PKA phosphorylates the p75 receptor and regulates its localization to lipid rafts

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Although a large number of studies have been carried out on the diverse effects mediated by the common neurotrophin receptor p75^{NTR}, little is known about the molecular mechanisms by which p75^{NTR} initiates intracellular signal transduction. We identified a variant of the β catalytic subunit of cAMP-dependent protein kinase (PKAC β) as a p75^{NTR}-interacting protein, which phosphorylates p75^{NTR} at Ser304. Intracellular cAMP in cerebellar neurons was accumulated transiently by ligand binding to p75^{NTR}. Activation of cAMP-PKA is required for translocation of p75^{NTR} to lipid rafts, and for biochemical and biological activities of p75^{NTR}, such as inactivation of Rho and the neurite outgrowth. Proper recruitment of activated p75^{NTR} to lipid rafts, structures that represent specialized signaling organelles, is of fundamental importance in determining p75^{NTR} bioactivity.

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Introduction

Neurotrophins mediate the survival, differentiation, growth and apoptosis of neurons by binding to two types of cell surface receptor, the Trk tyrosine kinases and the p75 neurotrophin receptor (p75^{NTR}) (Kaplan and Miller, 2000). The function of the neurotrophin receptors varies markedly. Although p75^{NTR} modulates the affinity of Trk receptors for neurotrophins (Barker and Shooter, 1994) and increases ligand specificity in cells that express both Trk and p75^{NTR} (Bibel *et al.*, 1999), it transduces its own signal in cells that express only p75^{NTR}. While Trk receptors transmit positive signals, such as enhanced survival and growth, p75^{NTR} transmits both positive and negative signals. The most prominent biological function of p75^{NTR} may be that it induces cell death, as it contains a death domain sequence similar to the intracellular domains of the Fas and tumor necrosis factor (TNF) receptors (Kaplan and Miller, 2000). Other p75^{NTR}-mediated activities have been proposed, including enhancing axonal outgrowth (Brann *et al.*, 1999; Yamashita *et al.*, 1999; Bentley and Lee, 2000), influencing Schwann cell migration (Anton *et al.*, 1994), modulating

synaptic transmission (Blöchl and Sirrenberg, 1996) and regulating the function of sensory neurons (Stucky and Koltzenburg, 1997) and calcium currents (Jiang *et al.*, 1999). Quite a few adaptor proteins that bind to p75^{NTR} have been identified (Kaplan and Miller, 2000), each trying to elucidate the molecular mechanisms that explain the diverse effects of p75^{NTR}. Furthermore, several signaling molecules have been shown to stay downstream of p75^{NTR}, such as JNK–p53–Bax (Casaccia-Bonofil *et al.*, 1996; Aloyz *et al.*, 1998), Rho (Yamashita *et al.*, 1999, 2002), Rac (Harrington *et al.*, 2002), ceramide (Dobrowsky *et al.*, 1994), NF- κ B (Carter *et al.*, 1996), mitogen-activated protein kinase (MAPK; Susen *et al.*, 1999) and phosphatidylinositol 3-kinase (PI3K; Roux *et al.*, 2001). Despite numerous studies, these findings failed to address fully the wide variety of biological functions of p75^{NTR}, suggesting the presence of as yet unknown molecules that are involved in the signaling pathways of p75^{NTR}.

In addition, a fundamental question regarding the diverse p75^{NTR} functions emerges. How can p75^{NTR} efficiently transduce these signals in the cells, or are there any mechanisms facilitating signal transduction? Lipid rafts are cholesterol- and sphingolipid-rich lipid microdomains in eukaryotic cell membranes, and have important functions (Simons and Ikonen, 1997). Recent evidence suggests that these rafts act in signal transduction in immunocytes such as T lymphocytes and basophils (Xavier and Seed, 1999). Rafts are believed to function in cellular signaling by concentrating or separating specific molecules in a unique lipid environment, not only in immunocytes but also in neurons (Galbiati *et al.*, 2001). For example, glial cell line-derived neurotrophic factor (GDNF) transduces its signal through RET, a transmembrane receptor tyrosine kinase, and a glycosylphosphatidylinositol (GPI)-anchored co-receptor GFR α 1, which localizes in lipid rafts. GFR α 1 recruits RET to lipid rafts after GDNF stimulation and results in strong and continuous signal transduction (Tansey *et al.*, 2000). We hypothesized that there might be a similar regulatory mechanism for p75^{NTR}. If that is the case, the next question would be what is the molecular event regulating the shift of p75^{NTR} to lipid rafts?

In the course of our effort to elucidate fully the mechanisms governed by p75^{NTR}, we found that the catalytic subunit of cAMP-dependent protein kinase (PKA) interacted with and phosphorylated p75^{NTR}. Interestingly, cAMP accumulation, which is responsible for activation of PKA, was observed by the ligand binding to p75^{NTR}. Activation of PKA turned out to be a necessary component that regulates the specific membrane localization and the downstream signals of p75^{NTR}.

Fig. 1. Isolation of PKACβ4ab as a p75^{NTR} interactor. (A) Structure of the splice variants of the human PKA catalytic subunit β (PKACβ). The sequence of the positive clone in yeast two-hybrid analysis is identical to that of PKACβ4ab. PKACβ4ab uses the exons 1-4, 1-a and 1-b instead of exon 1-1 in PKACβ1. (B) p75^{NTR} interacts with PKACβ4ab in the yeast two-hybrid system. The complementarity of the *cdc25-H* temperature-sensitive mutation was tested. The colonies that expressed p75ICD and PKACβ4ab grew on the galactose plates. The yeast transformed with Ras family protein grew independently of protein-protein interactions. (C) Interaction of p75^{NTR} with PKACβ4ab in transfected 293T cells. HA-p75^{NTR} or FLAG-PKACβ4ab was immunoprecipitated from the transfected cells, and the precipitated proteins were immunoblotted with anti-FLAG or anti-HA antibody, respectively. (D and E) Interaction of HA-p75^{NTR} with endogenous PKACβ in 293T cells. HA-p75^{NTR} (D) or endogenous PKACβ (E) was immunoprecipitated, and the precipitated proteins were immunoblotted with anti-PKACβ antibody or anti-HA antibody, respectively. (F) Interaction of the endogenous proteins in cerebellar neurons. Precipitation was done by anti-p75^{NTR} antibody, and the precipitated proteins were immunoblotted by anti-PKACβ antibody.

Results

PKACβ interacts with p75^{NTR}

A major difficulty in clarifying the role of p75^{NTR} is the non-enzymic nature of its cytoplasmic domain. Like all other members of this family, p75^{NTR} needs to associate with cytoplasmic proteins to exert its signaling functions. Although the classical yeast two-hybrid system has been a powerful tool for the purpose of searching for interactors, it is based on a transcriptional readout and may not be suitable for identifying membrane-associated proteins or proteins that do not localize in the nucleus. To identify proteins that interact with the p75^{NTR} intracellular domain

(p75ICD) in a more natural environment, we used a yeast two-hybrid assay based on the Son of sevenless (Sos) recruitment system. The bait consisted of the complete intracellular domain of human p75^{NTR} fused in-frame to the human Sos protein (Sos-p75ICD). This construct does not activate the Ras signaling pathway on its own in yeast (our unpublished data). We screened a human fetal brain cDNA library and isolated 13 candidate clones, one of which encoded a splice variant of the β catalytic subunit of PKA (PKACβ4ab) (Figure 1A). To assess specificity, yeast cells containing either a non-relevant bait (Sos-Coll3) or the original bait (Sos-p75ICD) were transformed together with the cDNA containing the open reading frame

of PKAC β 4ab. Suppression of the *cdc25-H* temperature-sensitive phenotype occurred in cells that expressed the Sos-p75ICD fusion protein and PKAC β 4ab on galactose plates (Figure 1B). These results indicate that PKAC β 4ab specifically interacts with the intracellular domain of p75^{NTR} in yeast.

Co-immunoprecipitation experiments were then performed using 293T cells transfected with hemagglutinin (HA)-tagged full-length p75^{NTR} and FLAG-tagged PKAC β 4ab. In line with the yeast two-hybrid results, HA-tagged p75^{NTR}, precipitated with an anti-HA antibody, efficiently co-precipitated transfected PKAC β 4ab (Figure 1C). FLAG-tagged PKAC β 4ab, precipitated with an anti-FLAG antibody, also co-precipitated transfected p75^{NTR} (Figure 1C). Next, we assessed interaction of endogenous proteins using an antibody against PKAC β . Precipitated HA-tagged p75^{NTR} efficiently co-precipitated endogenous PKAC β (Figure 1D) and, vice versa, endogenous PKAC β co-precipitated HA-tagged p75^{NTR} (Figure 1E). Using cerebellar neurons, which express all isoforms of catalytic subunits of PKA and p75^{NTR} endogenously (Supplementary figure 1 available at *The EMBO Journal* Online), we examined interaction of these molecules. As expected, endogenous p75^{NTR} co-precipitated endogenous PKAC β (Figure 1F). Other isoforms of the catalytic subunit of PKA, PKAC α and PKAC γ , were not co-precipitated by p75^{NTR} (Supplementary figure 1). These results suggest that PKAC β interacts with p75^{NTR} in mammalian cells.

Expression of the variants of PKAC β

Recently, six splice variants of the human PKAC β gene have been identified (Ørstavik *et al.*, 2001). The PKAC β cDNA we identified in the yeast two-hybrid screening was one of the splice variants (PKAC β 4ab), and diverged from the original human PKAC β sequence (PKAC β 1). These variants have unique exon 1 sequences, while the sequences of the 3' portion are identical and encompass the entire catalytic regions (Figure 1A). The 351 amino acid PKAC β 4ab contained a unique N-terminus of 17 amino acids encoded by a unique 5' exon (Figure 2A).

We determined the tissue distribution of PKAC β 4ab to gain insight into possible specific functional roles for this variant. A 370 bp fragment corresponding to exons 1-4, 1-a and 1-b of PKAC β 4ab was used to detect PKAC β 4ab mRNA specifically. Northern blot analysis of a panel of tissues from humans revealed an ~4.6 kb mRNA species with high levels of expression in the brain. However, no PKAC β 4ab mRNA expression was found in the other tissues examined (Figure 2B). These results showed the specific localization of PKAC β 4ab in the brain.

p75^{NTR} is phosphorylated by PKA

Interaction of p75^{NTR} with the catalytic subunit of PKA suggests that p75^{NTR} may be a substrate for PKA. To test this possibility, GST-fused p75ICD was generated in *Escherichia coli* and purified by high-pressure liquid chromatography (HPLC). The purified proteins were incubated with the catalytic subunit of PKA and [γ -³²P]ATP. Since each splice variant of PKAC β contains the complete catalytic domain (Figure 1A), suggesting that it has similar enzymic activities, we used commercially available catalytic subunit of PKA purified from bovine

Fig. 2. Analysis of PKAC β 4ab expression. (A) Structure of the human genomic sequence encoding PKAC β . The exons 1-4, 1-a and 1-b for PKAC β 4ab are located 86, 94 and 96 kb downstream, respectively, of exon 1-1, which is specific for PKAC β 1. There are several other splice variants (C β 2, C β 3, C β 4 and C β 4abc), which include exons 1-2, 1-3 and 1-c. The amino acid sequences derived from different exon 1s are shown. PKAC β 4ab has eight serine residues in its N-terminal end. (B) Tissue distribution of PKAC β 4ab mRNA. A human multiple tissue northern blot (Clontech) was probed with a 370 bp ³²P-labeled fragment corresponding to exons 1-4, 1-a and 1-b of PKAC β 4ab.

heart for *in vitro* phosphorylation assay. Incubation of wild-type p75ICD resulted in the appearance of a ³²P-labeled species at 22 kDa (the expected molecular mass of p75ICD), and this radioactivity was completely diminished by PKI, a specific inhibitor of PKA (Figure 3A). This indicates that p75ICD is phosphorylated by PKA *in vitro*.

The amino acid sequence R/K X X S/T is the consensus PKA recognition site, and either S or T is the phosphorylation site (Kemp *et al.*, 1977). The human p75ICD sequence has one putative PKA phosphorylation site containing Ser304 in the juxtamembrane linker region. This PKA recognition site is conserved in mouse and chicken p75ICD (Figure 3B). To test whether this conserved site could be phosphorylated by PKA, we introduced a serine to glycine substitution mutation in this site of the p75ICD sequence to generate the p75ICD mutant (p75ICDm) protein. This mutant protein failed to be phosphorylated by PKA (Figure 3A), indicating that this site is the only site phosphorylated by PKA in p75ICD.

To determine whether p75^{NTR} is phosphorylated *in vivo*, we transfected HA-tagged full-length p75^{NTR} (HA-p75^{NTR} WT) with 293T cells, which do not express p75^{NTR}, labeled the cells with [³²P]orthophosphate, and immunoprecipitated p75^{NTR} from cell lysates with an anti-HA antibody. Although the basal level of phosphorylation can be detected in these cells before the ligand stimulation, the addition of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) at 50 ng/ml (Figure 3C) for

Fig. 3. Phosphorylation of p75^{NTR} by PKA. (A) *In vitro* phosphorylation of p75^{NTR}. Bacterially expressed wild-type (p75ICD) or mutant (p75ICDm) p75^{NTR} intracellular domain were incubated with or without the catalytic subunit of PKA and PKI in the presence of [γ -³²P]ATP for 30 min. Reaction mixtures were separated by SDS-PAGE, and the gels were autoradiographed. Histone protein was used as a positive control. (B) The amino acid sequence of the PKA phosphorylation site of p75ICD. p75^{NTR} has one PKA phosphorylation consensus site (KLHG; highlighted in bold) in the juxta-membrane linker region, and the sequences around this site are highly conserved among mouse, chick and human. p75ICD mutant (p75ICDm) was made by replacement of Ser304 by glycine. (C) *In vivo* phosphorylation of p75^{NTR}. 293T cells were transfected with wild-type (HA-p75^{NTR} WT) or mutated (HA-p75^{NTR} MT) p75^{NTR}. The cells labeled with ³²P were stimulated by NGF or BDNF for 10 min, and the proteins were immunoprecipitated. Autoradiographs of phosphorylated p75^{NTR} recovered after immunoprecipitation and SDS-PAGE are shown. Signal intensities relative to the mean basal phosphorylation level (left, lane 2 or right, lane3) are calculated and normalized to total p75^{NTR} level. The mean value and standard error from three experiments are shown. Asterisks indicate statistical significance (**P* < 0.05, Student's *t*-test).

10 min significantly increased the phosphorylation of p75^{NTR}. The highly selective inhibitor of PKA, KT5720 (Kase *et al.*, 1987), abolished the increase in the phosphorylation level. In addition, phosphorylated p75^{NTR} in 293T cells was increased by overexpression of PKACβ4ab or stimulation with 8-bromoadenosine-cAMP (8-Br-cAMP; membrane-permeable cAMP analog). However, NGF or BDNF treatment of cells expressing mutated full-length p75^{NTR} (HA-p75^{NTR} MT), which lacks the PKA phosphorylation site, showed no increase of phosphorylation (Figure 3C). These results indicate that p75^{NTR} is a target of PKA and is phosphorylated by the ligand stimulation in mammalian cells.

Activated p75^{NTR} translocates to lipid rafts

Phosphorylation of p75^{NTR} may play a role in the efficiency of signal transduction or its membrane localization. We hypothesized that p75^{NTR} utilizes a compartment in which particular downstream signal molecules assemble to regulate a wide range of signals. We tested whether activated p75^{NTR} clustered into lipid rafts, struc-

tures that may represent highly specialized signaling organelles. Mouse cerebellar neurons, which express p75^{NTR} but not TrkA, were used to evaluate the effect of p75^{NTR} on its membrane localization without taking TrkA activation into consideration when using NGF. We separated Brij 58-insoluble, low-density membranes (raft-associated proteins) from fully soluble, high-density membranes (non-raft-associated proteins) using flotation gradients and analyzed them by SDS-PAGE for p75^{NTR} immunoreactivity. We observed white bands corresponding to detergent-resistant membrane fractions near the top of a 20% sucrose gradient in the centrifuge tube (Supplementary figure 2). Each fraction was subjected to SDS-PAGE and assessed by western blotting using anti-flotillin-1 antibody. Flotillin-1, known as a raft marker (Galbiati *et al.*, 2001), partially accumulates in the soluble fraction, which is dependent on the detergents used (Kimura *et al.*, 2002; Marchand *et al.*, 2002). Although we observed the basal level of the signal for p75^{NTR} in insoluble raft fractions, stimulation with NGF (200 ng/ml) resulted in movement of a significant amount of p75^{NTR} to

Fig. 4. NGF induces p75^{NTR} association with lipid rafts in a PKA-dependent manner. (A) p75^{NTR} becomes associated with detergent-resistant membrane fractions after NGF stimulation. Mouse cerebellar neurons (P6) or rat hippocampal neurons (E18) were treated or not with NGF for 10 min. The neurons were then detergent extracted and the lysate was subjected to sucrose density gradient centrifugation. Twelve fractions of the gradient were collected from the top to the bottom (from 1 to 12, see Supplementary figure 2). Equal volumes of insoluble rafts fractions (Nos 2 and 3 are shown for cerebellar neurons, and Nos 2, 3 and 4 are shown for hippocampal neurons) and soluble high-density fractions (Nos 11 and 12 are shown) were subjected to SDS-PAGE. Western blots for p75^{NTR}, flotillin-1 and TrkB (only in the case of cerebellar neurons) are shown. In unstimulated cells, p75^{NTR} mainly fractionates to soluble fractions. After NGF stimulation, a significant portion of p75^{NTR}, especially the middle molecular weight band (arrowhead), floats to the raft fraction, where flotillin-1 (raft marker) fractionates. TrkB in cerebellar neurons did not translocate in parallel with p75^{NTR}. (B) RT-PCR analysis of RNA extracted from E18 hippocampal neurons. While the *trkA* gene is not expressed in these neurons, p75^{NTR} can clearly be detected 18 h after culturing on dishes. The level is lower in the neurons before plating. Control samples include mRNA from PC12 cells. (C) Localization of transfected p75^{NTR} in 293T cells. Large amounts of p75^{NTR} accumulated in lipid rafts when these cells were transfected with p75^{NTR} alone, whereas a significantly reduced amount of p75^{NTR} was seen in the raft fraction if p75^{NTR} and TrkA were co-expressed. A 10 min incubation with 200 ng/ml BDNF resulted in the translocation of wild-type p75^{NTR} (p75^{NTR}WT, upper panel) to lipid rafts, but did not affect the distribution of mutated p75^{NTR} (p75^{NTR}MT, lower panel). (D) Localization of mutated p75^{NTR} to lipid rafts. BDNF (50 ng/ml) induced translocation of wild-type p75^{NTR} into lipid rafts in 293T cells (upper panel), whereas no accumulation of mutated p75^{NTR} (S304A and S304D) in the raft fraction was seen after BDNF stimulation (middle and bottom panels). (E) PKA-dependent translocation of p75^{NTR} into lipid rafts. P6 mouse cerebellar neurons were either not treated (Control) or treated with NGF (NGF) for 10 min with or without pre-treatment with PKA inhibitor KT5720 at 200 nM for 1 h. Insoluble raft fractions (nos 2–4 are shown) and soluble high-density fractions (Nos 11 and 12 are shown) were isolated and analyzed by western blot. The signal intensity of the band representing p75^{NTR} or flotillin-1 in the insoluble fractions (Nos 2 and 3, left two lanes) was measured, and the total intensity of p75^{NTR} is shown as the ratio to that of flotillin-1, a marker for lipid rafts. Data are mean and standard errors from three experiments. An asterisk indicates statistical significance (**P* < 0.01, Student's *t*-test).

the top of the flotation gradient (Figure 4A and E), confirming its association with low-density lipids. NGF at 50 ng/ml was sufficient to elicit this reaction (unpublished data). E18 rat hippocampal neurons express p75^{NTR} mRNA but not TrkA mRNA after 18 h in culture (Figure 4B), as reported previously (Brann *et al.*, 1999). The basal amount and ligand-dependent accumulation of p75^{NTR} in lipid rafts were also observed in these neurons 10 min after the addition of NGF at 200 (Figure 4A) or 50 ng/ml (our unpublished data). These data indicate that NGF induces p75^{NTR} translocation to lipid rafts in neurons that express only p75^{NTR} as an NGF receptor.

As overexpression of p75^{NTR} in 293T cells resulted in assembly of the protein in the lipid rafts (Figure 4C), we could not assess the effect of NGF in these cells (our unpublished data). In 293T cells co-transfected with TrkA and p75^{NTR}, however, the basal amount of p75^{NTR} in lipid rafts was low, and BDNF, which was used to activate only p75^{NTR} but not TrkA in these cells, induced significant translocation of wild-type p75^{NTR} (p75^{NTR} WT) to the lipid rafts (Figure 4C). BDNF at 50 ng/ml was sufficient to elicit this reaction (Figure 4D). Like TrkB in cerebellar neurons, transfected TrkA in 293T cells was present only in the soluble fraction (Figure 4C). To substantiate the importance of phosphorylation at Ser304, we employed a phosphorylation-defective mutant (p75^{NTR}S304A; Ser304 to alanine) as well as a phosphorylation mimic mutant (p75^{NTR}S304D; Ser304 to aspartate). BDNF had no effect on the redistribution of p75^{NTR}S304A or p75^{NTR}S304D (Figure 4D), as was the case with the serine to glycine mutant (p75^{NTR} MT) (Figure 4C). These results demonstrate that the phosphorylation of p75^{NTR} is required for the ligand-induced accumulation of the receptor in the lipid rafts.

To test the hypothesis that cAMP-PKA was a necessary component for p75^{NTR} to translocate to lipid rafts, the highly selective PKA inhibitor KT5720 (200 nM) was added to the culture medium of cerebellar neurons before flotation gradient analysis. In the presence of KT5720, stimulation with NGF no longer increased the amount of p75^{NTR} moving to lipid rafts (Figure 4E). These data clearly show that the activation of PKA is essential for NGF-induced movement of p75^{NTR} to lipid rafts.

PKA is required for p75^{NTR}-mediated activities

A previous study demonstrated that ligand binding to p75^{NTR} promoted neurite outgrowth of cultured hippocampal neurons (Brann *et al.*, 1999). As hippocampal neurons express PKAC β (our unpublished data), we used these cells to assess whether the biological activities of p75^{NTR} are dependent on cAMP-PKA. Although the addition of 50 ng/ml NGF to the culture of hippocampal neurons significantly enhanced outgrowth of neurites, KT5720 (200 nM) completely blocked this activity of NGF (Figure 5A).

RhoA activity has been shown to be downstream of p75^{NTR}. NGF causes a loss of RhoA activation in cells that express endogenous as well as transfected p75^{NTR} (Yamashita *et al.*, 1999), and we chose this GTPase as one of the biochemical consequences elicited by activation of p75^{NTR}. The activity of RhoA was measured using the Rho-binding domain of the effector protein Rhotekin (Ren *et al.*, 1999). Rhotekin, a downstream target of RhoA,

binds specifically to the GTP-bound form of RhoA, thus enabling us to precipitate and quantify the active form of RhoA. The level of the active form of RhoA was decreased within 10 min following the addition of NGF (50 ng/ml) to cerebellar neurons (Figure 5B), consistent with the previous report. This inactivation was completely blocked when the cells were incubated with KT5720 (200 nM) 1 h prior to the addition of NGF, while KT5720 itself had no effect on RhoA activity. These results show that activation of PKA is essential for p75^{NTR}-mediated RhoA inactivation. Although p75^{NTR}-mediated RhoA inactivation, which was inhibited by KT5720, could also be observed in 293T cells transfected with wild-type p75^{NTR}, neither p75^{NTR} S304A nor p75^{NTR} S304D could elicit RhoA inactivation after 50 (Figure 5B) or 200 ng/ml (our unpublished data) NGF treatment. These data suggest that the phosphorylation of p75^{NTR} as well as the activation of PKA is essential for this signal pathway.

Elevated intracellular cAMP levels trigger signal transduction pathways through activation of PKA. To examine whether activation of cAMP-PKA can be triggered by p75^{NTR} itself, we measured the intracellular level of cAMP in primary cultured cerebellar neurons. Measured by competitive immunoassay, the basal level of cAMP was 33.8 ± 5.3 fmol per 10^6 cells. The amount of cAMP in the cells was increased ~ 1.6 times 10 min after the addition of NGF at 200 ng/ml, and returned towards the baseline level after 30 min (Figure 5C). Elevation of cAMP levels seems to be saturated by NGF at a concentration of ~ 50 ng/ml (Figure 5C). These results demonstrate that PKA can be activated by ligand binding to p75^{NTR}.

Taken together, our data demonstrate that PKA modulates p75^{NTR}-mediated biological and biochemical activities, presumably by phosphorylating p75^{NTR}.

Discussion

Splice variants of the catalytic subunit of PKA

We have screened the human fetal brain cDNA library using a yeast two-hybrid system, and identified a splice variant of the catalytic subunit of PKA as a p75^{NTR}-interacting protein. The inactive PKA holoenzyme exists as a heterotetramer of two regulatory (R) and two catalytic (C) subunits. The activity of PKA is dependent on the concentration of intracellular cAMP, and the activation is elicited when four molecules of cAMP bind to the R subunit dimer, two to each subunit, in a positive cooperative fashion, followed by the release of the C subunits from the R subunits (Skålhegg and Tasken, 2000). Thus, our data show that p75^{NTR} is a substrate of PKA, but not a regulator of PKA. The C subunit family consists of three characterized isoforms (C α , C β and C γ) that have been described in various species (Skålhegg and Tasken, 2000). The C α isoform is expressed ubiquitously, whereas the C β isoform is highly expressed in brain (Skålhegg and Tasken, 2000). In cattle and mice, two and three splice variants of C β have been identified, respectively (Wiemann *et al.*, 1996; Guthrie *et al.*, 1997). A recent study demonstrated that the C β gene in humans encodes at least six different gene products, designated C β 1, C β 2, C β 3, C β 4, C β 4ab and C β 4abc (Ørstavik *et al.*, 2001). The variant we identified as a p75^{NTR}-interacting protein was

Fig. 5. cAMP-PKA accumulates by ligand binding to p75^{NTR} and affects signal transduction. (A) Neurite outgrowth through p75^{NTR} is dependent on cAMP-PKA. NGF (50 ng/ml) was added to the cultures of dissociated hippocampal neurons with or without 200 nM KT5720. After 18 h, cells were fixed and stained for TuJ1. The length of the longest neurite per neuron was measured. Data are means ($\pm$ SEM) for 30 individual neurons that have neurites $>30 \mu\text{m}$. An asterisk indicates statistical significance ($*P < 0.01$, Student's *t*-test). Scale bar, 50 μm . (B) Affinity precipitation of RhoA in cerebellar neurons and in transfected 293T cells. NGF decreases the activity of RhoA not only in cerebellar neurons (top) but also in 293T cells transfected with wild-type p75^{NTR} (middle). The inactivation was lost in cells pre-treated with KT5720, or transfected with mutated p75^{NTR} (bottom). (C) Time course measurement and dose response of intracellular cAMP in cerebellar neurons. A total of 5×10^6 neurons were dissociated in individual wells of a six-well dish. Neurons were treated with NGF (200 ng/ml) for 0–30 min (left) or with different concentrations of NGF for 10 min (right), as indicated. The levels of cAMP were measured and compared with the control. The results are the means ($\pm$ SEM) of three experiments, each done in quadruplicate.

C β 4ab. As is the case with the murine and bovine splice variants, all the human C β splice variants vary in the N-terminal region but have the same sequences in the 3' portion, which encodes the binding site for a regulatory subunit and the catalytically active domain (Figure 1A), suggesting that all these variants have similar catalytic activities. Although the C β 1 isoform in mouse was found not only in brain but also in spleen, kidney and liver (Guthrie *et al.*, 1997), human C β 4ab mRNAs were expressed only in brain (Figure 2B).

Phosphorylation of p75^{NTR} by PKA

We found that the catalytic subunit of PKA could bind to p75^{NTR} in both yeast and mammalian cells. In the yeast

system, a faint growth of the yeast expressing the irrelevant control protein and PKAC β 4ab could be found (Figure 1B), presumably due to the growth-promoting activity of overexpressed PKA. A previous study revealed that SOX9, a high mobility group domain-containing transcription factor, was a target of PKA in a study using the same method as we used (Huang *et al.*, 2000). We found that a consensus PKA phosphorylation site within the p75^{NTR} juxtamembrane linker region could be phosphorylated by PKA *in vitro* and that both NGF and BDNF induced the phosphorylation of p75^{NTR}, which is inhibited by the highly specific PKA inhibitor, at least in p75^{NTR}-overexpressing 293T cells (Figure 3C). Phosphorylation of p75^{NTR} has been described in several conditions. A

Fig. 6. Model of p75^{NTR} recruitment to lipid rafts. In neurons expressing p75^{NTR} but not Trk receptors, ligands induce p75^{NTR}-mediated transient accumulation of intracellular cAMP. PKA is then activated and phosphorylates p75^{NTR} itself. Phosphorylated p75^{NTR} may translocate to lipid rafts, where a number of downstream signaling molecules such as RhoA and ceramide accumulate, thus facilitating the efficiency of the signal transduction.

previous report (Grob *et al.*, 1985) showed that p75^{NTR} in human melanoma cells, which express p75^{NTR} but not TrkA, was constitutively serine phosphorylated, consistent with our result. However, no detectable change in the phosphorylation level after NGF stimulation could be observed. The ligand-dependent phosphorylation of p75^{NTR} by PKA might be masked in these experiments. It should also be noted that there is a difference in the methods used for the precipitation of p75^{NTR}. Since the affinity of the antibody used for immunoprecipitation of p75^{NTR} might be influenced by the phosphorylation state, we introduced HA-tagged full-length p75^{NTR} in 293T cells and used anti-HA antibody for the *in vitro* phosphorylation assay to immunoprecipitate all the p75^{NTR} expressed. Another report (Taniuchi *et al.*, 1986) showed there was no difference in the p75^{NTR} phosphorylation level when NGF was added to PC12 cells or rat sympathetic neurons. As both cells used express TrkA as well as p75^{NTR}, this result may reflect the function of p75^{NTR} as a co-receptor for NGF, whereas we assessed p75^{NTR} function independently of Trk. In PCNA cells, which express p75^{NTR} but not TrkA, p75^{NTR} is tyrosine-phosphorylated by NGF stimulation and can be co-precipitated with MAPK (Susen *et al.*, 1999). Although a protein of ~80 kDa immunoprecipitated by anti-p75^{NTR} antibody was recognized by anti-phosphotyrosine antibody after NGF stimulation, it is not clear from their experiments which kinase phosphorylates p75^{NTR} and which site is the target of the enzyme. In contrast to their indirect evidence for the tyrosine phosphorylation of p75^{NTR}, our assay revealed that the phosphorylation of p75^{NTR} induced by the ligand stimulation was totally dependent on PKA, suggesting no ligand-stimulated tyrosine phosphorylation. In addition, p38 β 2 kinase interacts with the fifth and sixth helices of p75^{NTR} and phosphorylates its intracellular domain (Wang *et al.*, 2000). Although dimerization of p75^{NTR} and mimicry of ligand stimulation activate p38 β 2, the mechanism by which phosphorylated p75^{NTR} transduces the downstream signal was not described fully.

Biological actions elicited by cAMP-PKA

Several lines of evidence show that cAMP-PKA is an important signal in axonal guidance. Studies of cultured *Xenopus* spinal neurons have shown that the level of cytosolic cAMP or the activity of PKA is critical in

determining whether the turning response is attractive or repulsive (Song *et al.*, 1997). For example, activation of PKA converts repulsion induced by myelin-associated glycoprotein (MAG), which is known as a potent inhibitor of nerve regeneration (McKerracher *et al.*, 1994; Mukhopadhyay *et al.*, 1994), into attraction. Both cerebellar neurons and dorsal root ganglion neurons were shown to acquire the ability to extend their axons, even in the presence of MAG, if these cells were pre-treated with neurotrophins for more than several hours (Cai *et al.*, 1999). The cAMP-PKA signal cascade is responsible for this priming effect. Developmental regulation of the endogenous neuronal cAMP level was also shown to play an important role in the switch of neuronal response to myelin or MAG (Cai *et al.*, 2001). They reported accumulation of cAMP 30 min after exposure to BDNF, but not NGF, in cerebellar neurons. However, we observed transient accumulation of cAMP when NGF was added in the same cells (Figure 5C). There is also other evidence that shows that NGF-induced cAMP accumulation is elicited rapidly by p75^{NTR} (Knipper *et al.*, 1993). Therefore, a more detailed analysis will be required to define clearly which receptor is involved in this priming effect. If p75^{NTR} plays a role in this priming effect, it may be possible to find a way to make neurons regenerate against the inhibitory molecules adequately by showing the mechanisms by which p75^{NTR} is activated.

Downstream signals of p75^{NTR}

Rho family GTPases control the formation of distinct actin-based structures in the growth cone, and transmit the signals from extracellular guidance cues (Dickson, 2001). In cultured chick ciliary neurons, inactivation of Rho proteins mimicked the effect of neurotrophins by increasing the rate of neurite elongation (Yamashita *et al.*, 1999). Activation of RhoA by MAG in cerebellar neurons was shown to be mediated by p75^{NTR} (Yamashita *et al.*, 2002). Here we showed that NGF induced inactivation of RhoA in cerebellar neurons and 293T cells, and that this effect was PKA dependent (Figure 5B). PKA phosphorylates many target proteins, and one such target identified is RhoA. When Ser188 is phosphorylated, RhoA becomes inactive (Lang *et al.*, 1996). Taken together, it is possible that inactivation of RhoA is the downstream component of cAMP-PKA. However, another interpretation of our data is that the inhibition of the PKA signal blocked the translocation of the receptor to lipid rafts and might result in failure of transduction of the downstream signal.

The mechanism that regulates the receptor localization

Immunoblot analysis revealed that p75^{NTR} localized both in raft fractions and soluble fractions in cerebellar neurons and hippocampal neurons without NGF addition (Figure 4). This is consistent with a previous study using p75^{NTR}-overexpressing NIH-3T3 or PC12 cells (Bilderback *et al.*, 1997). Our data support a model in which some parts of p75^{NTR} move to lipid rafts after ligand stimulation. However, as the raft association of p75^{NTR} was only shown by biochemical experiments, it is possible that p75^{NTR} had higher detergent insolubility by binding to some raft-associated proteins such as Src family or cytoskeletal proteins. Further analysis using multiple

strategies will be required to obtain firm evidence. Rafts organize the multitude of signals impinging on the cell surface into distinct signaling cascades by including or excluding key signaling molecules (Galbiati *et al.*, 2001). Activation of the T-cell receptor is followed by the rapid phosphorylation of multiple intracellular proteins by the Src family tyrosine kinase Lck and the cytoplasmic tyrosine kinase ZAP-70, all of which accumulate in lipid rafts (Galbiati *et al.*, 2001). A small GTPase TC10, Rho, Ras, c-Src and FAK in rafts fraction have been described (Iwabuchi *et al.*, 1998; Watson *et al.*, 2001). Furthermore, ceramide, one of the downstream signaling molecules of p75^{NTR}, can strongly stabilize raft formation (Xu *et al.*, 2001), and also mediates selective clustering of molecules into lipid rafts (Cremesti *et al.*, 2001). Our observations, together with these reports, provide a comprehensive model that explains how p75^{NTR} transduces the signals, such as Rho GTPases, Ras-MAPK, PI3K and ceramide (Figure 6). Interestingly, only middle molecular weight p75^{NTR} species existed in rafts fraction on biochemical analysis (Figure 4A), suggesting that protein modification is required for the translocation of p75^{NTR} to rafts. The PKA inhibitor attenuated the p75^{NTR} localization to lipid rafts (Figure 4E), and phosphorylation of p75^{NTR} is necessary for translocation of the receptor (Figure 4C and D). In future research, it will be interesting to ask whether cell type- or cell status-specific signals elicited by p75^{NTR} are due, at least partly, to localization of the receptor.

In conclusion, the findings that PKA plays roles in receptor localization on the membrane and affects signaling cascades reveal an aspect of the biology of p75^{NTR} that is in line with its diverse signals.

Materials and methods

Yeast two-hybrid screening

A yeast Sos recruitment two-hybrid screen was performed essentially as described previously (Aronheim *et al.*, 1997). Approximately 5×10^6 transformants were screened, and positive clones were isolated from the temperature-insensitive colonies. Plasmids were extracted from surviving colonies and amplified in *E. coli* for DNA sequence analysis. Verification of the specificity of interaction between the bait and target proteins was then performed by co-transformation. MAFB was used as a positive control since MAFB proteins can bind to each other. Irrelevant bait (Coll3) and target (LaminC) were used as negative controls.

Co-immunoprecipitation of p75^{NTR} and PKAC β

HA-tagged human p75^{NTR} (p75FL-HA) and FLAG-tagged PKAC β 4ab (PKAC β -FLAG) were cloned into pcDNA3.1 expression plasmids (Invitrogen). Transfection was performed by lipofection using Lipofectamine 2000 (Gibco-BRL). The cells were lysed with 0.1% NP-40 in buffer A containing 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 100 μ M sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate and protease inhibitor cocktail (Roche Biochemicals). The lysates were incubated with a monoclonal anti-HA antibody (Covance) or a polyclonal anti-FLAG antibody (Sigma) for 16 h, followed by incubation with protein G-Sepharose (Amersham Biosciences) for 2 h at 4°C. The beads were washed three times with buffer A, and subjected to SDS-PAGE, followed by immunoblot analysis. To immunoprecipitate endogenous p75^{NTR}, monoclonal anti-p75^{NTR} antibody (Chemicon) was used. Polyclonal anti-PKAC α , PKAC β or PKAC γ (Santa Cruz Biotechnology) antibody was used for the detection of each endogenous PKA isoform.

Mutagenesis and production of recombinant proteins

To make p75ICD or p75^{NTR} mutants, the Ser304 codon was converted through site-directed mutagenesis to either a glycine (G), alanine (A) or

aspartate (D) codon. All mutations were confirmed by DNA sequencing. The p75ICD coding sequence was cloned into the pGEX-5X bacterial expression vectors (Amersham Biosciences) to generate a GST fusion protein. Recombinant proteins were treated with factor Xa (Amersham Biosciences) and purified by ion exchange HPLC on a DEAE-cellulose column.

In vitro phosphorylation

Recombinant wild-type and mutant p75ICD proteins (40 μ g each) were added to PKA reaction buffer containing 20 mM HEPES pH 7.5, 10 mM MgCl₂, 5 mM dithiothreitol, 100 mM NaCl and 1 mM [γ -³²P]ATP (0.5 Ci/mmol), and incubated with 50 U of PKA catalytic subunit (Calbiochem) at 30°C for 30 min in a total volume of 50 μ l. To test the specificity, 1 μ g of PKI (Sigma) was added to the reaction buffer to inhibit specifically the activity of PKA. A 100 ng aliquot of histone protein (Sigma) was used as a positive control. Each sample was resolved by electrophoresis on a SDS-7% polyacrylamide gel, and the gel was dried and autoradiographed.

In vivo phosphorylation

293T cells were transfected with p75FL-HA and/or PKAC β -FLAG as described above. After 24 h, the cells were labeled with [³²P]orthophosphate in phosphate-free Dulbecco's modified Eagle's medium (DMEM) for 4 h in the presence or absence of the specific PKA inhibitor, KT5720. Then, the cells were treated with 50 ng/ml NGF (Upstate Biotechnology) or 200 μ M 8-Br-cAMP (Calbiochem) for 10 min and lysed with 1% Triton X-100 in buffer A. p75^{NTR} was immunoprecipitated using polyclonal anti-HA antibody (Sigma) and protein A-Sepharose (Amersham Biosciences). Quantification of the signal intensity was performed by comparing densitometry readings using NIHImage software. 293T cells transfected with p75FL-m were also analyzed by the same method using a monoclonal anti-p75^{NTR} antibody (Upstate Biotechnology) for immunoprecipitation.

Isolation of detergent-resistant membrane fractions

Cells were lysed on ice with 0.5 ml of 0.5% Brij-58 (Sigma) in buffer A. Extracts were adjusted to 40% sucrose by adding 0.5 ml of 80% sucrose, then placed in an SW41Ti ultracentrifuge tube (Beckman), and overlaid with 8 ml of 30% sucrose and 1 ml of distilled water. All of these steps were performed in a 4°C cold room and on ice. After centrifugation (16 h, 200 000 g, 4°C), 12 fractions of 0.83 ml each were collected from the top (Nos 1–12), and equal volumes of them were prepared for SDS-PAGE and immunoblot analysis. For detection of p75^{NTR}, TrkB, flotillin-1 and HA-tagged TrkA, polyclonal anti-p75 antibody (1:2000, Promega), polyclonal anti-TrkB antibody (1:1000, Santa Cruz Biotechnology), monoclonal anti-flotillin-1 antibody (1:1000, Transduction Laboratory) and polyclonal anti-HA antibody (1:1000 Santa Cruz Biotechnology) were used, respectively. Measurement of signal intensity was performed as described above.

Neurite outgrowth assay

Hippocampal neurons were cultured at low density as described above. For outgrowth assays, plated cells were incubated for 18 h with or without NGF and KT5720 (200 nM), fixed in 4% (w/v) paraformaldehyde/phosphate-buffered saline and immunostained with TuJ1 antibody (Research Diagnostics). The length of the longest process for each β -tubulin III-positive neuron, if it was >30 μ m, was determined.

Affinity precipitation of GTP-Rho

Cells were lysed in 50 mM Tris pH 7.5 containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl₂ and protease inhibitor cocktail. Cell lysates were clarified by centrifugation at 13 000 g at 4°C for 10 min, and the supernatants were incubated with 20 μ g of the GST-Rho-binding domain of Rhotekin beads at 4°C for 45 min (Ren *et al.*, 1999). The beads were washed four times with washing buffer (50 mM Tris pH 7.5 containing 1% Triton X-100, 150 mM NaCl, 10 mM MgCl₂ and protease inhibitor cocktail). Bound Rho proteins were detected by western blotting using monoclonal antibody against HA (Covance).

cAMP assay

Cerebellar neurons were plated onto a 6-well dish, and incubated for at least 3 days. NGF was added at various concentrations and incubated for 0–30 min before the cells were lysed. Intracellular cAMP was measured by competitive immunoassay, according to the manufacturer's instructions (Amersham Biosciences).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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