The common neurotrophin receptor p75NTR has been shown to initiate intracellular signaling that leads either to cell survival or to apoptosis depending on the cell type examined; however, the mechanism by which p75NTR initiates its intracellular transduction remains unclear. We show here that the tumor necrosis factor receptor-associated death domain protein (TRADD) interacts with p75NTR upon nerve growth factor (NGF) stimulation. TRADD could be immunodetected after p75NTR immunoprecipitation from MCF-7 breast cancer cells stimulated by nerve growth factor. In addition, confocal microscopy indicated that NGF stimulation induced the plasma membrane localization of TRADD. Using a dominant negative form of TRADD, we also show that interactions between p75NTR and TRADD are dependent on the death domain of TRADD, thus demonstrating its requirement for binding. Furthermore, the p75NTR-mediated activation of NF-κB was inhibited by transfection with a dominant negative TRADD, resulting in an inhibition of NGF antiapoptotic activity. These results thus demonstrate that TRADD is involved in the p75NTR-mediated antiapoptotic activity of NGF in breast cancer cells.

Nerve growth factor (NGF) is the archetypal member of the neurotrophin family of proteins (including brain-derived neurotrophic factor, NT-3, and NT-4/5), which display a broad range of survival and trophic activities for neuronal cells (1). In addition to its neurotrophic function, other activities of NGF have been described. For example, NGF can modulate gene expression in monocytes (2), is chemotactic for melanocytes (3), and is motogenic for Schwann cells (4). NGF also stimulates the proliferation of lymphocytes (5), keratinocytes (6), and both prostate (7) and breast cancer (8) cells.

Cellular responses to NGF are elicited via two specific surface receptors: TrkA tyrosine kinase receptors (p140TrkA) and the common neurotrophin receptor, p75NTR, which belongs to the tumor necrosis factor (TNF)-receptor gene family. Although NGF binding to p140TrkA is known to activate its kinase domain, thus triggering various downstream Ras signaling pathways such as the mitogen-activated protein kinases, the function of p75NTR and how it signals remain controversial. p75NTR has been shown to regulate the activation of TrkA receptors but is also capable of triggering cellular responses independent of them (9–11). In breast cancer cells, the mitogenic activity of NGF is mediated through activation of p140TrkA, whereas p75NTR is required for its antiapoptotic effect, independent of p140TrkA (12). There is evidence that NGF can both positively and negatively regulate cell death and differentiation, depending on the cell type examined. In some cases, p75NTR is an inducer of apoptosis, even without NGF stimulation (13), whereas in other cases, the activation of p75NTR results in a protection from cell death (14). The mechanism by which p75NTR initiates such signaling is poorly described, but the tumor necrosis factor receptor-associated factor (TRAF) proteins, particularly TRAF2 and TRAF6, as well as the receptor-interacting protein 2, have been shown to interact with p75NTR and differentially modulate both the activation of the transcription factor NF-κB and cell survival in response to NGF (15, 16).

The first protein recruited to TNF-receptor 1 is the TNF-R1-associated death domain protein (TRADD), which serves as a platform to recruit additional mediators such as the TRAF proteins (17). Although the involvement of TRAF proteins in the p75NTR signaling complex has been described, the requirement for TRADD has not been reported. Here we show that TRADD functionally associates with p75NTR in breast cancer cells. The interaction between p75NTR and TRADD is required for activation of NF-κB, which controls the antiapoptotic effect of nerve growth factor.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture reagents were provided by Bio-Whittaker except insulin (Organon, Transferrin (Sigma), and Fibronectin (Phal-losotech). Recombinant NGF and other neurotrophins (BDNF, NT-3, and NT-4) were purchased from R&D Systems. C2 ceramide analogue (N-acetyl-r-sphingosine), Hoechst 33258, and electrophoresis reagents were from Sigma. The ExGen 500 transfectant and the Opti-MEM were from Euroclone. Protein A-agarose was purchased from Transduction Laboratories (Lexington, KY). The mouse monoclonal IgG anti-NGF receptor p75NTR antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). The goat and rabbit polyclonal antibodies (IgG) raised against TRADD and TRAF2, respectively, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the corresponding sec-

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¶ To whom correspondence should be addressed: UPRES-EA 1033, Bâtiment SN3, Université des Sciences et Technologies de Lille, Villeneuve d’Ascq, France. E-mail: hubert.hondermarck@univ-lille1.fr.

1 The abbreviations used are: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; p75NTR, p75-neurotrophin receptor; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factor; TRADD, TNF receptor-associated death domain protein; CMV, cytomegalovirus; PBS, phosphate-buffered saline.
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Ordinary antibodies were from Sigma. The mouse monoclonal anti-Myc antibody was from Jackson Laboratories. Polyclonal goat anti-rabbit IgG and donkey anti-goat IgG, both coupled to Alexa 568, and polyclonal goat anti-mouse IgG coupled to Alexa 488. The secondary antibodies were from Santa Cruz Biotechnology, Inc. (Dallas, TX). Mouse monoclonal anti-Myc (Sigma) and donkey anti-goat IgG, both coupled to Alexa 568, and polyclonal goat anti-rabbit IgG (Vector Laboratories) served as primary or control vectors (pCMV-β-galactosidase vector was from Clontech.

Cell culture and apoptosis measurement—The MCF-7 breast cancer cell line was obtained from the American Type Culture Collection and routinely grown as monolayers. Cells were maintained in minimal essential medium (Earle’s salts) supplemented with 20 mM HEPES, 2 g/liter sodium bicarbonate, 2 mM L-glutamine, 1% nonessential amino acids, 276 amino acids (corresponding to the extracellular and plasma membrane region) of human p75<sub>NTR</sub> was cloned into an episomal vector under CMV promoter control; the membrane association has been assessed in Madin-Darby canine kidney cells using immunocytochemistry. To investigate possible interactions between p75<sub>NTR</sub> and TRADD; a pCMV-β-galactosidase vector was from Clontech.

NF-κB activity measurement—After 24 h of NGF treatment, transfectected cells (TRADD, TRADD, PRK5, NF-κB-Luc, pLuc-MCS, and pCMV-β-galactosidase) were harvested with a reporter lysis buffer (Promega). NF-κB activity was determined using a luciferase assay kit (Promega) and measured with a luminometer (Lumat 9501, Berthold). The induction of NF-κB was calculated by measuring the luciferase expression by luminometer in cells expressing NF-κB promoter element present in the control vector (pLuc-MCS from Stratagene) lacking the NF-κB binding sequences. NF-κB activity was normalized with the pCMV-β control vector coding the β-galactosidase protein.

renders the following buffer: 150 mM NaCl, 50 mM Tris, pH 7.5, 1% Nonidet P-40, 1 mM sodium orthovandate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium pyrophosphate, 10 μg/ml leupeptin and aprotinin, and 1 μg/ml pepstatin. Cell lysate (1.5 mg) was preclarified with protein A-agarose (5 μl, 1:500) for 1 h, and then incubated with appropriate antibodies (1 μg/ml anti-p75<sub>NTR</sub>, 1:500) for 16 h at 4°C. The immunocomplexes were precipitated with protein A-agarose beads (10 μl, 1 h, 4°C), which were then pelleted by centrifugation (10,000 × g, 2 min). The pellet was rinsed three times with lysis buffer and boiled for 5 min in Laemmli buffer. After SDS-PAGE and electrophoretic transfer, nitrocellulose membranes (Schleicher & Schuell) were blocked with 3% bovine serum albumin. Membranes were then incubated with appropriate antibodies (1 μg/ml anti-p75<sub>NTR</sub>, 1:500) for 16 h at 4°C. The immunocomplexes were then washed with PBS and incubated with appropriate secondary antibodies diluted at 1:500 for 2 h at room temperature. To assess the requirement of TRADD death domain interactions with p75<sub>NTR</sub>, endogenous p75<sub>NTR</sub> was immunoprecipitated from 1.5 mg of total protein extract from MCF-7 cells transfected with 1 μg of Myc-tagged-TRADD, Myc-tagged-TRADD, or PRK5 empty vector. Immunoblotting was done as described above with 1:50 anti-Myc antibody. Membranes were washed extensively at room temperature, and the antibody complexes were visualized using the ECL system (Amer sham Biosciences) with Eastman Kodak Co. X-Omat AR film.

Immunofluorescent labeling and confocal microscopy—Cells, cultured onto glass coverslips coated with collagen, were treated for 15 minutes with NGF (10 ng/ml) and washed with PBS (10 min) and blocked for 10 min in permeabilizing buffer (PBS, 0.1% triton x-100). Cells were then fixed with PBS containing 4% paraformaldehyde for 30 min at room temperature. After washing with ammonium chloride (10 min at room temperature), cells were permeabilized with 0.5% saponin in PBS (10 min) and blocked for 10 min in permeabilizing buffer (PBS containing 2% BSA and 0.05% saponin). Cells were then successively incubated with 1 μm at room temperature, with primary antibodies (anti-p75<sub>NTR</sub> and anti-TRADD); after five washes with permeabilizing buffer, cells were incubated with both the secondary antibodies (30 min at 37°C at a 1:100 dilution). Cells were washed twice with permeabilizing buffer, twice with PBS plus 2% BSA, and then with PBS alone. Slides were mounted using Vectorshield mounting medium (Vector Laboratories).

Fluorescence-stained slides were examined under a Leica (TCS NT) laser-scanning confocal microscope comprising a krypton/argon laser. Simultaneous two-channel recording was performed. Frame scanning was performed at ×1000 magnification, and a single optical section was collected per field.

RESULTS

Interaction of TRADD with p75<sub>NTR</sub>—To investigate possible interactions between p75<sub>NTR</sub> and TRADD, MCF-7 cell lysates were prepared from NGF-treated cells followed by immunoprecipitation with p75<sub>NTR</sub> antibody and Western blotting with TRADD antibody. The results (Fig. 1) indicate that TRADD co-immunoprecipitates with p75<sub>NTR</sub> when cells have been stimulated with NGF. NGF concentration of 5–50 ng/ml was sufficient to induce the interaction between p75<sub>NTR</sub> and TRADD; a clear dose-dependent effect was observed (Fig. 1A). A study in which TRADD immunoprecipitates were Western blotted with p75<sub>NTR</sub>, was performed at 100 ng/ml NGF. The time course study (Fig. 1B) indicated that p75<sub>NTR</sub> /TRADD interaction can be detected as soon as 5 min of NGF stimulation; the maximum effect was observed after 10 min of stimulation. Other neurotrophins (BDNF, NT-3, NT-4) also mediate p75<sub>NTR</sub>/TRADD interaction (Fig. 1C); 50 ng/ml was a sufficient dose for each tested neurotrophin. Confocal microscopy analysis revealed that TRADD shifted to a plasma membrane localization upon NGF stimulation (Fig. 1D). This co-localization was demon-
TRADD Involvement in p75<sup>NTR</sup>-mediated Antia apoptotic Activity

**Fig. 1. Interaction of TRADD with p75<sup>NTR</sup>.** A, dose dependence. Immunoblot detection of TRADD associated with p75<sup>NTR</sup> is shown. MCF-7 cells were cultured in 10% fetal calf serum-containing medium before serum starvation for 24 h. Cells were then stimulated or not with 5, 50, 100, and 200 ng/ml NGF for 10 min. Total proteins were extracted, 1.5 μg was immunoprecipitated with anti-p75<sup>NTR</sup> (or anti-TRADD) and immunoblotted (Western blotting (WB)) against anti-p75<sup>NTR</sup> and anti-TRADD. NGF stimulation enhanced the association of endogenous TRADD with endogenous p75<sup>NTR</sup> receptor. Similar results were obtained in three separate experiments. B, time course of p75<sup>NTR</sup>/TRADD interaction. Immunoprecipitation with anti- p75<sup>NTR</sup> followed by immunoblot against anti-p75<sup>NTR</sup> and anti-TRADD were performed after 5, 10, 15, and 30 min of stimulation by 100 ng/ml NGF. C, stimulation of p75<sup>NTR</sup>/TRADD interaction by BDNF, NT-3, and NT-4. Immunoprecipitations and Western blotting were realized as described above. D, fluorescence confocal micrographs showing paraformaldehyde-fixed MCF-7 cells treated with or without NGF. Cellular localization of p75<sup>NTR</sup> and TRADD was assessed using secondary antibodies directed against anti-p75<sup>NTR</sup> and anti-TRADD coupled to Alexa 488 (green) and Alexa 568 (red), respectively. NGF induced a shift of TRADD to the plasma membrane.

...strated by the appearance of a merged fluorescence at the plasma membrane. The nuclear translocation of TRADD has recently been reported (20), and in order to investigate this possibility in the case of NGF stimulation, a measurement of the intensity of staining for TRADD in both the cytoplasm and the nucleus has been performed using the LSM-Image Browser (Zeiss) software. Our results show that the ratio of nuclear to cytoplasmic staining does not vary significantly upon NGF stimulation (data not shown). Altogether, these results indicate a p75<sup>NTR</sup> interaction with TRADD, which is regulated by NGF; in addition, no interaction between TRADD and TrkA was ever detected (data not shown). To further confirm the p75<sup>NTR</sup>/TRADD interaction, MCF-7 cells were transfected with a vector coding Myc-TRADD or Myc-TRADD<sup>ΔDD</sup>, the latter a dominant negative form lacking the death domain (Fig. 2A), and the association was determined as above. The results (Fig. 2B) demonstrated that the association between p75<sup>NTR</sup> and TRADD requires the death domain of TRADD, since its deletion clearly impairs the association.

**TRADD Is Required for p75<sup>NTR</sup>-induced Activation of NF-κB—Stimulation of p75<sup>NTR</sup> is known to activate the transcription factor NF-κB in breast cancer cells so that it translocates to the nucleus (12). Since TRADD is known to be a mediator of TNFR1-induced NF-κB activation (17), it was important to determine whether TRADD was also involved in the activation of NF-κB mediated through p75<sup>NTR</sup>. The results (Fig. 3) demonstrated that TRADD wild type control vector strongly enhanced the NGF-mediated induction of NF-κB. Furthermore, this induction was inhibited by p75<sup>NTR</sup> dominant negative (lacking the cytoplasmic tail) or by TRADD<sup>ΔDD</sup> dominant negative, thus demonstrating the involvement of TRADD in the activation of NF-κB in breast cancer cells.
TRADD Is Required for p75NTR-mediated Antiapoptotic Effect of NGF—We have previously shown that p75NTR-mediated activation of NF-κB results in the survival of breast cancer cells (12). We show here that transfection with TRADDΔDD prevents the antiapoptotic effect of NGF in MCF-7 breast cancer cells (Fig. 4A). However, the antiapoptotic activity of NGF was not totally inhibited with TRADDΔDD, since transfection with a vector coding a mutated form of IkB suggests that TRADD is not the only intermediate associating with p75NTR to activate NF-κB. In contrast, transfection with TRADD enhances the antiapoptotic effect of NGF, confirming the involvement of TRADD in p75NTR-mediated survival activity. The morphology of cell nuclei observed after Hoescht staining is shown in Fig. 4B.
DISCUSSION

We have shown here for the first time that nerve growth factor stimulation of the common neurotrophin receptor p75NTR results in the recruitment of TRADD. It has previously been shown that p75NTR interacts with TRAF protein family members (15, 21) and that this interaction modulates p75NTR-induced cell death and NF-κB activation with contrasting effects (15). TRAF4 inhibited the NF-κB response, whereas TRAF2 and TRAF6 enhanced p75NTR-induced NF-κB activation. However, these experiments were carried out in immortalized human epithelial kidney HEK 293T cells in which p75NTR stimulation by NGF resulted in cell death induction. In contrast, for breast cancer cells, p75NTR stimulation by NGF has been shown to be antiapoptotic and mediated by the nuclear translocation of NF-κB (12). TRADD is a multifunctional intracellular signaling adaptor protein that is recruited by TNFR1, leading to IKK activation and so to NF-κB nuclear translocation (22). Our results indicate that, in breast cancer cells, TRADD is also recruited by p75NTR, resulting in an activation of NF-κB, similar to the signaling initiated by TNFR1. Cell survival induced by NGF was observed from 50 ng/ml, and the maximum effect was obtained at 200 ng/ml, providing a coherence between the p75NTR/TRADD interaction and the biological effect of NGF in breast cancer cells. In addition, it should be emphasized that our experiments were performed without transfection of p75NTR, addition, it should be emphasized that our experiments were performed without transfection of p75NTR or TRADD, strengthening the physiological relevance of the p75NTR/TRADD interaction in breast cancer cells.

Two intracellular domains have been identified within p75NTR. The first one is homologous to the binding domain for TRAF proteins, and the second is homologous but distinct from the death domain of TNFR1 (23). TRADD death domain has been shown to bind to the death domain of TNFR1 (17). In immortalized striatal neurons, p75NTR was found to induce apoptosis without the participation of TRADD as an adaptor protein (28). Whether or not the interaction of TRADD with p75NTR provides a more specific pathway for NGF-induced antiapoptotic activity remains to be determined, but TRADD should now be considered as a significant influence in the balance between proapoptotic and antiapoptotic signaling pathways initiated by p75NTR.

In conclusion, the findings reported here provide new insights into the proximal elements of the p75NTR signaling pathway by demonstrating the involvement of TRADD as a critical intermediary for NF-κB activation. In immortalized striatal neurons, p75NTR was found to induce apoptosis without the participation of TRADD as an adaptor protein (28). Whether or not the interaction of TRADD with p75NTR provides a more specific pathway for NGF-induced antiapoptotic activity remains to be determined, but TRADD should now be considered as a significant influence in the balance between proapoptotic and antiapoptotic signaling pathways initiated by p75NTR.

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Tumor Necrosis Factor Receptor-associated Death Domain Protein Is Involved in the Neurotrophin Receptor-mediated Antiapoptotic Activity of Nerve Growth Factor in Breast Cancer Cells

Ikram El Yazidi-Belkoura, Eric Adriaenssens, Laurent Dollé, Simon Descamps and Hubert Hondermarck

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