Functional interaction between p75<sup>NTR</sup> and TrkA: the endocytic trafficking of p75<sup>NTR</sup> is driven by TrkA and regulates TrkA-mediated signalling

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INTRODUCTION

The mammalian neurotrophin family, which includes NGF (nerve growth factor), brain-derived neurotrophic factor, neurotrophin 3 and neurotrophin 4/5, was initially characterized based on the ability of its members to promote the survival and differentiation of neurons in the peripheral and central nervous systems [1,2]. These effects result from the activation by the specific neurotrophin of the high-affinity tyrosine kinase receptors TrkA, TrkB and TrkC, and/or of the low-affinity neurotrophin receptor p75<sup>NTR</sup>. Neurotrophin binding to the Trk receptors results in dimerization and activation of their intrinsic tyrosine kinase activity, leading in turn to activation of the p21<sup>B</sup>ck/ERK, extracellular-signal-regulated kinase; FRT cells, Fischer rat thyroid cells; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor κB; NGF, nerve growth factor; NHS, N-hydroxysuccinimido; NHS-SS-biotin, sulphosuccinimido-6-(biotinamide) hexanoate; NTR, neurotrophin receptor.

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The topology and trafficking of receptors play a key role in their signalling capability. Indeed, receptor function is related to the microenvironment inside the cell, where specific signalling molecules are compartmentalized. The response to NGF (nerve growth factor) is strongly dependent on the trafficking of its receptor, TrkA. However, information is still scarce about the role of the cellular localization of the TrkA co-receptor, p75<sup>NTR</sup> (where NTR is neurotrophin receptor), following stimulation by NGF. It has been shown that these two receptors play a key role in epithelial tissue and in epithelial-derived tumours, where the microenvironment at the plasma membrane is defined by the presence of tight junctions. Indeed, in thyroid carcinomas, rearrangements of TrkA are frequently found, which produce TrkA mutants that are localized exclusively in the cytoplasm. We used a thyroid cellular model in which it was possible to dissect the trafficking of the two NGF receptors upon neurotrophin stimulation. In FRT (Fischer rat thyroid) cells, endogenous TrkA is localized exclusively on the basolateral surface, while transfected p75<sup>NTR</sup> is selectively distributed on the apical membrane. This cellular system enabled us to selectively stimulate either p75<sup>NTR</sup> or TrkA and to analyse the role of receptor trafficking in their signalling capability. We found that, after binding to NGF, p75<sup>NTR</sup> was co-immunoprecipitated with TrkA and was transcytosed at the basolateral membrane. We showed that the TrkA–p75<sup>NTR</sup> interaction is necessary for this relocation of p75<sup>NTR</sup> to the basolateral side. Interestingly, TrkA-specific stimulation by basolateral NGF loading also induced the TrkA–p75<sup>NTR</sup> interaction and subsequent p75<sup>NTR</sup> transcytosis at the basolateral surface. Moreover, specific stimulation of p75<sup>NTR</sup> by NGF activated TrkA and the MAPK (mitogen-activated protein kinase) pathway. Our data indicate that TrkA regulates the subcellular localization of p75<sup>NTR</sup> upon stimulation with neurotrophins, thus affecting the topology of the signal transduction molecules, driving the activation of a specific signal transduction pathway.

Key words: compartmentalization, polarity, p75<sup>NTR</sup>, trafficking, TrkA.
thyroid carcinomas, and these chimaeric proteins show an altered subcellular localization [20]. Moreover, mutant Trks also show association with particular downstream signalling molecules in the thyroid. In fact, the fusion proteins TRK-T1 and TRK-T3 (based on the receptor for NGF encoded by \textit{NTRK1} gene) activate FRS2 (fibroblast growth factor receptor substrate 2) and FRS3 in thyroid cells [21]. Thus the cellular localization of these receptors is a key element in the interaction and activation of downstream signalling molecules.

FRT (Fischer rat thyroid) epithelial cells have been used previously to analyse protein trafficking [22,23]. When grown in filter chambers, they form polarized monolayers in which it is possible to discriminate between the apical and basolateral surfaces, which are physically separated by the tight junctions [24,25]. Interestingly, we show here that FRT cells express endogenous and functional TrkA, which is localized exclusively on the basolateral surface, while transfected p75NTR is distributed selectively on the apical membrane [26]. Therefore this cellular system enables us to selectively stimulate either p75NTR or TrkA and to analyse their intracellular trafficking upon stimulation. We demonstrate that, after binding to NGF added from the apical surface, p75NTR co-immunoprecipitates with TrkA, and then the two receptors co-localize on the basolateral surface. Moreover, the TrkA–p75NTR interaction is necessary for relocation of p75NTR to the basolateral side. TrkA-specific stimulation induced by the basolateral addition of NGF also induces the TrkA–p75NTR interaction and p75NTR transcytosis at the basolateral surface. Our data indicate that TrkA drives the endocytic trafficking and subcellular localization of p75NTR upon stimulation with neurotrophins. Interestingly, p75NTR-specific stimulation from the apical surface with NGF induces TrkA activation and the subsequent signalling cascade. A p75NTR mutant lacking the cytoplasmic tail is not transcytosed at the basolateral surface upon stimulation by NGF and does not activate TrkA. These data demonstrate that the TrkA–p75NTR interaction upon endocytosis induces the formation of a signalling complex.

MATERIALS AND METHODS

Reagents and antibodies

Cell culture reagents were purchased from Gibco, Protein A–Sepharose was from Pharmacia and Protein G–Sepharose was from Sigma. Sulpho-NHS (\textit{N}-hydroxysuccinimido) derivatives and streptavidin–agarose beads were from Pierce (Rockford, IL, U.S.A.). Anti-p75NTR monoclonal antibody and anti-p75NTR rabbit polyclonal antibody were gifts from Andrè Le Bivic (IBDM, Marseille, France). A rabbit polyclonal antibody that specifically recognizes phosphorylated TrkA in FRT cells was also detected using anti-TrkA rabbit polyclonal antibodies from Upstate Biotechnology (Lake Placid, NY, U.S.A.).

7S NGF (a mixture of several different subunits of NGF, i.e. \(\alpha_3\beta_2\)) was from Upstate Biotechnology. K252a was from Calbiochem–Novabiochem Corp. (La Jolla, CA, U.S.A.). Monoclonal antibodies against rat DPPIV (dipeptidyl peptidase IV) (CLB4/40), the B-subunit of rat Na/K-ATPase (IEC1/48) and Ag 35–40 (antigen of 35–40 kDa) (CLB1/41) have all been described previously [22].

Cell culture and neurotrophin treatment

FRT cells stably expressing p75NTR (E1A5) [26] were grown for 5 days on Transwell filters (Costar Corp., Cambridge, MA, U.S.A.) in F12 Coon’s modified medium containing 5% (v/v) fetal bovine serum. Cells were serum-starved in F12 Coon’s modified medium containing 0.2% (w/v) BSA for 24 h before neurotrophin treatment, and then stimulated with NGF (100 ng/ml) in either the apical or the basolateral medium. In TrkA-phosphorylation inhibition experiments, K252a was added 2 h before neurotrophin stimulation and was present throughout the stimulation time.

Immunofluorescence and confocal microscopy

FRT cells cultured on 12 mm-diameter Transwells for 5 days were fixed in 2% (v/v) paraformaldehyde in Dulbecco’s PBS solution containing 1.8 mM Ca\(^{2+}\) and 0.5 mM Mg\(^{2+}\) (CM-PBS). Cells were treated with 0.075% (w/v) saponin in CM-PBS containing 0.2% (w/v) gelatin and were incubated with the specific antibodies in the same buffer. After washes with CM-PBS containing saponin and gelatin, filters were incubated with fluorescein- or rhodamine-conjugated goat anti-mouse or anti-rabbit secondary antibodies, each diluted 1:50. Immunofluorescent images were collected using a confocal microscope (Zeiss LSCM 410).

Biotinylation assays

Confluent monolayers on Transwells were labelled overnight with 0.5 mCi/ml [\(^{35}\)S]Met/Cys and 1 mCi/ml [\(^{35}\)S]Cys (Amersham, Arlington Heights, IL, U.S.A.) and were biotinylated and processed for immunoprecipitation, as described previously [23]. Biotinylated antigens were then precipitated with streptavidin–agarose beads. After boiling the beads in Laemmli buffer, supernatants were analysed by SDS/PAGE and fluorography.

Immunoprecipitation of p75NTR and p75NTR-associated proteins

Confluent cell monolayers on Transwells were starved for 24 h before NGF treatment, stimulated with neurotrophins from the apical or basolateral side as indicated, washed two times with ice-cold PBS containing 1 mM Na\(_2\)VO\(_4\), and lysed in 500 \(\mu\)l of buffer A. Equal amounts of proteins were incubated for 2 h at 4°C with anti-p75NTR monoclonal antibody at a dilution of 1:200, as described [26].

Inhibition of endocytosis

Cells were incubated in hypertonic medium [120 mM NaCl, 12 mM MgSO\(_4\), 1 mM EDTA, 15 mM sodium acetate, 1% (w/v) BSA, 100 mM Hepes, pH 7.0, 5 mM KCl, 0.4 M sucrose] for 30 min before and during stimulation, and then lysed as described previously [27].

Biotin internalization assay

Cells grown on filters were biotinylated using NHS-SS-biotin [sulphosuccinimidyl-6-(biotinamide) hexanoate] from the apical or basolateral plasma membrane domain. Sample filters were incubated at 37°C for 15 min in the presence and or in the absence of NGF, while control filters were kept at 4°C. After washing all filters with ice-cold PBS containing 0.1 mM CaCl\(_2\) and 1 mM MgCl\(_2\), reduction with glutathione (50 mM) was carried out twice from the apical or basolateral side at 4°C. Subsequent lysis, precipitation with streptavidin beads, and SDS/PAGE were performed as described above. Western blot analysis was carried out using the appropriate antibody.
Surface immunoprecipitation and endocytosis assay

Cells grown on filters were washed with culture medium and incubated for 2 h at 4°C with anti-p75NTR antibody diluted 1:200 in the culture medium added to the apical surface. Filters were then washed five times on ice with culture medium containing 5% (v/v) fetal bovine serum. Sample filters were incubated at 37°C for 15 min in the presence or absence of NGF, while control filters were kept at 4°C. Cells were lysed as described above. Protein G-Sepharose was added to the lysate and rocked for 1 h at 4°C. The immunocomplexes were subjected to SDS/PAGE, then blotted on to nitrocellulose. The supernatant of the surface immunoprecipitation was incubated overnight with anti-p75NTR monoclonal antibody at a dilution of 1:200. Protein G-Sepharose was added to the lysate for 1 h. The immunocomplexes, representing the total amount of p75NTR in each sample, were recovered and subjected to Western blot analysis using the appropriate antibody.

Electrophoretic mobility shift assay

Following 30 min of stimulation with NGF, cells were washed in ice-cold PBS, scraped and pelleted by centrifugation at 2300 g for 5 min at 4°C. The cells were lysed in hypotonic buffer (20 mM Hepes, pH 7.9, 1 mM MgCl2, 0.1 mM EGTA, 15 mM KCl, 1 mM PMSF). Nuclei were pelleted at 400 g for 10 min at 4°C and then lysed in high-salt buffer (20 mM Hepes, pH 7.9, 1 mM MgCl2, 0.1 mM EGTA, 15 mM KCl, 400 mM NaCl, 1 mM PMSF). The insoluble material was recovered at 16500 g for 20 min at 4°C. The supernatants containing the nuclear proteins were frozen at −80°C until used in the binding reaction. The binding of activated NFκB (nuclear factor κB) in the lysates to an oligonucleotide corresponding to the κ light chain immunoglobulin enhancer was assessed as described in [7].

RESULTS

Internalization of p75NTR following stimulation by NGF from the apical membrane

To determine whether TrkA is expressed in polarized epithelial FRT cells, we used both an anti-TrkA antibody against the entire ectodomain and an anti-TrkA antibody against a C-terminal peptide. We found that FRT cells contained a protein of the same molecular mass as endogenous TrkA from PC12 cells, and that the level of expression in these two cell lines was comparable (Figure 1A, upper panel).

By confocal analysis, we found that TrkA was localized exclusively on the basolateral domain of the plasma membrane (Figure 1A, lower panels). To exclude the possibility that a mutation in the gene encoding TrkA in FRT cells was responsible for its exclusively basolateral localization, we transfected these cells with a cDNA encoding wild-type rat TrkA and analysed its distribution in stably expressing clones. The transfected receptor was also expressed exclusively on the basolateral membrane (results not shown).

It has been demonstrated that TrkA is internalized upon stimulation by NGF, and that this event plays a key role in TrkA function [15,17]. In contrast, the role of p75NTR internalization is still unclear. To investigate this point, we first investigated whether p75NTR is internalized following stimulation by NGF. To this end, we grew FRT cells expressing transfected p75NTR and endogenous TrkA (E1A5 clone) on filters and used an endocytosis assay based on the use of a cleavable biotin analogue (NHS-SS-biotin) [28]. p75NTR at the apical surface was biotinylated, and then NGF was added from the apical surface for 15 min. We found that approx. 20% of p75NTR was internalized after stimulation with NGF from the apical side (Figure 1B).

p75NTR co-localizes with TrkA upon stimulation by NGF

Since p75NTR was internalized upon apical loading of NGF, we decided to analyse p75NTR trafficking in polarized FRT cells following neurotrophin stimulation. Surprisingly, by indirect immunofluorescence, we found that, within 15 min of apical NGF addition, a significant amount of p75NTR was recolocalized from the apical to the basolateral domain, where it co-localized with TrkA (Figure 1C). This behaviour was specific for p75NTR in response to NGF, as localization of the apical marker DPPIV and the basolateral markers Na/K-ATPase (Figure 1D) and Ag 35–40 (not shown) [22,23] did not change following apical stimulation with NGF. To confirm these data, we quantified the membrane localization of p75NTR, DPPIV and Ag 35–40 before and after NGF addition by using selective domain biotinylation (Figure 1D).

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After its internalization, p75NTR co-immunoprecipitates with TrkA

To examine whether p75NTR and TrkA interact following apical stimulation with NGF in FRT cells, we performed co-immunoprecipitation experiments before and after treatment with NGF. No co-immunoprecipitation was observed in polarized E1A5 cells in the absence of stimulation (Figure 3A). However, after 15 min of p75NTR-specific stimulation with NGF from the apical side, TrkA co-immunoprecipitated with p75NTR (Figure 3A). To demonstrate that p75NTR bound TrkA after being internalized from the apical surface following NGF stimulation, we performed
Figure 1  Stimulation of p75NTR by the apical addition of NGF induces p75NTR transcytosis at the basolateral surface

(A) Upper panel: Immunoblotting with anti-TrkA antibodies against the N-terminus (left) and against a C-terminal peptide (right). Lower panel: FRT cells expressing p75NTR (E1A5 clone) were grown on filters for 5 days and then subjected to indirect immunofluorescence using anti-TrkA or anti-p75NTR antibodies as indicated in the Materials and methods section. A, apical membrane; BL, basolateral membrane. (B) E1A5 cells were biotinylated with NHS-SS-biotin (BIOT.) as described in the Material and methods section. Immunocomplexes and 1/10 of the total extracts were subjected to Western blot analysis using an anti-p75NTR antibody. Glut., glutathione. (C) Treated and control filters were divided into four parts and subjected to indirect immunofluorescence carried out using the appropriate antibodies as indicated. (D) Proteins were labelled in vivo using [35S]Met and [35S]Cys for 14 h. After p75NTR-specific stimulation with NGF, surface proteins were labelled from the apical or basolateral surfaces with biotin as described in the Materials and methods section. Proteins were immunoprecipitated with the appropriate antibody, boiled in 0.5% SDS, diluted in lysis solution, and immunoprecipitated again with streptavidin to identify the biotinylated fraction. Proteins were separated by SDS/PAGE, dried and exposed to autoradiography. Aliquots containing 1/10 of the total immunoprecipitated proteins are shown as a control on the right-hand side. These experiments are representative of at least three independent experiments.

We analysed whether TrkA is internalized at steady state in FRT cells. Using an endocytosis assay based on the use of a cleavable biotin analogue (NHS-SS-biotin), we demonstrated that, in the absence of stimulation, 25% of TrkA was internalized, and that apical addition of NGF did not change this rate of internalization (Figure 3C), suggesting that in FRT cells TrkA recycles continuously at the basolateral surface.

p75NTR is internalized following activation of TrkA by NGF stimulation from the basolateral membrane

We next assessed whether stimulation of TrkA by the basolateral addition of NGF induced p75NTR transcytosis at the basolateral surface. First we analysed whether basolateral addition of NGF affected TrkA internalization. Using the endocytic assay, we demonstrated that the endocytic pool of TrkA increased following 15 or 30 min of stimulation by NGF (Figure 4A). Indirect immunofluorescence analysis showed that, after 15 min of NGF basolateral loading, p75NTR was not completely apical; an intracellular and basolateral signal was also detected (Figure 4B). Indeed, following 30 min of NGF basolateral addition, p75NTR was transcytosed at the basolateral surface (Figure 4B). We next investigated whether the p75NTR-TrkA interaction occurred following basolateral stimulation by NGF. Immunoprecipitation experiments showed that TrkA was associated with p75NTR after 15
TrkA drives p75NTR endocytic trafficking

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Figure 2 Blockade of endocytosis inhibits p75NTR transcytosis at the basolateral surface

(A) Cells were incubated in hypertonic medium for 30 min before and during stimulation with NGF. Treated and control filters were subjected to indirect immunofluorescence using anti-p75NTR monoclonal antibody. (B) Following apical addition of NGF, electrophoretic mobility shift assay analysis of nuclear extracts was performed as described in the Materials and methods section. C indicates competition of protein–probe binding with unlabelled specific double-stranded DNA oligonucleotides; U–C indicates competition carried with unspecific unlabelled oligonucleotides; N.S. indicates not stimulated. (C) E1A5 cells were stimulated by the apical (A) or basolateral (BL) addition of NGF. Western blot analysis was carried out using antibodies against phosphorylated TrkA (P-TrkA) and total TrkA. These experiments were repeated more than three times, with similar results.

Figure 3 Stimulation of p75NTR by the apical addition of NGF induces p75NTR–TrkA interaction

(A) E1A5 cells were grown and stimulated from the apical medium, as described for Figure 1. Protein extracts from treated and control cells were immunoprecipitated with anti-p75NTR antibody. Western blot analysis was carried out using an anti-TrkA (upper) or an anti-p75NTR (lower) antibody. (B) Surface p75NTR was labelled using an anti-p75NTR antibody as described in the Materials and methods section. Immunocomplexes and 1/10 of the total extract were separated on SDS/8%-PAGE. Western blot analysis was carried out using anti-(phosphorylated TrkA) (P-TrkA), anti-TrkA or anti-p75NTR antibodies. ‘endo block’ indicates that endocytosis was blocked. (C) E1A5 cells were grown and biotinylated with NHS-SS-biotin as described in the Materials and methods section. Protein extracts from treated and control cells were immunoprecipitated with streptavidin. Immunocomplexes (BIOT.) and 1/10 of the total extracts were separated on SDS/8%-PAGE. Western blot analysis was carried out using an anti-TrkA antibody. Glut., glutathione. These experiments are representative of at least three independent experiments.

and 30 min of NGF basolateral loading (Figure 4C). These data suggest that the TrkA–p75NTR interaction occurred before p75NTR transcytosis at the basolateral surface. Indeed, we detected a very low amount of TrkA associated with p75NTR after 10 min of NGF basolateral loading (Figure 4C). Thus we hypothesized that when the endocytic pool of either TrkA or p75NTR is increased by stimulation with NGF, the two receptors interact in an endocytic compartment.

We decided to investigate whether the p75NTR–TrkA interaction that occurs upon basolateral NGF addition takes place at the plasma membrane or in an intracellular compartment. To discriminate between the surface and intracellular localization of p75NTR following 15 min of NGF basolateral loading, we performed indirect immunofluorescence experiments in both the absence and the presence of saponin detergent. In the absence of saponin treatment, the antibodies could bind only the proteins localized at the cell surface, while proteins localized in intracellular compartments were undetectable. Under these conditions p75NTR showed only apical localization (Figure 4D). In contrast, in the presence of the detergent we detected p75NTR also on the basolateral side of the cells (Figure 4D), demonstrating that following 15 min of basolateral NGF loading, p75NTR was localized in an intracellular compartment.

p75NTR-specific stimulation activates TrkA and the MAPK pathway

Since we found that TrkA associated with p75NTR was phosphorylated after 15 min of apical stimulation by NGF (Figure 3B),
we investigated further whether p75NTR-specific stimulation by NGF could affect TrkA signalling. We analysed TrkA activation following either apical or basolateral addition of NGF by Western blot using an anti- (phosphorylated TrkA) antibody. We found that TrkA was activated by 2 min of NGF stimulation from the basolateral side (Figure 5A). Surprisingly, we also observed TrkA phosphorylation after 15 min of apical addition of NGF (Figure 5A). The Trk-specific inhibitor K252a (10 nM) blocked TrkA phosphorylation (Figure 5A). Therefore we analysed whether the MAPK pathway was activated following apical stimulation of p75NTR. Phosphorylation of ERK1/2 occurred within 2 min of direct TrkA activation by NGF addition from the basolateral side and also upon 15 min of NGF stimulation from the apical surface (Figure 5B). K252a at 10 nM completely abolished ERK1/2 activation following p75NTR-specific stimulation with NGF (Figure 5B), demonstrating that ERK1/2 phosphorylation was TrkA-dependent.

To confirm further that the formation of an intracellular complex between p75NTR and TrkA was responsible for TrkA activation, we transfected FRT cells with a p75NTR mutant (NTRPLAP) carrying a glycosylphosphatidylinositol anchor instead of the transmembrane and cytoplasmic domains. This chimaera was also
localized exclusively on the apical surface. This mutant did not transcytose to the basolateral surface after 15 min of NGF apical loading (Figure 5C). Indeed, TrkA activation did not occur in these cells upon apical stimulation by NGF, whereas basolateral addition of NGF did activate TrkA (Figure 5D). These data clearly demonstrate that both p75NTR transcytosis at the basolateral surface and TrkA activation were not a consequence of the presence of small amounts of TrkA on the apical surface that was undetectable by indirect immunofluorescence.

**p75NTR** is transcytosed to the basolateral surface following interaction with TrkA

To further confirm that the p75NTR–TrkA interaction was necessary for p75NTR transcytosis at the basolateral surface, we used increasing concentrations of K252a, which has been shown to inhibit the p75NTR–TrkA interaction in a dose-dependent manner [29]. In the presence of 10 nM K252a, p75NTR and TrkA still co-immunoprecipitated, while at higher concentrations K252a blocked the p75NTR–TrkA interaction (Figure 6A). Using confocal microscopy we found that high concentrations of K252a totally blocked p75NTR transcytosis (Figure 6B). In contrast, in the presence of low K252a concentrations that did not impair formation of the complex, p75NTR was transcytosed at the basolateral surface (Figure 6B). These data demonstrate that TrkA activation was not necessary for p75NTR transcytosis at the basolateral surface. Indeed, TrkA activation did not occur in the presence of 10 nM K252a (Figure 6C).

**DISCUSSION**

Two new aspects have emerged recently in the study of neurotrophin receptor signalling. First, it has been demonstrated that these receptors play a key role not only in nervous system differentiation, but also in the correct differentiation of other tissues [10,30]. Interestingly, these two receptors also play a key role in the progression of non-neural carcinogenesis [31–33]. Secondly, numerous analyses have demonstrated that TrkA signalling is regulated by its intracellular localization [15,17]. Although it has been shown that the low-affinity NGF receptor p75NTR co-operates with TrkA and enhances its responsiveness to NGF [6,34], the role of p75NTR is still debated because its function is cell-type-dependent.

The present study has demonstrated that in polarized epithelial FRT cells p75NTR and TrkA are compartmentalized in the apical and basolateral domains respectively of the plasma membrane. This enabled us to selectively stimulate either p75NTR or TrkA. Our new findings are summarized as follows: (1) upon p75NTR-specific stimulation by NGF, this receptor binds TrkA; (2) this interaction occurs after receptor endocytosis, and leads to the transcytosis of p75NTR, which then co-localizes with TrkA on the basolateral surface; (3) the p75NTR–TrkA interaction is necessary for p75NTR transcytosis at the basolateral surface; (4) coated pit-mediated endocytosis is involved in p75NTR internalization and transcytosis at the basolateral surface; (5) TrkA stimulation by the basolateral addition of NGF also leads to p75NTR–TrkA association and p75NTR transcytosis; and (6) in FTR cells, the p75NTR–TrkA interaction induced by p75NTR-specific stimulation leads to TrkA phosphorylation and MAPK activation.

We show that p75NTR and TrkA are co-immunoprecipitated following either apical or basolateral addition of NGF. Indeed, blocking the p75NTR–TrkA interaction by using high concentrations of the TrkA-specific inhibitor K252a prevents p75NTR transcytosis at the basolateral membrane. We demonstrate clearly for the first time that p75NTR–TrkA binding induces the transcytosis of p75NTR at the basolateral side, showing that TrkA drives the endocytic trafficking of p75NTR, leading to a change in its topology in the cell. In fact, it is highly unlikely that p75NTR would recycle by itself to the basolateral membrane after neurotrophin binding, as we and others have shown that this receptor does not contain any basolateral sorting signal [26,35]. In polarized epithelial cells, early apical and basolateral endosomes are functionally and topologically distinct [36]; however, it has been proposed that they can be interconnected via a basolateral sorting machinery [37]. Therefore, after apical internalization, p75NTR could transcytose to the basolateral side only if driven by specific signals or interactions, suggesting that the same molecular mechanism could be used in other cell types. These data are in agreement with and help to explain further previous studies on the role of the p75NTR–TrkA complex. It has been demonstrated that TrkA immobilizes p75NTR molecules at the membrane, and that the change in p75NTR mobility requires the intact cytoplasmic domains of both...
p75NTR and TrkA [38]. Conversely, it has also been shown that TrkA mobility at the membrane is regulated by p75NTR [39].

The present study demonstrates that both p75NTR and TrkA are internalized in FRT cells. We also show that endocytosis is necessary for the p75NTR–TrkA interaction. Indeed, the two receptors do not co-immunoprecipitate upon the apical addition of NGF when endocytosis is inhibited. Thus we hypothesize that the two receptors interact following internalization. Our hypothesis is supported by a recent study which found that a green fluorescent protein–TrkA chimaera shows highly dynamic trafficking between the cell surface and the endocytic compartment [40].

Interestingly, our results characterize further the relationship between receptor trafficking and activity. We show for the first time that the physical interaction between TrkA and p75NTR induces TrkA activation and the MAPK signalling cascade. Indeed, TrkA phosphorylation did not occur when the p75NTR–TrkA interaction was abolished by blocking endocytosis. These data further demonstrate that the p75NTR–TrkA interaction occurs in an endocytic compartment, because we show that blockade of endocytosis did not impair signalling starting from the plasma membrane. These results indicate that the p75NTR–TrkA interaction results in the formation of a signalling complex. In agreement with the present study, it has been shown that forced dimerization of TrkA by cross-linking with anti-TrkA antibodies induces TrkA auto-phosphorylation and a TrkA-dependent signalling cascade [41]. Lee and Chao [42] also reported that activation of TrkA can occur in the absence of NGF. Moreover, it is known that a mutation in the cytoplasmic tail of p75NTR blocks the phosphorylation of intracellular substrates of TrkA upon stimulation by NGF [43]. Indeed, it has been demonstrated recently that p75NTR interacts with Src, enhancing its TrkA-mediated activation [44]. Moreover, in sympathetic vesicles, co-expression of p75NTR induces 8-fold higher tyrosine phosphorylation of TrkA [45]. Our data clearly demonstrate that, upon activation, p75NTR can recruit signalling molecules in a complex with TrkA, inducing the activation of a downstream signalling cascade.

The present study has relevance for understanding the role of p75NTR and TrkA in non-neuronal carcinogenesis, especially in epithelial-derived tumours. The signalling proteins that are recruited in FRT cells by the p75NTR–TrkA complex upon stimulation by NGF remain to be elucidated, and we are investigating this further. Interestingly, FRT cells expressing p75NTR represent a useful cellular model for identifying signalling molecules involved in either p75NTR or TrkA signalling. This cellular system enables us to dissect the endocytic trafficking of these receptors and the activation of downstream signalling molecules, shedding new light on the function of these receptors in non-neuronal carcinogenesis.

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REFERENCES

29 Bibel, M., Hoppe, E. and Barde, Y.-A. (1999) Biochemical and functional interactions between the neurotrophin receptors Trk and p75NTR. EMBO J. 18, 616–622

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TrkA drives p75<sup>NTR</sup> endocytic trafficking


