Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR

Miriam Bibel1, Edmund Hoppe2 and Yves-Alain Barde

Max-Planck Institute of Neurobiology, Department of Neurobiochemistry, 82152 Planegg-Martinsried, Germany
2Present address: Hoechst Marion Roussel, Fraunhoferstraße 22, 82152 Martinsried, Germany
1Corresponding author
e-mail: miriam@neuro.mpg.de

Neurotrophins bind to two structurally unrelated receptors, the trk tyrosine kinases and the neurotrophin receptor p75NTR. Ligand activation of these two types of receptor can lead to opposite actions, in particular the prevention or activation of programmed cell death. Many cells co-express trk receptors and p75NTR, and we found that p75NTR was co-precipitated with trkA, trkB and trkC in cells transfected with both receptor types. Co-precipitation of p75NTR was not observed with the epidermal growth factor receptor. Experiments with deletion constructs of trkB (the most abundant trk receptor in the brain) and p75NTR revealed that both the extracellular and intracellular domains of trkB and p75NTR contribute to the interaction. Blocking autophosphorylation of trkB substantially reduced the interactions between p75NTR and trkB constructs containing the intracellular, but not the extracellular, domains. We also found that co-expression of p75NTR with trkB resulted in a clear increase in the specificity of trkB activation by brain-derived neurotrophic factor, compared with neurotrophin-3 and neurotrophin-4/5. These results indicate a close proximity of the two neurotrophin receptors within cell membranes, and suggest that the signalling pathways they initiate may interact soon after their activation.

Keywords: BDNF/NT3/NT4/neurotrophins/receptors

Introduction

All known members of the nerve growth factor (NGF) family, designated the neurotrophins, bind to two different types of receptors, the trk tyrosine kinases and the neurotrophin receptor p75NTR (Bothwell, 1991; Chao, 1992; Meakin and Shooter, 1992). In mammals, three different trk receptors have been identified and are activated by one or more of the four neurotrophins (for review see Barbacid, 1994). Binding of the neurotrophins to the trk receptors leads to receptor tyrosine phosphorylation, triggering the activation of pathways leading to the prevention of programmed cell death during development (Kaplan and Miller, 1997). Studies with antibodies and with mouse mutants have established that each neurotrophin and trk receptor is required during neural development, the elimination of any one of these components leading to specific deficits in the nervous system (Snider, 1994; Lewin and Barde, 1996).

All neurotrophins also bind to the neurotrophin receptor p75NTR, a member of the tumour necrosis factor (TNF) receptor and FAS/Apo-1/CD95 family. Following the discovery of the trk receptors as mediators of the trophic effects of the neurotrophins, the role of p75NTR was mostly discussed as that of an accessory receptor modulating the signalling of the trk receptors (Chao and Hempstead, 1995). Recently however, p75NTR has also been shown to mediate cell death in a ligand-dependent fashion (Casaccia-Bonnefil et al., 1996; Bamji et al., 1998; Davey and Davies, 1998), a function similar to that demonstrated previously with the structural parents of p75NTR, namely the TNF receptor 1 and CD95. Evidence for this new function of p75NTR has also been obtained in vivo in the avian retina (Frade et al., 1996; Frade and Barde, 1998), in mouse sympathetic ganglia (Bamji et al., 1998) and in the developing spinal cord of mice carrying a mutation in the ngf or the p75 gene (Frade and Barde, 1999).

In view of these observations and the fact that many neuronal populations co-express trk receptors and p75NTR, we tested to see whether an interaction between both receptor types could be demonstrated by immunoprecipitation in transfected cells. While this question has been addressed in previous studies, work has focused on p75NTR and trkA using either cross-linking (Huber and Chao, 1995; Gargano et al., 1997) or co-patching techniques (Ross et al., 1996). As trkB can be activated by three different neurotrophins, namely brain-derived neurotrophic factor (BDNF), neurotrophin-4/5 (NT4/5) and neurotrophin-3 (NT3), we also investigated the extent to which co-expression of p75NTR with trkB modulates the ligand specificity of this receptor.

Results

Co-immunoprecipitation of p75NTR with trkA, trkB and trkC

Comparable conditions for immunoprecipitation of the rat receptors trkA, trkB and trkC were established by tagging the three receptors at their N-terminal ends using a nine amino acid hemagglutinin (HA) epitope. The ability of the tagged receptors to bind neurotrophins was determined in cross-linking experiments using radiolabelled neurotrophins and by ligand-induced receptor phosphorylation (data not shown). These three cDNAs were used to transfet A293 cells together with rat p75NTR at a ratio of 1:1. Following cell lysis with 1.0% Triton X-100, the trk receptors were precipitated with anti-HA antibodies and the immunoprecipitates analysed by Western blotting with anti-p75 antibodies. These experiments revealed that
of p75NTR could be detected under these conditions (data after mixing the cell extracts. No co-immunoprecipitation of cells and the immunoprecipitation performed as above (Figure 1B).

trk tissue. Also, previous studies have established that trk most abundant of the receptors. When similar experiments were performed with the chick receptors expressed in brain (Figure 2). Deletion mutants were constructed for each receptor lacking either most of the intracellular domain (ΔICD) or the extracellular domain (ΔECD). trkB ΔICD essentially corresponds to the naturally occurring trkB splice variant designated T1 and lacking the tyrosine kinase domain. p75NTR ΔICD, as well as p75NTR ΔECD, co-immunoprecipitated with full-length trkB (Figure 3A). Likewise, trkB ΔICD and trkB ΔECD co-immunoprecipitated with full-length p75NTR. The weakest interaction was found between p75NTR and trkB ΔECD (Figure 3B). Also, p75NTR ΔECD and trkB ΔECD, as well as p75NTR ΔICD and trkB ΔICD, co-immunoprecipitated, but no detectable interactions could be seen when p75NTR ΔICD was expressed together with trkB ΔECD (Figure 3C). In sum, these mapping studies indicate that interaction between p75NTR and trkB involves the extracellular, as well as intracellular, domains of both receptors. However, the transmembrane sequence (which is common to all the constructs tested) does not seem to be sufficient for a stable interaction under our experimental conditions.

The trkB–p75NTR interaction is K-252a sensitive

The demonstration by immunoprecipitation of an interaction between trk receptors and p75NTR necessitates receptor over-expression. This leads to phosphorylation of the trk receptors due to their propensity to dimerize in a ligand-independent fashion. As our results indicate that the intracellular domain of trkB interacts with p75NTR (see above), the question arises as to whether the phosphorylation status of trkB influences the formation of the trkB–p75NTR complex. This possibility was tested using the kinase inhibitor K-252a. A dose-dependent reduction of the trkB–p75NTR interaction was observed, with a slight inhibition at 500 nM K-252a and a marked reduction at 1 μM K-252a (Figure 4). To test whether the effects of K-252a result from an action of the alkaloid on the intracellular domain of trkB, we used the trkB ΔECD construct with p75NTR. The interaction between the two receptors was inhibited almost completely with 500 nM K-252a. In contrast, the interaction of trkB T1 with p75NTR was not influenced by K-252a. Therefore these experiments indicate that the K252a-mediated inhibition of receptor interaction is due to its action on the intracellular domain of trkB, and that the phosphorylated form of trkB is predominantly involved in the interaction with p75NTR.

p75NTR influences the specificity of ligand-dependent phosphorylation of trkB

The close proximity of the two receptors may affect their ability to be activated by different ligands. As trkB can be activated in fibroblasts by three different neurotrophins (see below).

Mapping trkB and p75NTR interacting domains

We were interested in delineating the receptor domains involved in the interaction and designed various deletion constructs of p75NTR and trkB. The trkB receptor also exists as a splice-variant form lacking the tyrosine kinase domain, raising the question of whether the truncated form of this receptor would also interact with p75NTR.

To facilitate comparisons between the constructs, the HA epitope was preserved in the trkB constructs, as was the p75NTR detection epitope (Figure 2). Deletion mutants were constructed for each receptor lacking either most of the intracellular domain (ΔICD) or the extracellular domain (ΔECD). trkB ΔICD essentially corresponds to the naturally occurring trkB splice variant designated T1 and lacking the tyrosine kinase domain. p75NTR ΔICD, as well as p75NTR ΔECD, co-immunoprecipitated with full-length trkB (Figure 3A). Likewise, trkB ΔICD and trkB ΔECD co-immunoprecipitated with full-length p75NTR. The weakest interaction was found between p75NTR and trkB ΔECD (Figure 3B). Also, p75NTR ΔECD and trkB ΔECD, as well as p75NTR ΔICD and trkB ΔICD, co-immunoprecipitated, but no detectable interactions could be seen when p75NTR ΔICD was expressed together with trkB ΔECD (Figure 3C). In sum, these mapping studies indicate that interaction between p75NTR and trkB involves the extracellular, as well as intracellular, domains of both receptors. However, the transmembrane sequence (which is common to all the constructs tested) does not seem to be sufficient for a stable interaction under our experimental conditions.

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Deletion constructs of p75\textsuperscript{NTR} and HA-\textit{trkB}

Fig. 2. Deletion constructs of p75\textsuperscript{NTR} and HA-\textit{trkB}.

<table>
<thead>
<tr>
<th>p75</th>
<th>p75\textsuperscript{ΔICD}</th>
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<th>HA-\textit{trkB}</th>
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\textsuperscript{1} Antibody epitope
\textsuperscript{*} HA-Tag

Fig. 3. Co-immunoprecipitation of the deletion constructs of p75\textsuperscript{NTR} and \textit{trkB}. (A) Deletion constructs of p75\textsuperscript{NTR} co-immunoprecipitated with full-length HA-\textit{trkB}. (B) Full-length p75\textsuperscript{NTR} co-immunoprecipitated with deletion constructs of HA-\textit{trkB}. (C) Co-immunoprecipitation of deletion constructs of both receptors. The experiments were performed as described in Figure 1, except that 10% acrylamide SDS gels were used. Note that p75\textsuperscript{ΔICD} and HA-\textit{trkB}\textsuperscript{ΔECD} do not interact. The interaction of p75\textsuperscript{NTR} full-length with HA-\textit{trkB}\textsuperscript{ΔECD} is significantly weaker.

be activated by BDNF, NT4/5 and NT3, we asked whether p75\textsuperscript{NTR} increases the ligand selectivity of \textit{trkB}. Ligand-dependent \textit{trkB} phosphorylation was investigated in A293 cells stably transfected with a \textit{trkB} construct that can be readily activated by three different neurotrophins (\textit{trkB}-L, Strohmaier \textit{et al}., 1996). These cells were transiently transfected with various p75\textsuperscript{NTR} constructs. Whereas the BDNF-induced phosphorylation of \textit{trkB} remained unchanged with p75\textsuperscript{NTR} co-expression (Figure 5), that induced by NT4/5 and NT3 was clearly reduced. This increased selectivity was dose-dependent, being more prominent at lower neurotrophin concentrations and not readily apparent at 100 ng/ml neurotrophin (data not shown). p75\textsuperscript{NTR} \textsuperscript{ΔICD} also mediated the increased ligand
cell death or cell survival. Our study provides evidence
that this signalling may lead to results as different as
pressing since both receptors are now known to signal,
to bind the neurotrophins. This question is all the more
been raised ever since two different receptors were shown
signalling.

Discussion

The possibility of trk–p75NTR receptor association has
been raised ever since two different receptors were shown
to bind the neurotrophins. This question is all the more
pressing since both receptors are now known to signal,
and that this signalling may lead to results as different as
cell death or cell survival. Our study provides evidence
for a direct association between the trk receptors and
p75NTR, as demonstrated by co-immunoprecipitation. This
association is relevant to the trk function in intact cells,
as it leads to an increase in ligand specificity.

Previous biochemical studies on trk–p75NTR
interactions

The question of an association between the trk receptors
and p75NTR has already been addressed in previous studies
with trkA. Chemical cross-linking was used, either with
radiolabelled NGF (Huber and Chao, 1995), or by revers-
ibly cross-linking receptors expressed in Sf9 insect cells
(Gargano et al., 1997). Also, co-patching studies with fluoroescently labelled receptors (Wolf et al., 1995; Ross
et al., 1996) suggested a co-localization of the receptors
in cell membranes using Sf9 cells. Curiously, no such
evidence could be obtained in similar experiments using
trkB instead of trkA (Wolf et al., 1995; Ross et al., 1996).
Functionally, co-operation between p75NTR and all three
trk receptors has been obtained in transfected cells by
showing that co-expression increases responsiveness to
low neurotrophin concentrations, but in these and other
experiments the demonstration of co-immunoprecipitation
of p75NTR and trkB failed (Hantzopoulos et al., 1994). The
explanation as to why we eventually succeeded in
demonstrating a biochemical interaction between p75NTR
and all three trk receptors may lie in the tagging procedure.
It is possible that antibodies used previously to test
receptor association may have either interfered with the
formation of a trk–p75NTR receptor complex or did not
recognise the receptor complex. In particular, we note that
Gargano and colleagues documented the observation that,
whereas antibodies to p75NTR efficiently co-precipitated p75NTR
were insufficient to co-precipitate trkB (Gargano et al., 1997).
Also, it is conceivable that the A293 cells used in this
study offer a favourable context to study the interactions
between p75NTR and trk receptors, for example by provid-
ing cytoplasmic proteins that stabilise the interaction.
However, merely mixing detergent extracts of cells
expressing only one receptor type does not lead to receptor
association (see Results).

Functional evidence for a trk–p75NTR association

Whereas there is no doubt that the trk receptors play a
role in mediating the trophic effects of neurotrophins,
it is also clear that the expression of trk receptors does
not account for all neurotrophin receptor properties on
neurons. For example, NGF binds with high affinity to
sensory neurons (~10–11 M; Sutter et al., 1979), and most
of the sites formed by trkA are of a low-affinity type
(Mahadeo et al., 1994). Similar observations were made
with BDNF and trkB [compare for example Rodriguez-
Tébar and Barde (1988) with Dechant et al. (1993)]. In
addition, co-expression of both p75NTR and trkA in the
same cells leads to the formation of high-affinity receptors
(Hempstead et al., 1991; Mahadeo et al., 1994). That the
formation of high-affinity binding sites is of functional
significance is well established for NGF. For example,
studies with PC12 cells indicate a reduced activation of
trkA at low NGF concentrations when binding to p75NTR
is prevented (Barker and Shooter, 1994; see also Verdi
et al., 1994). Moreover, in mice carrying a deletion in
the NGF-binding domain of p75NTR, substantial sensory deficits have been observed, and neurons isolated from such mice display a decreased sensitivity to low concentrations of NGF (Lee et al., 1992, 1994; Davies et al., 1993). In line with this, two recent studies have shown that a mutated form of NGF unable to bind to p75NTR, but capable of activating trkA, is less active than wild-type NGF in supporting the survival of p75NTR/trkA expressing neurons at low ligand concentrations (Horton et al., 1997; Rydén et al., 1997).

Of special relevance to the present study are previous indications that an additional function of the trk–p75NTR association may be an increased ligand specificity. Thus, sympathetic neurons isolated from p75NTR−/− animals are more responsive to NT3 than wild-type neurons (Lee et al., 1994) and PC12 cells have an increased responsiveness to NT3 when p75NTR levels are reduced (Benedetti et al., 1993). Finally, there is evidence for a modulation of p75NTR, mediated sphingomyelin hydrolysis by trkA (Dobrowsky et al., 1995).

**Properties of the trk–p75NTR association**

Our experiments indicate that the interaction of p75NTR with all three trk receptors is stable enough to resist detergent solubilization in a buffer containing 1% Triton X-100. It is thus unlikely that the association results from hydrophobic interactions within the membrane, and co-transfection with constructs only able to interact with their transmembrane domains are in line with this interpretation. In this context, it is interesting to note that a previous study using a reversible cross-linker of trkA and p75NTR also concluded that transmembrane interaction is unlikely to participate significantly in receptor interactions (Gargano et al., 1997). Our mapping experiments indicate that both the extracellular and intracellular domains of the receptors seem to be sufficient to drive a stable association of the two receptors. This result is in agreement with that of Gargano et al. (1997), who used deletion constructs of trkA and p75NTR. However, the finding that the association with the intracellular domain of trkB is K252a-sensitive raises the possibility that in the absence of receptor phosphorylation, the interaction may be driven mainly by the extracellular domains of the receptor. The possibility therefore exists that the state of phosphorylation of the trk receptors modulates the strength of the interactions of the trk receptor with p75NTR, and our findings suggest that phosphorylation of trk would reinforce the interaction with p75NTR. Clearly not all tyrosine kinase receptors interact with p75NTR as no immunoprecipitation could be demonstrated with the EGF receptor.

Beyond co-immunoprecipitation, the most direct indication for an interaction between trk receptors and p75NTR is the increase in ligand specificity of trkB when p75NTR is co-expressed with trkB. The details of the mechanisms by which p75NTR increases ligand specificity are unclear. It is conceivable that the association of p75NTR with the trk receptors changes their conformation leading to increased ligand-binding specificity. Such a mechanism could also be responsible for the enhanced binding of NGF to trkA in the presence of p75NTR (Barker and Shooter, 1994; Verdi et al., 1994). In theory, signalling through p75NTR following ligand binding could also be a mechanism leading to increased ligand specificity. In particular, a recent report indicates that BDNF can activate p75NTR to cause serine phosphorylation of trkB (MacPhee and Barker, 1997). But as our results with p75NTR carrying a large deletion in the intracellular domain indicate, this construct is equally effective in increasing ligand specificity, compared with full-length p75NTR, we consider this possibility unlikely. Previously we have reported that a chick trkB splice variant lacking exon 9 coding for 11 amino acids in the extracellular juxtamembrane domain of trkB also shows an increased selectivity for BDNF, compared with NT4/5 and NT3, even in the absence of expression of p75NTR (Strohmaier et al., 1996). A similar variant of trkB with increased selectivity for BDNF was also found in human retinal pigmented epithelium (Hackett et al., 1998). Taken together, these results indicate that there are at least two different ways by which the selectivity of trkB for BDNF can be increased. This is particularly relevant in the context of numerous previous reports indicating that BDNF has trophic activities on a variety of neurons that differ from those of NT4/5, even though both ligands bind to trkB.

**Conclusion**

The results of this study point to a close association of trk and p75NTR receptors in cellular membranes and that one outcome of receptor association is an increased ligand specificity. Close association of these two different receptor types suggests that their signalling pathways may interact with each other as soon as they are activated.

**Materials and methods**

**Cell culture and reagents**

A293 cells (Graham et al., 1977) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco-BRL) supplemented with 10% fetal calf serum (Boehringer Mannheim) at 37°C and 5% CO2. The A293 cell line stably expressing chick trkB had been established previously and was used as described in Dechant et al. (1993). K-252a was from the Alexis Corporation.

**Construction of p75NTR- and trk-expression plasmids and deletion mutants**

Rat p75NTR full-length and the p75NTR extracellular domain (ΔECD) were cloned in the mammalian expression vector pcDNA3 (Invitrogen). The intracellular domain of p75NTR (ΔICD), rat trkB and the trkB constructs, as well as trkA and trkC, were cloned in the pRC/CMV AC7 vector, a derivative of the pRC/CMV vector (Invitrogen), which contains the BM40 signal peptide (Mayer et al., 1994). In all cases expression was driven from the CMV promoter.

Rat p75NTR was isolated from the pGEM1 vector by cloning it with EcoRI and Apal in a pcDNA3 expression vector leaving the 5′ and part of the 3′ untranslated region (UTR) intact. Using rat p75NTR subcloned in pcDNA3 as template, a one-step PCR strategy was performed to delete the 118 C-terminal amino acid residues (p75NTRΔICD). Primers were: 5′-CTGGAATTCGCGGCCGCTCAG; and the 3′ antisense oligonucleotide with a translation stop codon inserted: 3′-GCCAGGCCGCCCT- CAGCCACTGTCGCTGTGCAGTT. The resulting cDNA for the ΔICD construct was generated by performing PCR with the primers 5′-GCCCCCTGAGTCGCCGCCACACCGAGC- AAC and 3′-GATCGATGCGGCCCTCAGCCTGGATTGCG- AG, which deletes the 245 N-terminal amino acid residues. The construct was subcloned into pKH1 and NotI into pRC/CMV AC7 and sequenced.

Rat trkB with the fused HA tag was cloned in the pRC/CMV AC7 vector and used as a template to generate the construct trkBΔECD, in which the 398 N-terminal amino acid residues are deleted. The primers used were: 5′-GCCGCCCTGAGTCGCCGCCACACCGAGC- AAC and 3′-GATCGATGCGGCCCTCAGCCTGGATTGCG- AG, which deletes the 245 N-terminal amino acid residues. The construct was subcloned into pKH1 and NotI into pRC/CMV AC7 and sequenced.
Interactions between trk and p75NTR

GTGCAGCCGGCTACGCTGTTAGGGTCAGAGTA. Nhel and NotI were used for subcloning into pRC/CMV AC7. The T1 isoform of trkB was cloned from a pCMV-5 trkB.1 construct using HindIII and Xbal to fuse it to the HA-tagged N-terminus of trkB in the vector pB-KS followed by subcloning into pRC/CMV AC7 with Nhel and NotI. Rat trkA and rat trkC were similarly cloned with the HA tag into the vector pRC/CMV AC7. The EGF receptor construct pRk5 was kindly provided by A.Ullrich.

Neurotrophins and antibodies

Recombinant BDNF, NT3 and NT4/5 produced in Chinese hamster ovary cells (CHO) were a gift from Genentech, Inc. In some experiments Escherichia coli-derived recombinant neurotrophins were also used (Regeneron Amgen Partners). The antibodies used included anti-human p75NTR pAb (Promega) as well as a rabbit polyclonal antiserum #17 generated against a peptide corresponding to amino acids 248–274 of the cytoplasmic domain of chick p75NTR (kindly provided by A.Rodriguez-Tebar). Monoclonal anti-HA for immunoprecipitation was used from hybridoma supernatants (a kind gift from S.Werner). Western blotting was performed with anti-HA mouse mAb clone 12CA5 1 μg/ml (Boehringer Mannheim). A rabbit anti-trk antiserum (pantrk) recognizing all trk proteins (raised against a peptide corresponding to the last 14 amino acids of the chick trkA sequence) had been established previously (Schröpel et al., 1995). Monoclonal anti-phosphotyrosine (clone 4G10) was purchased from Upstate Biotechnology. Anti-EGF receptor antibodies were #108 (Waterfield et al., 1982) and RK2 (Kris et al., 1985).

Transfection of cell lines

A293 cells were transfected by the calcium phosphate precipitation protocol using the method of Chen and Okayama (1987). Within one experiment, the amount of DNA was kept constant by supplementing samples with pcDNA3 vector DNA. For transient expression, cells were lysed 2 days after transfection. Transfection efficiency was analysed by parallel transfection with a pCMV-GFP vector (pEGFP-N1, Clontech).

Immunoprecipitation

Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed at 4°C in 1 ml lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 10 μg/ml Leupeptin and 1 mM PMSF) followed by centrifugation at 4°C with 10,000 g for 10 min. The supernatants were incubated with 200 μl anti-HA antibody for 2–5 h at 4°C followed by precipitation with protein A-Sepharose 6MB beads (Pharmacia Biotech) overnight at 4°C. The EGF receptor was precipitated with 2 μl antibody #108. After washing the beads three times with lysis buffer, containing 0.1% Triton X-100 instead of 1% and no Leupeptin and PMSF, the proteins were eluted by boiling in 30 μl Laemmli loading buffer for 5 min. Samples were subsequently processed by Western blotting.

Receptor phosphorylation studies

A293–citrB-L cells were grown in serum-free medium overnight, incubated in fresh serum-free medium for another 3–5 h and neurotrophins were added for 5 min at 37°C. Immunoprecipitation was performed as described above, except that the lysis buffer was supplemented with 5 mM orthovandate and 3 mM EDTA. The beads were washed with a buffer containing 1 mM orthovandate. triB was immunoprecipitated with 2 μl anti-trk antisemum for 1–2 h at 4°C followed by the addition of protein A-Sepharose beads.

Western blotting

Proteins were separated on 7% or 10% polyacrylamide gels and subsequently transferred onto Immobilon (Millipore) membranes. Following incubation with 2% nonfat milk powder in Tris-buffered saline-Tween (TBST; 50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween 20), the membranes were incubated overnight at 4°C with anti-human p75NTR pAb (Promega), diluted at 1:2000 in blocking buffer or with the p75NTR antisemur #17, diluted at 1:500 in blocking buffer, followed by incubation for 1 h at room temperature with goat anti-rabbit IgG-POD (Pierce, 1:10 000 in TBST). The EGF receptor was similarly detected with the antibody RK2, diluted at 1:1000.

After blocking with a gelatin solution (0.5% gelatin, 5 mM EDTA in TBST), phosphorylated triB receptors were detected using the anti-phosphotyrosine antibody 4G10 overnight at room temperature, diluted at 1:400 in gelatine blocking buffer, followed by incubation for 1 h at room temperature with goat anti-mouse IgG-POD (Pierce, 1:10 000 in TBST). triB receptors were detected following overnight incubation at 4°C with the pantrk antibody, diluted at 1:1000 in TBST/2% milk or with the anti-HA antibody (Boehringer Mannheim), diluted at 1:1000 in TBST/2% milk.

The immune complexes were detected using the ECL detection system (Amersham) and exposure to autoradiographic film (Fuji).

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References


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