Polyubiquitination of the neurotrophin receptor p75 directs neuronal cell survival

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ABSTRACT

Specific binding of nerve growth factor (NGF) to p75 neurotrophin receptor (p75NTR) leads to p75NTR polyubiquitination and its subsequent interaction with TRAF6 resulting in neuronal cell survival. However, when the binding of NGF to p75NTR was blocked with p75 antiserum, p75NTR polyubiquitination and neuronal cell survival were impaired. Results showed that tyrosine phosphorylation of p75NTR increased the polyubiquitination of p75NTR and contributed to the observed apparent neuroprotective effects. Similar to p75NTR polyubiquitination, interaction of TRAF6 with p75NTR was NGF/tyrosine phosphorylation dependent suggesting that TRAF6 might function as an E3 ubiquitin ligase. In sum, the results show that specific binding of NGF to p75NTR mediates neuronal cell survival.

1. Introduction

Nerve growth factor (NGF) is a neurotrophin that regulates survival, differentiation, and maintenance of sensory and sympathetic neurons [1]. Neurotrophins can bind to two classes of cell-surface receptors, a high-affinity tyrosine kinase Trk receptor and a low-affinity p75NTR [2,3], p75NTR is a member of the tumor necrosis factor (TNF) super family of receptors [4–6], and is involved in cellular apoptosis, cell survival, differentiation, neurite outgrowth [7–9]. Schwann cell myelination, and cell development [5,10]. p75NTR contains four ligand-binding pockets in a cysteine-rich extracellular domain and its intracellular region possesses the chopper domain and TNFR-like death domain [11]. p75NTR has no intrinsic enzymatic activity; however, NGF leads to tyrosine phosphorylation of p75NTR and activates the MAPK pathway [12]. The tyrosine phosphorylation of p75NTR can also be induced by the phosphatase inhibitor pervanadate [13].

p75NTR regulates both neuronal cell survival and apoptosis [5,14,15], and can bind to several interacting proteins involved in its functions. The neurotrophin receptor interacting factor (NRIF), p75NTR-associated death executor (NADE), and neurotrophin receptor interacting MAGE homologue (NRAGE) proteins bind to different regions of the p75NTR cytoplasmic domain and are involved in the induction of apoptosis [16–18]. p75NTR promotes the activation of sphingomyelinase and ceramide production that functions to activate the JNK kinase [19,20]. Interaction of TRAF6 with p75NTR enhances the cell survival [14,21], but the mechanism for this is unknown.

Ubiquitin is a 76 amino acid polypeptide; it covalently binds proteins through an isopeptide bond formed between its C-terminal glycine and the lysine residues of the substrate proteins. Three enzymes are required for ubiquitination. First, E1 (ubiquitin-activating enzyme) forms a high-energy thioester bond with the C-terminus of ubiquitin. Second, the activating enzyme (E2) mediates the formation of an isopeptide bond with the lysine residue in the substrate proteins. Third, ubiquitin is transferred to the substrate proteins through an isopeptide bond formed between its C-terminal glycine and the lysine residues of the substrate proteins [22]. Substrates can be mono-ubiquitinated [23–25] or polyubiquitinated, [26–28] linked through each of the seven lysine residues present in ubiquitin (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) [29]. The function of Lys48-linked ubiquitin chains is to target proteins for proteasomal degradation, whereas Lys63-linked ubiquitin chains play a role in endocytosis, protein sorting, and receptor trafficking [30]. Previously, it was shown that p75NTR interacts with TRAF6 [14]. TRAF6 is an E3 RING ubiquitin ligase [31] that directs the synthesis of Lys63-linked polyubiquitin chains [32] and is itself activated by Lys63-linked polyubiquitination [32]. Moreover, p75NTR signaling is deficient in traf6−/− mice [15], suggesting that p75NTR/TRA6 may play a role in the regulation of p75NTR signaling.
This prompted us to examine whether NGF binding to p75NTR and tyrosine phosphorylation of p75NTR could stimulate p75NTR polyubiquitination and lead to neuroprotection.

2. Materials and methods

2.1. Materials

Rabbit p75NTR and ubiquitin antibodies were purchased from Santa Cruz Biotechnology (La Jolla, CA). p75NTR antibody was purchased from Millipore (Billerica, MA) and rabbit TRAF6 antibody for Western blotting was obtained from Abcam (Cambridge, MA). Anti-phosphotyrosine (PY20) was purchased from BD Transduction Laboratories (San Diego, CA). NGF (2.5S) was from Bioproducts for Science (Indianapolis, IN). p75NTR antiserum (antibody 9561) directed against the third and fourth cysteine-rich repeats of the extracellular domain of mouse p75NTR was generously provided by Dr. Moses Chao, New York University School of Medicine.

2.2. Cell culture

The mouse hippocampal cell line HT-22 was a generous gift from Dr. David Schubert (The Salk Institute, La Jolla, CA) [33]. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Sigma, St. Louis, MO) supplied with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) as previously described [34]. Cells were incubated in a 5% CO2 atmosphere at 37°C. Cells were lysed with Triton lysis buffer to detect protein–protein interactions (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin and aprotinin) or SDS lysis buffer to detect covalent interaction of ubiquitin and p75NTR (Triton lysis buffer containing 1% SDS) [35]. Protein was estimated using the Bradford procedure (Bio-Rad) and with bovine serum albumin (BSA) as a standard for all samples except those that contained SDS. Protein was estimated in those samples using the DC assay (Bio-Rad).

2.3. Immunoprecipitation and Western blotting analysis

Cell lysates (1 mg) were diluted in lysis buffer and incubated with 4 μg of primary antibody at 4°C for 3 h. The immunoprecipitates were collected with agarose-coupled secondary antibody for 2 h at 4°C and then were washed three times with lysis buffer. Samples were boiled in sodium dodecyl-sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and resolved on 7.5–12% SDS–PAGE, transferred onto nitrocellulose membranes, and analyzed by Western blotting with the appropriate antibodies.

2.4. Cell survival assay

Cell survival was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, HT-22 cells were seeded in six-well plates in DMEM containing 10% FBS. The next day, cells were serum-starved and treated according to the experimental design. The cells were incubated in the presence of MTT, a substrate that is converted to a water-soluble formazan by a dehydrogenase enzyme present in metabolically active cells. The quantity of formazan product was determined by spectrophotometry at 490 nm. The results are shown as the percentage of live cells remaining under experimental conditions relative to that of controls (untreated) specified in the experiment.

3. Results and discussion

3.1. NGF induces the ubiquitination of p75NTR

We sought to explore whether NGF might induce p75NTR ubiquitination. HT-22 cells were treated with 100 ng/ml NGF for different times. The p75NTR ubiquitination was determined by immunoprecipitation with p75NTR antibody and Western blotting with anti-ubiquitin and anti-p75NTR. NGF treatment for 10 and 20 min resulted in maximum p75NTR polyubiquitination (Fig. 1A), however, polyubiquitination was sustained above basal levels in the presence of NGF. Antiserum (antibody 9561) directed against the third and fourth cysteine-rich repeats of the extracellular domain of mouse p75NTR inhibits NGF neurotrophic activity by interfering with the NGF-p75NTR interaction [36]. HT-22 cells were treated with p75NTR antiserum and control non-immune serum (final dilutions of 1:100) in the presence or absence of NGF for 10 min. Results showed that p75NTR antiserum abrogated the p75NTR polyubiquitination (Fig. 1B). These suggest that p75NTR is ubiquitinated through a pathway that is activated by the binding of NGF to p75NTR.

3.2. p75NTR blocking antibody impairs the interaction of p75NTR with TRAF6

TRAF6 functions as ubiquitin ligase and catalyzes the formation of noncanonical K63-linked polyubiquitin chains [32]. Because TRAF6 has been shown to interact with p75NTR [14,37], we sought to determine if that interaction could be recapitated in vivo in the HT-22 cell line. Cells were stimulated with NGF followed by immunoprecipitation of p75NTR and Western blotting to detect p75NTR and TRAF6. Lysates were also Western blotted for p75NTR and TRAF6. As shown in Fig. 2A, p75 and TRAF6 showed maximum transient co-interaction at 10 and 20 min of NGF stimulation. Interestingly, we observed that this co-interaction (complex) of p75NTR and TRAF6 existed at the same time that p75NTR ubiquitination

![Image](https://via.placeholder.com/150)

**Fig. 1.** NGF stimulates p75NTR polyubiquitination. (A) HT-22 cells were treated with NGF (100 ng/ml) for 0, 5, 10, 20, 30, and 60 min at 37°C. Cells were lysed and ubiquitination was examined by immunoprecipitation (IP) with anti-p75NTR followed by Western blot (WB) analysis with anti-ubiquitin (Ub) and anti-p75NTR. (B) HT-22 cells were treated with p75NTR antiserum (p75NTR blocking antibody) directed against the extracellular domain of p75NTR (1:100 final dilution) along with without NGF (100 ng/ml) for 10 min. Ubiquitination was examined by immunoprecipitation (IP) with anti-p75NTR followed by Western blot (WB) analysis with anti-ubiquitin and anti-p75NTR.
would enhance its own interaction with TRAF6. The determined whether an increase in tyrosine phosphorylation of 10 min. The cell lysate was immunoprecipitated (IP) with anti- p75NTR and Western partion of NGF involved in the interaction of p75NTR and TRAF6. was treated to HT-22 cells in the presence or absence of NGF for 10 min. This was followed by p75NTR immunoprecipitation and ubiquitination seen in the presence of the p75NTR blocking antibody. Next, we evaluated the contri- tion of TRAF6 with p75NTR increased (Fig. 3B). The cell extracts were also Western blotted (WB) with anti-p75NTR and anti-TRAF6. The cell lysate was also blotted with the same antibodies to verify the expression. was demonstrated. These results indicate that TRAF6 interaction may lead to p75NTR ubiquitination. Next, we evaluated the contribu- bition of NGF involved in the interaction of p75NTR and TRAF6. p75NTR antiserum that blocks the binding of NGF to the p75NTR was treated to HT-22 cells in the presence or absence of NGF for 10 min. This was followed by p75NTR immunoprecipitation and Western blotting with anti-p75NTR and anti-TRAF6 (Fig. 2B). In control, p75NTR interacts with TRAF6 upon NGF treatment. When the cells were treated with p75NTR blocking antibody along with NGF, the co-interaction of p75NTR with TRAF6 was impaired as shown in Fig. 2B. The results parallel the impairment of p75NTR ubiquitination seen in the presence of the p75NTR blocking antibody (Fig. 1B). These findings further support a model whereby TRAF6 interaction is necessary for p75NTR ubiquitination.

### 3.3. Tyrosine phosphorylation of p75NTR leads to p75NTR ubiquitination and interaction with TRAF6.

Next, we analyzed whether p75NTR tyrosine phosphorylation would lead to p75NTR ubiquitination. Tyrosine phosphorylation of p75NTR was induced by the phosphatase inhibitor pervanadate [13]. HT-22 cells were treated with increasing concentrations of pervanadate for 10 min, and the cell lysates were immunoprecipitated with anti-p75NTR and Western blotted with anti-ubiquitin and anti-p75NTR. Pervanadate (20 and 40 μM) treatment increased the p75NTR ubiquitination as shown in Fig. 3A. In addition, we also determined whether an increase in tyrosine phosphorylation of p75NTR would enhance its own interaction with TRAF6. The pervanadate treated cells were immunoprecipitated with p75NTR antibody and analyzed for the co-interaction of p75NTR with TRAF6 by Western blotting. As the pervanadate concentration increased, the tyrosine phosphorylation of p75NTR increased as shown by Western blotting with phosphotyrosine (PY20) antibody. In addition, as the phosphorylation of p75NTR increased, the co-interaction of TRAF6 with p75NTR increased (Fig. 3B). The cell extracts were also Western blotted with anti-p75NTR and anti-TRAF6 to check the expression of these proteins (Fig. 3C). The results suggested that p75NTR ubiquitination and the subsequent interaction with TRAF6 depend upon the tyrosine phosphorylation of p75NTR.

### 3.4. p75NTR ubiquitination is a neuronal cell survival factor

p75NTR can lead to neuronal cell survival or cell death [8,11]. To investigate the role of p75NTR ubiquitination and its interaction with TRAF6, we determined whether NGF binding to p75NTR is involved in neuroprotection. Under normal conditions, HT-22 cells are grown in DMEM containing 10% FBS, but serum starvation for 24 h induces cell death. However, we found that NGF treatment protected serum-starved HT-22 cells from death, perhaps by exerting a neuroprotective effect (Fig. 4A). When NGF binding to p75NTR was blocked specifically by adding p75NTR antisera, the neuro-protective effect of NGF was abolished as shown in Fig. 4A. Additionally, an increase in the tyrosine phosphorylation of p75NTR enhanced the p75NTR ubiquitination and TRAF6 interaction (Fig. 3). Based on these observations, we set out to investigate whether tyrosine phosphorylation of p75NTR also enhances the neuroprotective activity. Tyrosine phosphorylation of p75NTR was induced by the addition of increasing concentrations of pervana- date to the serum-withdrawal media and cell death was monitored biochemically using the MTT assay (Fig. 4B). We discovered that the addition of pervanadate protected serum-starved HT-22 cells from cell death. In addition, as the concentration of pervanadate increased, it enhanced the ability of the cells to survive in parallel with the degree of p75NTR tyrosine phosphorylation. Collectively, these results demonstrate that NGF and pervanadate enhance p75NTR ubiquitination and the subsequent interaction with TRAF6 while also providing a neuroprotective effect.
In the present study, we provide evidence that NGF induces p75\textsuperscript{NTR} polyubiquitination and the subsequent p75\textsuperscript{NTR} interaction with TRAF6 to regulate neuronal cell survival. NGF is known to bind both Trk receptor and p75\textsuperscript{NTR} [35,38]. To confirm that NGF binding specifically to p75\textsuperscript{NTR} can lead to p75\textsuperscript{NTR} polyubiquitination and neuroprotection, we blocked the binding of NGF to p75\textsuperscript{NTR} with p75\textsuperscript{NTR} antiserum, which does not block NGF binding to the Trk receptor [36]. Interaction of TRAF6 with p75\textsuperscript{NTR} is NGF dependent, as is p75\textsuperscript{NTR} polyubiquitination, suggesting that TRAF6 might function as E3 ubiquitin ligase. Our results parallel those of Powell et al. [10], who suggested that p75\textsuperscript{NTR} is a substrate for the Trk receptor [36]. Interaction of TRAF6 with p75\textsuperscript{NTR} signaling function for the p75 receptor death domain mediated via the caspase recruitment domain receptor-interacting protein 2, J. Neurosci. 21 (2001) 5854–5863.

Fig. 4. NGF and pervanadate enhance cell survival. (A) Neuronal HT22 cells were serum-starved and incubated with NGF (30 ng/ml) for 24 h in the presence or absence of p75 blocking antibody. (B) HT22 cells were incubated with increasing concentrations of pervanadate in serum-starved media for 24 h. Cell death was assessed biochemically using the MTT assay. The mean and standard deviation of three experiments is shown (*P < 0.05).

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