Mechanisms of neurotrophin receptor signalling

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Abstract
Regulation of cell survival decisions and neuronal plasticity by neurotrophins are mediated by two classes of receptors, Trks (tropomyosin receptor kinases) and p75, the first discovered member of the tumour necrosis factor receptor superfamily. The p75 receptor participates with the TrkA receptor in the formation of high-affinity nerve growth factor-binding sites to promote survival under limiting concentrations of neurotrophins. Activation of Trk receptors leads to increased phosphorylation of Shc (Src homology and collagen homology), phospholipase C-γ and novel adaptor molecules, such as the ARMS (ankyrin-rich membrane spanning)/Kidins220 protein. Small ligands that interact with G-protein-coupled receptors can also activate Trk receptor kinase activity. Transactivation of Trk receptors and their downstream signalling pathways raise the possibility of using small molecules to elicit neuroprotective effects for the treatment of neurodegenerative diseases. Like amyloid precursor protein and Notch, p75 is a substrate for γ-secretase cleavage. The p75 receptor undergoes an α-secretase-mediated release of the extracellular domain followed by a γ-secretase-mediated intramembrane cleavage. Cleavage of p75 may represent a general mechanism for transmitting signals as an independent receptor and as a co-receptor for other signalling systems.
and Notch may be relevant to neurodegenerative diseases, such as Alzheimer’s dementia and multiple sclerosis. In this paper, we describe the behaviour of p75 cleavage-resistant receptors with respect to associations with Trk and known p75 adaptor proteins.

Material and methods

Immunoprecipitation and immunoblotting

Cell lysates from transiently transfected HEK-293 cells (human embryonic kidney cells) were prepared by incubating in lysis buffer (1% Nonidet P40, 150 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8.0, 10% glycerol, 10 mM NaF, 1 mM sodium orthovanadate, 1 mg/ml aprotinin, 1 µg/ml leupeptin and 25 µg/ml PMSF) for 30 min on ice. Clarified lysates were immunoprecipitated by incubating overnight at 4°C with anti-pan-Trk polyclonal antibody (Trk C-14), anti-FLAG or anti-Myc antibodies followed by incubation with Protein A–Sepharose beads. Equivalent amounts of protein were analysed for each condition. The beads were washed three times with lysis buffer, and the immune complexes were boiled in SDS-sample buffer and loaded on to SDS/polyacrylamide gels for immunoblot analysis using anti-p75 (9992) serum. The immunoreactive protein bands were detected by enhanced chemiluminescence (Amersham).

Luciferase assays

For luciferase assays, HEK-293 cells in 24-well plates were transfected with Lipofectamine™ 2000 in complete media with 1050 ng of total DNA per well. Each well contained 200 ng of NF-κB (nuclear factor κB)-luciferase reporter, 50 ng of Renilla for normalization, 800 ng of full-length p75, p75–ICD, p75–FasTM and p75–FasS. Cells were harvested for luciferase assay 24 h after transfection using the Dual-Luciferase® Reporter Assay system (Promega). Results shown represent the averages of triplicate wells per condition, normalized for transfection efficiency. Statistical significance was determined using a two-tailed Student’s t test. The results shown are means ± S.D. for triplicate assays normalized to Renilla expression.

Cross-linking of cell-surface receptors

HEK-293 cells transiently transfected with either p75, p75–FasTM or p75–FasS were rinsed twice with ice-cold PBS (pH 8.0) to remove amine-containing culture media and protein from the cells and then incubated with BS3 (bis(sulphosuccinimidyl) suberate; Pierce) solution in PBS at a final concentration of 1 mM for 45 min at room temperature. Cells were then lysed in RIPA buffer (1% Nonidet P40, 0.1% SDS, 0.1% deoxycholate, 150 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8.0, 10 mM NaF, 1 mM sodium orthovanadate, 2 µg/ml aprotinin, 1 µg/ml leupeptin and 25 µg/ml PMSF) for 30 min on ice.

Results

Interactions of p75 with TrkA

In order to circumvent the problems of using pharmacological inhibitors of α- and γ-secretase activities, we sought to define
sequences involved in the cleavage of p75. As cleavage by α- and γ-secretases is quite promiscuous, a series of chimaeric receptors were generated. Two p75 chimaeras, p75–FasTM and p75–FasS, were found to be resistant to γ-secretase and α-secretase cleavage [13]. After introduction into neural cells, each chimaeric receptor was localized at the plasma membrane and was capable of interacting with the NgR [14].

Previous experiments indicated that p75 and Trk could be found in complexes [17–19]. After transfection of HEK-293 cells with wild-type TrkA receptors, a strong interaction was observed with full-length p75. The cleavage-resistant chimaera p75–FasTM showed a qualitatively comparable level of interaction, whereas the p75–FasS receptor displayed a weaker interaction. This suggests that the stalk domain of p75 might have a critical role in controlling association with TrkA, while the transmembrane sequence of p75–FasTM does not significantly modify this association between p75 and TrkA (Figure 2A).

The differential effects of the chimaeric receptors appear to be specific for Trk receptors, because the p75–FasS receptor retains the ability to interact strongly with the NgR [14]. Hence, sequences in the stalk domain of the receptor may not only be responsible for the initial α-secretase cleavage [12,13], but might also play a pivotal role in controlling the association of p75 with its co-receptor partners.

**Interaction with intracellular adaptors**

Many intracellular proteins that bind to p75 have been reported [6,9]. Among the many proteins are RIP2 (receptor-interacting protein 2) [20] and TRAF-6 (TNF-receptor-associated factor-6) [21], which are TNF receptor adaptor proteins involved in the regulation of the NF-κB activity. RIP2 contains a death effector domain and a protein kinase activity. Enhanced association of RIP2 with p75 results in an increase in NF-κB activity and a pro-survival outcome [20]. TRAF-6 is a zinc finger RING protein that contains an E3 ubiquitin ligase capable of inducing the degradation of IκB (inhibitory κB) [22]. We analysed the ability of the chimaeric constructs to interact with these two adaptor proteins.

Both proteins have been previously shown to interact with p75 at low basal levels that are increased upon nerve growth factor stimulation [20,21]. In order to investigate the impact of the stalk and transmembrane domains of p75, a series of transient transfection experiments were performed with full-length FLAG-tagged TRAF-6 along with wild-type p75 or the p75–FasR chimaeras. Lysates were immunoprecipitated using anti-FLAG antibody and co-immunoprecipitation was assessed with antibodies against the cytoplasmic domain of p75 (Figure 2B).

As described previously, there is a little basal interaction between p75 and TRAF-6 [21]. A similar result was observed for p75–FasTM. In contrast, the p75–FasS receptor displayed increased constitutive association with TRAF-6 (Figure 2B). Interestingly, when an analogous experiment was performed to study the interaction between the different p75 receptors and RIP2, we observed exactly the opposite result.

A strong constitutive association between RIP2 and p75–FasTM was observed, but the p75–FasS receptor displayed a lower basal level of interaction, similar to that observed with wild-type p75 (Figure 2C).

**Effects on NF-κB activity**

To determine whether the differences observed in the interaction with TRAF-6 and RIP2 have an effect on signal transduction, we assessed whether the p75 chimaeric constructs had any effect on NF-κB activity. The effects of 75, p75–FasTM and p75–FasS expression on NF-κB activity were assayed using a luciferase assay (Figure 3). A 4-fold increase in the reporter activation was observed in the presence of p75–FasS, while p75 and p75–FasTM showed comparable basal levels of activity.
Figure 3 | NF-κB activity and receptor cross-linking

(A) Activation of NF-κB in response to p75-Fas constructs. Using an NF-κB luciferase reporter, the p75-FasS construct provided high constitutive activation. RLU, relative luciferase units. (B) Cross-linking of p75 receptors using BS3. p75-FasS forms higher order complexes, as detected by anti-p75 (9992) antibody.

Both RIP2 and TRAF-6 have been implicated in the activation of NF-κB. Overexpression of RIP2 or TRAF-6 along with p75 results in enhanced reporter gene activity for NF-κB [11,20]. Similar to these published observations, we also found low basal activity of NF-κB in the presence of p75 alone. Interestingly, p75–FasS promoted constitutive activation of NF-κB activity. This finding correlated well with the co-immunoprecipitation experiments shown above, indicating that p75–FasS associates constitutively with TRAF-6 (Figure 2A). It is indeed possible that the ability of p75–FasS to interact constitutively with TRAF-6 might result in the activation of downstream pathways that trigger NF-κB activity.

Formation of multimeric complexes

Many of the TNF receptor superfamily members signal through the formation of homotrimeric complexes in response to binding to their trimeric ligand [23]. Furthermore, it has been demonstrated that they can also exist on the cell surface as preformed trimers mediated by sequences found in the extracellular domain [24]. Multimerization of TNF receptor family members has been shown to promote activation of signalling platforms with intracellular adaptor molecules through homotypic interaction with proteins containing death domains. Therefore we are interested in assessing the status of the chimaeric receptors at the cell surface.

It is possible that the strong interaction we observed for p75–FasS with TRAF-6 and the subsequent activation of signalling is the result of constitutive multimerization of the receptor, mimicking TNF receptor superfamily activation models. To address this possibility, we performed chemical cell-surface protein cross-linking using a membrane-impermeable reagent, BS3. HEK-293 cells were transiently transfected with p75, p75–FasS or p75–FasTM and then incubated with the BS3 cross-linker at 4°C in order to avoid receptor internalization. After quenching the cross-linking reaction, protein lysates were prepared and analysed by Western blotting (Figure 3B). The p75 and p75–FasTM receptors displayed similar patterns, with bands migrating at molecular masses consisting of monomeric and dimeric forms (Figure 3B, asterisk) of each receptor. On the other hand, p75–FasS migrated at much higher molecular masses, consistent with higher order multimeric forms of the receptor (Figure 3B, arrows). Hence, the presence of Fas receptor sequences in place of p75 sequence in the stalk domain had a dramatic effect on the higher order structure of the receptor.

Discussion

Many substrates of γ-secretase cleavage have been identified, including Notch, APP and ErbB4 receptor, indicating that there is broad substrate recognition by γ-secretase. The p75 receptor is a member of the TNF receptor superfamily that includes the Fas receptor, CD30 and CD40. The cleavage-resistant chimaeric receptors that we generated represent a powerful tool for studying the biological relevance of p75 proteolytic processing. Recent studies using the non-cleavable chimaeric receptors indicate that the p75 cleavage may be involved in nerve regeneration [14] and also neurotrophin-mediated apoptosis [15].

The characterization of the general signalling properties of the chimaeric receptors indicates that there are interesting changes in the ability of p75 to activate NF-κB and associate with interacting proteins. For example, p75–FasTM displayed an increased basal association with RIP2. The p75–FasS chimaera did not associate with TrkA, but there was increased association with NgR. Interestingly, an increased association with TRAF-6 correlated well with constitutive activation of an NF-κB reporter construct. The p75–FasS receptor is constitutively self-associated at the cell surface and is capable of forming multimeric complexes. Therefore specific sequences can determine aggregation of the receptor and increased association with adaptor proteins, such as TRAF-6, which can modulate NF-κB activity.

Another interesting property of the chimaeric receptors is the inability to be recognized by presenilin. The γ-secretase enzyme is a large protein complex with an unusual aspartyl protease activity and requires presenilins and other
components, such as nicastrin, Pen-2 and Aph-1. Although the wild-type p75 receptor could be found in a complex with presenilin after BDNF (brain-derived neurotrophic factor) binding, no association was observed between the p75–Fas chimaeric receptors by immunoprecipitation [15]. This observation provides a mechanism for the mode of action of the p75 chimaeric receptors in transfection experiments.

Cleavage of p75 by regulated intramembrane proteolysis is of particular importance for a number of reasons. First, each product of the cleavage may serve multiple functions. The release of the extracellular domain generates a binding protein for many potential ligands, including neurotrophins, pro-neurotrophin, β-amyloid and the rabies virus glycoprotein [9]. The CTF (C-terminal fragment) generated by α-secretase cleavage of p75 can influence Trk receptor binding affinity and signalling [25]. There is strong evidence that the CTF can preferentially form a complex with Trk receptors [10]. Secondly, the ICD of p75 has the potential of binding many intracellular proteins, including TRAF-6, SC-1 (Schwann cell factor 1), NADE (p75NTR-associated cell death executor), RIP2, NRAGE (neurotrophin receptor-interacting MAGE) and RhoA [7,8]. The p75 cytoplasmic domain may bring these intracellular proteins to function in different cellular compartments. The cleavage of the CTF fragment also gives rise to a small peptide, whose significance is unknown, but which is analogous to the Aβ (amyloid β-peptide) peptides generated from APP.

The cleavage of p75 may be physiologically important, since many cell types up-regulate p75 receptors under pathological or inflammatory conditions. For example, after seizures, there is prominent expression of p75 in cortical neurons [26]. Induction of p75\textsuperscript{NTR} has been observed in many cell types, including oligodendrocytes, Schwann cells, microglia, macrophages and smooth-muscle cells [6,7]. The early induction and cleavage of p75 in selective neurons that are destined to undergo intramembranous γ-secretase cleavage of APP suggests that p75 cleavage may be quite an important event during neurodegeneration.

References

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