR-1, ligand binding produces a conformational shift that enables specific signaling events, but does not alter the oligomeric nature of the receptor complex. Thus, the role of ligand is to shift the pre-assembled receptor complex to a different signaling mode. All three p75NTR constructs employed in our studies elicited Akt activation when produced at low expression levels, but much higher levels of expression were required for apoptotic signaling. We favor the hypothesis that these signaling events reflect distinct p75NTR signaling complexes and that oligomeric p75NTR exists in at least two distinct signaling complexes; in the ligand-free state, p75NTR will constitutively activate survival pathways that involve Akt, and when bound by ligand, it activates signaling pathways that result in cellular apoptosis.

In conclusion, we have identified a novel p75NTR signaling pathway that results in phosphorylation of Akt and that enhances cell survival. These data suggest that the autonomous signaling role of p75NTR may be broader than previously considered, with p75NTR capable of signaling pathways that support survival and death under different cellular circumstances.

Acknowledgments—We are grateful to Farid Arab Said and Sandra MacPherson for technical assistance with adenoviruses; Alain Boudreault for providing phosphatase assay reagents; Jane McGlade and David Kaplan for providing the anti-Shc and anti-TrkA antibodies, respectively; and Wayne Sossin and Sylvie La Boissière for careful reading of the manuscript.

REFERENCES

1. Lewin, G. R., and Barde, Y. A. (1996) *Annu. Rev. Neurosci.* **19,** 289–317
2. Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997) *Science* **275,** 661–665
3. Kaplan, D. R., and Miller, F. D. (2000) *Curr. Opin. Neurobiol.* **10,** 381–391
4. Bellacosa, A., Chan, T. O., Ahmed, N. N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J., and Tsichlis, P. (1998) *Oncogene* **17,** 313–325
5. Toker, A., and Newton, A. C. (2000) *J. Biol. Chem.* **275,** 8271–8274
6. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) *Cell* **96,** 857–868
7. Kennedy, S. G., Kandel, E. S., Cross, T. K., and Hay, N. (1999) *Mol. Cell. Biol.* **19,** 5800–5810
8. Fujita, E., Jinbo, A., Matuzaki, H., Konishi, H., Kikkawa, U., and Momoi, T. (1999) *Biochem. Biophys. Res. Commun.* **264,** 550–555
9. Tang, Y., Zhou, H., Chen, A., Pittman, R. N., and Field, J. (2000) *J. Biol. Chem.* **275,** 9106–9109
10. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) *Cell* **91,** 231–241
11. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999) *Nature* **401,** 82–85
12. Romashkova, J. A., and Makarov, S. S. (1999) *Nature* **401,** 86–90
13. Barker, P. (1998) *Cell Death Differ.* **5,** 346–356
14. Barrett, G. L. (2000) *Prog. Neurobiol.* **61,** 205–229
15. Barker, P. A., and Shooter, E. M. (1994) *Neuron* **13,** 203–215
16. Verdi, J. M., Birren, S. J., Ibanez, C. F., Persson, H., Kaplan, D. R., Benedetti, M., Chao, M. V., and Anderson, D. J. (1994) *Neuron* **12,** 733–745
17. Ryden, M., Hempstead, B., and Ibanez, C. F. (1997) *J. Biol. Chem.* **272,** 16322–16328
18. Brennan, C., Rivas-Plata, K., and Landis, S. C. (1999) *Nat. Neurosci.* **2,** 699–705
19. Bibel, M., Hoppe, E., and Barde, Y. A. (1999) *EMBO J.* **18,** 616–622
20. Dobrowsky, R. T., Werner, M. H., Castellino, A. M., Chao, M. V., and Hannun, Y. A. (1994) *Science* **265,** 1596–1598
21. Dobrowsky, R. T., Jenkins, G. M., and Hannun, Y. A. (1995) *J. Biol. Chem.* **270,** 22135–22142
22. Brann, A. B., Scott, R., Neuberger, Y., Abulafia, D., Boldin, S., Fainzilber, M., and Futerman, A. H. (1999) *J. Neurosci.* **19,** 8199–8206
23. Carter, B. D., Kaltschmidt, C., Kaltschmidt, B., Offenhauser, N., Bohm, M. R., Baeuerle, P. A., and Barde, Y. A. (1996) *Science* **272,** 542–545
24. Bhakar, A. L., Roux, P. P., Lachance, C., Kryl, D., Zeindler, C., and Barker, P. A. (1999) *J. Biol. Chem.* **274,** 21443–21449
25. Khursigara, G., Orlinick, J. R., and Chao, M. V. (1999) *J. Biol. Chem.* **274,** 2597–2600
26. Aloyz, R. S., Bamji, S. X., Pozniak, C. D., Toma, J. G., Atwal, J., Kaplan, D. R., and Miller, F. D. (1998) *J. Cell Biol.* **143,** 1691–1703
27. Bamji, S. X., Majdan, M., Pozniak, C. D., Belliveau, D. J., Aloyz, R., Kohn, J., Causing, C. G., and Miller, F. D. (1998) *J. Cell Biol.* **140,** 911–923
28. Yoon, S. O., Casaccia-Bonnefil, P., Carter, B., and Chao, M. V. (1998) *J. Neurosci.* **18,** 3273–3281
29. Casaccia-Bonnefil, P., Carter, B. D., Dobrowsky, R. T., and Chao, M. V. (1996) *Nature* **383,** 716–719
30. Frade, J. M., Rodriguez-Tebar, A., and Barde, Y. A. (1996) *Nature* **383,** 166–168
31. Frade, J. M., and Barde, Y. A. (1999) *Development* **126,** 683–690
32. Gu, C., Casaccia-Bonnefil, P., Srinivasan, A., and Chao, M. V. (1999) *J. Neurosci.* **19,** 3043–3049
33. Frade, J. M. (2000) *J. Cell Sci.* **113,** 1139–1148
34. Ye, X., Mehlen, P., Rabizadeh, S., Van Arsdale, T., Zhang, H., Shin, H., Wang, J. J., Leo, E., Zapata, J., Hauser, C. A., Reed, J. C., and Bredesen, D. E. (1999) *J. Biol. Chem.* **274,** 30202–30208
35. Bilderback, T. R., Grigsby, R. J., and Dobrowsky, R. T. (1997) *J. Biol. Chem.* **272,** 10922–10927
36. Chittka, A., and Chao, M. V. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 10705–10710
37. Casademunt, E., Carter, B. D., Benzel, I., Frade, J. M., Dechant, G., and Barde, Y. A. (1999) *EMBO J.* **18,** 6050–6061
38. Irie, S., Hachiya, T., Rabizadeh, S., Maruyama, W., Mukai, J., Li, Y., Reed, J. C., Bredesen, D. E., and Sato, T. A. (1999) *FEBS Lett.* **460,** 191–198
39. Mukai, J., Hachiya, T., Shoji-Hoshino, S., Kimura, M. T., Nadano, D., Suvanto, P., Hanaoka, T., Li, Y., Irie, S., Greene, L. A., and Sato, T. A. (2000) *J. Biol. Chem.* **275,** 17566–17570
40. Yamashita, T., Tucker, K. L., and Barde, Y. A. (1999) *Neuron* **24,** 585–593
41. Salehi, A. H., Roux, P. P., Kubu, C. J., Zeindler, C., Bhakar, A., Tannis, L. L., Verdi, J. M., and Barker, P. A. (2000) *Neuron* **27,** 279–288
42. Zundel, W., and Giaccia, A. (1998) *Genes Dev.* **12,** 1941–1946
43. Zhou, H., Summers, S. A., Birnbaum, M. J., and Pittman, R. N. (1998) *J. Biol. Chem.* **273,** 16568–16575
44. Schubert, K. M., Scheid, M. P., and Duronio, V. (2000) *J. Biol. Chem.* **275,** 13330–13335
45. Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A., and Shooter, E. M. (1987) *Nature* **325,** 593–597
46. Majdan, M., Lachance, C., Gloster, A., Aloyz, R., Zeindler, C., Bamji, S., Bhakar, A., Belliveau, D., Fawcett, J., Miller, F. D., and Barker, P. A. (1997) *J. Neurosci.* **17,** 6988–6998
47. Robbins, S. M., Quintrell, N. A., and Bishop, J. M. (1995) *Mol. Cell. Biol.* **15,** 3507–3515
48. Roux, P. P., Colicos, M. A., Barker, P. A., and Kennedy, T. E. (1999) *J. Neurosci.* **19,** 6887–6896
49. Laemmli, U. K. (1970) *Nature* **227,** 680–685
50. Boldin, M., Mett, I., Varfolomeev, E., Chumakov, I., Shemeravni, Y., Camonis, J., and Wallach, D. (1995) *J. Biol. Chem.* **270,** 387–391
51. Cochran, B. H. (1993) *Natl. Inst. Drug Abuse Res. Monogr.* **125,** 3–24
52. Mahadeo, D., Kaplan, L., Chao, M. V., and Hempstead, B. L. (1994) *J. Biol. Chem.* **269,** 6884–6891
53. Liu, L., Damen, J. E., Ware, M., Hughes, M., and Krystal, G. (1997) *Leukemia (Baltimore)* **11,** 181–184
54. Grob, P. M., Ross, A. H., Koprowski, H., and Bothwell, M. (1985) *J. Biol. Chem.* **260,** 8044–8049
55. Frade, J. M., and Barde, Y. A. (1998) *Neuron* **20,** 35–41
56. Blochl, A., and Sirrenberg, C. (1996) *J. Biol. Chem.* **271,** 21100–21107
57. MacPhee, I. J., and Barker, P. A. (1997) *J. Biol. Chem.* **272,** 23547–23551
58. Westwick, J. K., Bielawska, A. E., Dbaibo, G., Hannun, Y. A., and Brenner, D. A. (1995) *J. Biol. Chem.* **270,** 22689–22692
59. Verheij, M., van Blitterswijk, W. J., and Bartelink, H. (1998) *Acta Oncol.* **37,** 575–581
60. Sato, T., Irie, S., Kitada, S., and Reed, J. C. (1995) *Science* **268,** 411–415
61. Li, Y., Kanki, H., Hachiya, T., Ohyama, T., Irie, S., Tang, G., Mukai, J., and Sato, T. (2000) *Int. J. Cancer* **87,** 473–479
62. Yanagisawa, J., Takahashi, M., Kanki, H., Yano-Yanagisawa, H., Tazunoki, T., Sawa, E., Nishitoba, T., Kamishohara, M., Kobayashi, E., Kataoka, S., and Sato, T. (1997) *J. Biol. Chem.* **272,** 8539–8545
63. Wong, B. R., Besser, D., Kim, N., Arron, J. R., Vologodskaia, M., Hanafusa, H., and Choi, Y. (1999) *Mol. Cell* **4,** 1041–1049
64. Rabizadeh, S., Ye, X., Wang, J. J., and Bredesen, D. E. (1999) *Cell Death Differ.* **6,** 1222–1227
65. Rabizadeh, S., Oh, J., Zhong, L. T., Yang, J., Bitler, C. M., Butcher, L. L., and Bredesen, D. E. (1993) *Science* **261,** 345–348
66. Chan, F. K., Chun, H. J., Zheng, L., Siegel, R. M., Bui, K. L., and Lenardo, M. J. (2000) *Science* **288,** 2351–2354
67. Siegel, R. M., Frederiksen, J. K., Zacharias, D. A., Chan, F. K., Johnson, M., Lynch, D., Tsien, R. Y., and Lenardo, M. J. (2000) *Science* **288,** 2354–2357