p75 neurotrophin receptor regulates tissue fibrosis through inhibition of plasminogen activation via a PDE4/cAMP/PKA pathway

Benjamin D. Sachs,1 George S. Baillie,2 Julianne R. McCall,1 Melissa A. Passino,1 Christian Schachtrup,1 Derek A. Wallace,2 Allan J. Dunlop,2 Kirsty F. MacKenzie,2 Enno Klussmann,3 Martin J. Lynch,2 Shoana L. Sikorski,1 Tal Nuriel,1,4 Igor Tsigelny,1 Jin Zhang,1 Miles D. Houslay,2 Moses V. Chao,4 and Katerina Akassoglou1

1Department of Pharmacology, University of California, San Diego, La Jolla, CA 92039
2Molecular Pharmacology Group, Biochemistry & Molecular Biology, University of Glasgow, Glasgow G12 8QQ, Scotland, UK
3Leibniz-Institut für Molekulare Pharmakologie (FMP), Campus Berlin-Buch, 13125 Berlin, Germany
4Molecular Neurobiology Program, Skirball Institute of Biomolecular Medicine, Departments of Cell Biology, Physiology, and Neuroscience, New York University School of Medicine, New York, NY 10016

Clearance of fibrin through proteolytic degradation is a critical step of matrix remodeling that contributes to tissue repair in a variety of pathological conditions, such as stroke, atherosclerosis, and pulmonary disease. However, the molecular mechanisms that regulate fibrin deposition are not known. Here, we report that the p75 neurotrophin receptor (p75NTR), a TNF receptor superfamily member up-regulated after tissue injury, blocks fibrinolysis by down-regulating the serine protease, tissue plasminogen activator (tPA), and up-regulating plasminogen activator inhibitor-1 (PAI-1). We have discovered a new mechanism in which phosphodiesterase PDE4A4/5 interacts with p75NTR to enhance cAMP degradation. The p75NTR-dependent down-regulation of cAMP results in a decrease in extracellular proteolytic activity. This mechanism is supported in vivo in p75NTR-deficient mice, which show increased proteolysis after sciatic nerve injury and lung fibrosis. Our results reveal a novel pathogenic mechanism by which p75NTR regulates degradation of cAMP and perpetuates scar formation after injury.

Introduction

Tissue scarring, characterized by cell activation, excessive deposition of ECM, and extravascular fibrin deposition, is considered a limiting factor for tissue repair. Fibrin, the major substrate of the serine protease plasmin, is a provisional matrix deposited after vascular injury (Bugge et al., 1996). The two plasminogen activators (PAs), namely tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) and their inhibitors, such as plasminogen activator inhibitor-1 (PAI-1), are key modulators of scar resolution by spatially and temporally regulating the conversion of plasminogen to plasmin resulting in fibrin degradation and ECM remodeling (Lijnen, 2001). In the peripheral nervous system, previous work by us and others showed that inhibition of fibrinolysis in mice deficient in plasminogen or tPA exacerbated axonal damage (Akassoglou et al., 2000) and impaired functional recovery after nerve injury (Siconolfi and Seeds, 2001). In accordance, mice deficient for fibrinogen showed increased regenerative capacity (Akassoglou et al., 2002). Studies of fibrin deposition in human diseases, in combination with experiments from mice deficient in plasminogen and PAs, have provided information about a wide range of physiological and pathological conditions that are exacerbated by defective fibrin degradation, such as wound healing, metastasis, atherosclerosis, lung ischemia, rheumatoid arthritis, muscle regeneration, and multiple sclerosis (MS) (Degen et al., 2001; Adams et al., 2004).

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However, the molecular mechanisms that regulate proteolytic activity remain unclear.

In our current work, we focus on the mechanisms that regulate fibrinolysis after injury. Our previous studies demonstrated a correlation between fibrin deposition and expression of p75 neurotrophin receptor (p75NTR) after nerve injury (Akassoglou et al., 2002). Up-regulation of p75NTR is observed in MS (Dowling et al., 1999), stroke (Park et al., 2000), and spinal cord (Beattie et al., 2002) and sciatic nerve injury (Taniuchi et al., 1986), all of which are associated with fibrin deposition. p75NTR is also expressed in non-neuronal tissues (Lomen-Hoerth and Shooter, 1995) and is up-regulated in non-nervous system diseases associated with defects in fibrin degradation, such as atherosclerosis (Wang et al., 2000), melanoma formation (Herrmann et al., 1993), lung inflammation (Renzi et al., 2004), and liver disease (Passino et al., 2007). p75NTR has been primarily characterized as a modulator of cell death (Wang et al., 2000) and differentiation (Passino et al., 2007) in non-neuronal tissues. The expression of p75NTR by cell types such as smooth muscle cells and hepatic stellate cells, which actively participate in tissue repair by migration, and secretion of ECM and extracellular proteases, raises the possibility for a functional role of p75NTR in disease pathogenesis that extends beyond apoptosis and differentiation.

We find that p75NTR is involved in the regulation of proteolytic activity and fibrin degradation. Mice deficient for p75NTR (Lee et al., 1992) show increased proteolytic activity and decreased fibrin deposition in two disease models: sciatic nerve injury and lung fibrosis. p75NTR regulates proteolytic activity by simultaneously down-regulating tPA and up-regulating PAI-1 via a novel cAMP/PKA pathway. p75NTR decreases cAMP via interaction with the cAMP-specific phosphodiesterase (PDE) isozyme PDE4A4/5. This is of particular note, as selective PDE4 inhibitors have an anti-inflammatory action and have potential therapeutic utility in inflammatory lung disease, as well as in a wide range of neurologic diseases such as depression, spinal cord injury, MS, and stroke (Gretarsdottir et al., 2003; Nikulin et al., 2004; Houslay et al., 2005). Overall, the regulation of plasminogen activation by p75NTR identifies a novel pathogenic mechanism whereby p75NTR interacts with PDE4A4/5 to degrade cAMP and thus perpetuates scar formation that could possibly render the environment hostile for tissue repair.

**Results**

Fibrin deposition is reduced in p75NTR−/− mice

To examine whether p75NTR regulates fibrin deposition in the sciatic nerve we compared fibrin levels in wild-type (wt) and p75NTR−/− mice after injury. In wt mice, there is a dramatic increase of fibrin deposition (Fig. 1 c) and p75NTR expression (Fig. 1 d) after injury, when compared with uninjured nerves (Fig. 1 a and b). In contrast, p75NTR−/− mice show reduced fibrin deposition after injury (Fig. 1 e). Quantification of immunoblots reveals that p75NTR−/− mice have decreased fibrin by threefold 3 d and fourfold 8 d after injury (Fig. 1 g). Quantification of fibrin immunostaining also reveals that p75NTR−/− mice have significantly decreased fibrin (Fig. 1 h, P < 0.003). These results suggest that loss of p75NTR decreases the levels of fibrin in the sciatic nerve after injury.

p75NTR regulates expression of tPA in the sciatic nerve after crush injury

Analysis of total fibrinogen levels were similar in the plasma of wt and p75NTR−/− mice (unpublished data), suggesting the decrease in fibrin deposition is not the result of hypofibrinogenemia. Because fibrin removal depends on proteolytic activity (Bugge et al., 1996), we hypothesized that the decreased fibrin in the p75NTR−/− mice reflects an up-regulation of the proteolytic activity. p75NTR−/− mice have increased proteolytic activity (Fig. 2 b).
when compared with wt mice (Fig. 2 a) that is statistically significant (Fig. 3 i, P < 0.05). Uninjured nerves exhibit minimal proteolytic activity (Fig. 2 i), as expected (Akassoglou et al., 2000). Injured p75NTR−/− sciatic nerves do not show lysis of fibrin in the absence of plasminogen (Fig. 2 c), suggesting that the proteolytic activity is plasminogen dependent.

The tPA/plasmin system regulates fibrin clearance after nerve injury (Akassoglou et al., 2000). A specific tPA inhibitor, tPASTOP, blocks proteolytic activity in p75NTR−/− mice (Fig. 2 d). p75NTR is strongly activated by withdrawal of axons (Lemke and Chao, 1988) and its expression correlates with proliferating, non-myelin producing Schwann cells (SCs) (Zorick and Lemke, 1996). After sciatic nerve injury both p75NTR (Fig. 2 e, red) and tPA (Fig. 2 e, green) increase when compared with uninjured controls (Fig. 2 j), but show little colocalization (Fig. 2, e and h), suggesting that p75NTR-reexpressing SCs do not express tPA. Expression of tPA (Fig. 2 k, red) and p75NTR (Fig. 2 l, green) in SCs is confirmed using double immunofluorescence with the SC marker S100 (Fig. 2, k and l; green).

**Figure 2.** p75NTR regulates expression of tPA in the sciatic nerve after crush injury. In situ zymography in the presence of plasminogen on wt (a) and p75NTR−/− (b) mice and in the absence of plasminogen (c) or in the presence of plasminogen and tPASTOP (d) in p75NTR−/− mice. Arrows indicate the lytic zone. Double immunofluorescence for tPA (green) or p75NTR (red) on wt (e and h), p75NTR−/− (f) and p75NTR−/−/tPA−/− mice (g). Uninjured wt sciatic nerve exhibits minimal proteolytic activity (i) and minimal tPA and p75NTR immunoreactivity (j). Zymographies have been performed on n = 10 wt and n = 10 p75NTR−/− mice. Representative images are shown. tPA (k) and p75NTR (l) expression in SCs was verified by double immunofluorescence with an S100 (SC marker) antibody. Arrows indicate double-positive cells (k and l, yellow). The experiment was repeated at two different time points (4 and 8 d after crush injury) in n = 4 mice per genotype per time point and representative images are shown. Bar: 400 μm (a–d, i), 150 μm (e–g, j), 20 μm (h, k, and l).

**Genetic loss of tPA rescues the effects of p75NTR deficiency**

To examine genetically whether the increased proteolytic activity of p75NTR deficiency (Fig. 2, k and l; green) is shown in Fig. 3 i. The evidence derived from the genetic depletion of tPA in the p75NTR−/− mice (p75NTR−/−/tPA−/− mice, Fig. 3 g) are in accordance with the pharmacologic inhibition of tPA activity in the p75NTR−/− sciatic nerve using tPASTOP (Fig. 2 d). Overall, these results suggest that up-regulation of proteolytic activity in the sciatic nerve of p75NTR−/− mice is due to up-regulation of tPA.

**Expression of p75NTR inhibits tPA and fibrinolysis**

After finding a biological function for p75NTR in the regulation of tPA using SCs and sciatic nerves from p75NTR−/− mice, we used stable and transient transfections of p75NTR as well as...
siRNA against p75NTR to test the properties of p75NTR in heterologous systems. To examine whether p75NTR could inhibit fibrin degradation, we first used NIH3T3 fibroblasts stably transfected with p75NTR that exhibit high levels of p75NTR (10^5 receptors/cell) (Hsu and Chao, 1993). NIH3T3 cells on a 3D fibrin gel degrade fibrin (Fig. 5a), whereas NIH3T3p75NTR cells do not (Fig. 5b). Expression of p75NTR inhibits fibrin degradation by 12-fold (Fig. 5c; P < 0.001). NIH3T3 cells form lytic areas (Fig. 5d), whereas NIH3T3p75NTR cells grow uniformly on fibrin (Fig. 5e). NIH3T3 cells fully degrade the plasmin substrate casein (Fig. 5f) but NIH3T3p75NTR cells do not degrade casein (Fig. 5g), suggesting impaired proteolysis in NIH3T3p75NTR cells. Aprotinin, a general inhibitor of serine proteases, completely inhibits fibrin degradation by NIH3T3 cells (not depicted). In fibroblasts both tPA and uPA are involved in activation of plasminogen and fibrin degradation. tPA activity is significantly decreased in the NIH3T3p75NTR cells (Fig. 5h). In contrast, expression of p75NTR has no effect on uPA activity (Fig. 5i).

tPA is a transcriptionally regulated immediate-early gene (Qian et al., 1993). Indeed, expression of p75NTR down-regulates tPA transcripts (Fig. 5j). In addition, mRNA of PAI-1 is also up-regulated in NIH3T3p75NTR cells (Fig. 5j). Real-time quantitative PCR shows a 10.1-fold decrease in tPA mRNA, a fourfold increase in PAI-1 mRNA, and a twofold decrease in uPA mRNA in NIH3T3p75NTR cells. Upon expression of p75NTR, the decrease of uPA RNA does not affect uPA activity (Fig. 5i). In contrast, the decrease of tPA RNA in NIH3T3p75NTR cells results in a corresponding decrease in tPA activity (Fig. 5h; P < 0.01).

**Figure 3.** Loss of tPA rescues the effects of p75NTR deficiency in plasminogen activation and fibrin deposition in the sciatic nerve. Increased fibrin deposition in the crushed sciatic nerve of p75NTR−/− tPA−/− mice (c), when compared with crushed p75NTR−/− tPA−/− sciatic nerve (b). Wt (a) and tPA−/− (d) nerves are used for control. In situ zymography shows lack of proteolytic activity in the crushed p75NTR−/− tPA−/− sciatic nerves (n = 5) (g), when compared with crushed p75NTR−/− sciatic nerves (n = 20) (l). Crushed wt (e) and tPA−/− (h) nerves are used for control. Fibrin immunostainings and zymographies were performed on n = 5 p75NTR−/− tPA−/−, n = 20 p75NTR−/−, n = 20 wt, n = 5 tPA−/− mice. Representative images are shown. (i) Quantification of proteolytic activity 4 d after crush injury shows statistically significant increase for proteolytic activity in p75NTR−/− mice. Quantification results are based on n = 5 p75NTR−/−, n = 5 p75NTR−/− tPA−/−, n = 5 tPA−/− and n = 4 wt mice. Bar graph represents means ± SEM (*, P < 0.05; by ANOVA). Bar: 50 μm (a–d), 300 μm (e–h).

**Figure 4.** Regulation of tPA and fibrinolysis in SCs. Primary SC cultures from wt (a) or p75NTR−/− mice (b) on a 3D fibrin gel. Arrowheads indicate the border of fibrin degradation. Quantification of fibrin degradation (c) and tPA activity (d) from wt and p75NTR−/− SCs. Experiments were performed three times in duplicates. Representative images are shown. Bar graph represents means ± SEM (P < 0.01; by t test). Bar, 130 μm.
After injury, sciatic nerves of p75NTR−/− mice show a fourfold increase of tPA RNA when compared with wt (Fig. 5 k). Moreover, p75NTR−/− mice show an increase in tPA RNA in primary cerebellar granule neurons (CGNs) (Fig. S1 c, available at http://www.jcb.org/cgi/content/full/jcb.200701040/DC1), and increased proteolytic activity in the cerebellum (Fig. S1, a and b).

Overall, these data suggest that expression of p75NTR inhibits the tPA/plasmin system both in vivo in the cerebellum and after sciatic nerve injury, as well as in vitro in primary neurons, SCs, as well as fibroblasts.

p75NTR regulates tPA and PAI-1 via a PDE4/cAMP/PKA pathway

Transcriptional regulation of tPA depends on the cAMP/PKA pathway (Medcalf et al., 1990). Indeed, elevation of cAMP, using dibutyryl-cAMP (db-cAMP), overcomes the inhibitory effect of p75NTR (Fig. 6 a). Moreover, cAMP elevation, elicited using the general PDE inhibitor IBMX, elevates tPA activity in NIH3T3p75NTR to the levels seen in NIH3T3 cells (Fig. 6 b). IBMX does not affect basal levels of tPA in NIH3T3 cells (Fig. 6 b). These data suggest that expression of p75NTR inhibits the tPA/plasmin system both in vivo in the cerebellum and after sciatic nerve injury, as well as in vitro in primary neurons, SCs, as well as fibroblasts.

PKA activity is decreased in NIH3T3p75NTR cells (Fig. 6 c, lanes 3 and 4) compared with NIH3T3 cells (Fig. 6 c, lanes 1 and 2), suggesting that p75NTR expression reduces PKA activity. KT5720, a specific PKA inhibitor, decreases tPA activity in NIH3T3 cells (Fig. 6 b). Because the cAMP/PKA pathway enhances tPA transcription and suppresses PAI-1 secretion (Santell and Levin, 1988), we tested whether the cAMP/PKA pathway influences the p75NTR regulation of tPA and PAI-1. Forskolin-induced cAMP elevation increases, whereas KT5720-induced PKA inhibition decreases tPA RNA in NIH3T3 cells (Fig. 6 d). Forskolin treatment of NIH3T3p75NTR cells also increases both tPA RNA (Fig. 6 d) and activity (not depicted), whereas forskolin decreases PAI-1 RNA in both NIH3T3 and NIH3T3p75NTR cells (Fig. 6 e).

Similar to NIH3T3 cells, elevation of cAMP increases the activity of tPA in both wt and p75NTR−/− SCs (Fig. 6 f). Brain-derived neurotrophic factor (BDNF)/TrkB signaling has been shown to regulate tPA in primary cortical neurons (Fiumelli et al., 1999). In contrast to cortical neurons, SCs are known to express minute levels of TrkB but high levels of p75NTR (Cosgaya et al., 2002). We show here that treatment of SCs with either BDNF or nerve growth factor (NGF) has no effect on tPA (Fig. 6 f). Similar results are obtained after treatment of SCs with proNGF, the high-affinity ligand of p75NTR (Lee et al., 2001) (unpublished data). In addition, in NIH3T3 and NIH3T3p75NTR cells, which do not express Trk receptors, the p75NTR-mediated suppression of tPA activity occurs independent of neurotrophins or serum (unpublished data). In accordance, in NIH3T3 cells transient expression of the intracellular domain (ICD) of p75NTR decreases tPA similar to the full-length (FL) p75NTR (Fig. 6 g).
These data suggest that neurotrophin/p75NTR signaling is not involved in the regulation of tPA in SCs and fibroblasts and that regulation of tPA by p75NTR is independent of neurotrophins.

**p75NTR decreases cAMP via PDE4**

Because the effects of p75NTR were overcome by elevating cAMP, we examined whether p75NTR reduced cAMP levels. Indeed, cAMP is decreased 7.8-fold in NIH3T3p75NTR cells (Fig. 7 a; \( P < 0.0001 \)). Transient expression of p75NTR in NIH3T3 cells decreases levels of cAMP (Fig. 7 b; \( P < 0.0005 \)). Furthermore, siRNA knockdown against p75NTR leads to increased cAMP in both NIH3T3 and NIH3T3p75NTR cells (Fig. 7, c and e; \( P < 0.02 \) and primary SCs (Fig. 7, d and f; \( P < 0.03 \)). NIH3T3 cells transiently transfected with p75NTR express five-fold less p75NTR than the stably transfected NIH3T3p75NTR cells (unpublished data). Differences in expression between stably and transiently transfected cells may account for the differences in the fold-decrease of cAMP and tPA between these two systems. Moreover, immunostaining with an antibody against cAMP shows increased cAMP in injured sciatic nerves from p75NTR−/− mice (Fig. 7, g and h). In neurons BDNF elevates cAMP exclusively via TrkB (Gao et al., 2003). In NIH3T3p75NTR cells, which do not express TrkB, stimulation with NGF or BDNF does not affect the p75NTR-mediated suppression of cAMP (Fig. S2). Similarly, inhibition of neurotrophins by Fc-p75NTR or BDNF by Fc-TrkB does not alter cAMP levels in NIH3T3p75NTR cells (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200701040/DC1). In accordance, transient expression of the ICD of p75NTR decreases cAMP similar to the FL p75NTR in NIH3T3 cells (Fig. 7 b). Overall, these data suggest a neurotrophin-independent PDE4/cAMP pathway downstream of p75NTR, which consequently leads to decreases in extracellular proteolysis.

Down-regulation of cAMP can be mediated either by inhibition of cAMP synthesis via the action of Gi, a G protein that inhibits adenyl cyclase or via the action of PDEs. Treatment of cells with pertussis toxin (PTX) that blocks interactions between the Gi and G protein coupled receptors, does not rescue the p75NTR-mediated down-regulation of cAMP (Fig. 7 a; \( P > 0.5 \)). In contrast, the PDE inhibitor IBMX resulted in significant increase of cAMP in the NIH3T3p75NTR cells when compared with control NIH3T3p75NTR cells (Fig. 7 a; \( P < 0.000001 \)). Use of specific chemical inhibitors for PDE isoforms shows that only rolipram, a specific inhibitor of PDE4, significantly increases cAMP levels in NIH3T3p75NTR cells (Fig. 7 a; \( P < 0.000001 \)) to the levels of NIH3T3 cells (Fig. 7 a; \( P = 0.051 \)), suggesting that the p75NTR-induced cAMP decrease is mediated via PDE4.
p75NTR interacts with PDE4A4/5 and targets cAMP degradation to the membrane

Recruitment of PDE4 to subcellular structures such as the plasma membrane concentrates the activity of PDEs and reduces PKA activity by enhancing degradation of cAMP (Brunton, 2003; Houslay and Adams, 2003). We therefore examined whether p75NTR regulates cAMP via recruitment of PDE4. In NIH3T3p75NTR cells, p75NTR coimmunoprecipitates (co-IPs) with endogenous PDE4A (Fig. 8 a). No association is observed with the other three PDE4 sub-families, namely PDE4B, PDE4C, or PDE4D (unpublished data), suggesting that the effect was PDE4A specific. Based on the molecular weight of PDE4A at 109 kD, we determined that p75NTR co-IPs with the PDE4A5 isoform. Endogenous co-IP in CGNs (Fig. S3 a, available at http://www.jcb.org/cgi/content/full/jcb.200701040/DC1) and in injured sciatic nerve (Fig S3 b) shows that p75NTR and PDE4A5 interact at endogenous expression levels. Analysis of lysates shows that the levels of PDE4A are similar in NIH3T3 and NIH3T3p75NTR cells (Fig. S3 c). These results show that p75NTR forms a complex with PDE4A5.

A functional consequence of the p75NTR–PDE4A5 interaction would be recruitment of PDE4A5 to the membrane resulting in decreased membrane-associated cAMP/PKA signaling.

To investigate whether p75NTR reduces membrane-associated PKA activity, we modified the genetically encoded A-kinase activity reporter, AKAR2 (Zhang et al., 2005) and generated pm-AKAR2.2, a membrane-targeted fluorescent reporter of PKA activity that generates a change in fluorescence resonance energy transfer (FRET) when it is phosphorylated by PKA in living cells (Fig. S4 a). As expected, NIH3T3 cells show a dramatic emission ratio change for the pm-AKAR2.2 in response to forskolin (Fig. 8 b). In contrast, NIH3T3p75NTR cells show an attenuated response, revealing reduced PKA activity at the plasma membrane (Fig. 8 b). Transient transfection of p75NTR confirmed the results observed in the stable NIH3T3p75NTR cells using the latest generation of plasma membrane–specific PKA biosensor AKAR3 (Allen and Zhang, 2006) (Fig. S4 b, available at http://www.jcb.org/cgi/content/full/jcb.200701040/DC1). As expected, increased cAMP degradation at the plasma membrane results in decreased intracellular cAMP (Fig. S4 c; Fig. 7, a and b). Overall, our results showing reduced membrane-associated PKA activity upon expression of p75NTR suggest that p75NTR targets cAMP degradation to the membrane via its interaction with PDE4A5.

To verify the specificity of p75NTR–PDE4A5 association, a series of mapping studies were conducted using deletion mutants. PDE4A5 interacts with FL p75NTR, as well as deletions Δ3, Δ62,
Δ83, but not a deletion missing the distal 151 amino acids, Δ151 (Fig. 8 c), suggesting that the interaction between p75NTR and PDE4A5 occurs in the juxtamembrane region of p75NTR, requiring sequences between residues 275 and 343. To explain the specificity of the interaction of p75NTR with a single PDE4 isoform, we reasoned that p75NTR would interact with a unique region of PDE4A5 that is not present in other PDE4s. Although the PDE4 isoforms are highly homologous, PDE4A5 contains a unique C-terminal region with a yet unknown biological function (Houslay and Adams, 2003). Co-IP experiments in HEK293 cells using the PDE4A4 δCT mutant that is missing the C-terminal region (aa 721–886) abolishes the interaction with p75NTR (Fig. 8 d).

To examine whether p75NTR could interact with PDE4A5 in a direct manner, we performed in vitro pull-down assays using recombinant proteins. A GST fusion protein of p75NTR encoding the entire ICD interacts with both recombinant PDE4A5 and its human homologue PDE4A4 (Fig. 8 e). In contrast, p75NTR ICD does not interact with recombinant PDE4D3 (Fig. 8 e). These results are in accordance with both the endogenous co-IPs in cells (Fig. 8, a and c; Fig. S3) and the PDE4A4 mutagenesis data (Fig. 8 d) because similar to PDE4A4 δCT, PDE4D3 does not contain the unique C-terminal domain of PDE4A4/5. We have used peptide array technology to define sites of direct interaction in other PDE4s (Bolger et al., 2006). Screening a peptide array library of overlapping 25-mer peptides that scanned the sequence of PDE4A4 with GST-ICD p75NTR identified interactions with the LR1 domain, whose sequence is unique to the PDE4A subfamily (peptides 40 and 41), the catalytic domain (peptides 135 and 136) and the unique C-terminus (peptides 172 and 173). Alanine scanning mutagenesis shows that substitution of C862 abolishes the interaction of p75NTR with the 173 peptide that is unique to PDE4A.
p75NTR regulates plasminogen activation and fibrin deposition in a model of lipopolysaccharide-induced pulmonary fibrosis

Because expression of p75NTR inhibits fibrinolysis in fibroblasts, we hypothesized that the role of p75NTR as a modulator of fibrinolysis extends to tissues outside of the nervous system that express p75NTR after injury or disease. Because p75NTR is expressed in the lung (Ricci et al., 2004), we compared the levels of fibrin in the lung of wt and p75NTR−/− mice in a model of lipopolysaccharide (LPS)-induced lung fibrosis (Chen et al., 2004). LPS-treated wt mice showed widespread extravascular fibrin deposition (Fig. 9 b) and decreased proteolytic activity after LPS treatment (Fig. 9 e), when compared with saline-treated wt mice (Fig. 9 a and d). In contrast, p75NTR−/− mice show a 2.58-fold decrease of fibrin immunoreactivity (Fig. 9 c and j) and increased proteolytic activity (Fig. 9 f).

Decreased proteolytic activity in the lung after injury depends on the up-regulation of PAI-1 (Idell, 2003). Loss of PAI-1 protects from pulmonary fibrosis in LPS-induced airway disease, hyperoxia, and bleomycin-induced fibrosis (Savov et al., 2003). Because p75NTR increases PAI-1 (Fig. 5 j and Fig. 6 e), we examined whether p75NTR regulates expression of PAI-1 in vivo. PAI-1 is up-regulated in LPS-treated wt mice (Fig. 9 h) when compared with saline-treated wt mice (Fig. 9 g). In contrast, LPS-treated p75NTR−/− mice show similar immunoreactivity for PAI-1 (Fig. 9 i) as saline-treated wt mice (Fig. 9 g), suggesting that p75NTR induces up-regulation of PAI-1 after injury in the lung.

Western blots show a decrease in PAI-1 in the lungs of p75NTR−/− mice (Fig. 9 k). Similar to the p75NTR−/− mice, rolipram reduces fibrin deposition in the lung (Fig. S5, a and b; available at http://www.jcb.org/cgi/content/full/jcb.200701040/DC1) and sciatic nerve (Fig. S5, d–f), and decreases PAI-1 in the lung (Fig. S5 c), suggesting the involvement of PDE4 in p75NTR-mediated inhibition of fibrinolysis in vivo. Collectively, our data show that p75NTR increases fibrin deposition via a PDE4-mediated inhibition of plasminogen activation in both LPS-induced lung fibrosis and sciatic nerve crush injury. These data suggest a role for p75NTR/PDE4 signaling as a general regulator of plasminogen activation and fibrinolysis at sites of injury.

Discussion

Our study shows a novel direct interaction of p75NTR with PDE4A4/5, a specific PDE4 isoform, which results in the regulation of cAMP, a major intracellular signaling pathway, and mediates a major biological function of extracellular proteolysis and fibrinolysis (Fig. 10). p75NTR is expressed in a wide range of tissue injury models, where repair depends upon both cell differentiation and ECM remodeling. For example, we recently showed that in the absence of plasminogen the effects of p75NTR in tissue repair are protective due to its beneficial effects in cell differentiation (Passino et al., 2007). Similarly, in the flow-restricted carotid artery model of vascular injury that depends on uPA and not on tPA-mediated fibrinolysis (Kawasaki et al., 2001), p75NTR is protective due to the induction of smooth
We provide the first evidence for p75NTR in the regulation of cAMP by using genetic depletion, siRNA knockdown or up-regulation of the p75NTR. Our results suggest that p75NTR might exert the opposite function as Trk receptors by recruiting PDE4A4/5 and decreasing cAMP. Interestingly, PDE4A has been detected as the predominant PDE4 isoform at the corticospinal tract (Cherry and Davis, 1999). Because p75NTR can act as a coreceptor for NogoR, a mediator of the inhibition of nerve regeneration, PDE4A interaction with p75NTR could play an inhibitory role in nerve regeneration by competing with neurotrophin signaling via Trk receptors.

It is possible that the increased expression of p75NTR by neurons, glia, and brain endothelial cells could regulate the temporal and spatial pattern of tPA expression during brain injury or inflammation. p75NTR might also be upstream of other non-fibrinolytic functions associated with tPA, such as neurodegeneration, synaptic plasticity, and long-term potentiation (Samson and Medcalf, 2006). Given the dependence of p75NTR functions on the availability of ligands and coreceptors (Teng and Hempstead, 2004; Reichardt, 2006), further analysis will determine the role of p75NTR in extracellular proteolysis and ECM remodeling in different cellular systems. We show that expression of p75NTR can inhibit tPA in the absence of neurotrophin ligands. Constitutive expression of p75NTR may signal in a neurotrophin-independent manner to induce neuronal apoptosis (Rabizadeh et al., 1993), activation of Akt (Roux et al., 2001), and RhogTAPase (Yamashita et al., 1999). The regulation of cAMP identified here is an effect of expression of p75NTR that does not appear to depend on neurotrophin signaling. The cellular distribution of PDE4A4/5 would determine the involvement of p75NTR in the regulation of cAMP. It is possible that non-neurotrophin ligands that bind directly to p75NTR, such as β-amyloid (Teng and Hempstead, 2004), as well as the myelin/NogoR p75NTR-dependent inhibitors of nerve regeneration (Filbin, 2003), are able to regulate both cAMP and plasminogen activation by p75NTR. Because cAMP analogues decrease expression of p75NTR (Baron et al., 1997), it is possible that p75NTR by decreasing cAMP contributes to the positive regulation of its expression. Because PKA phosphorylates p75NTR and regulates its translocation to lipid rafts (Higuchi et al., 2003), p75NTR via regulation of PKA might regulate its own subcellular localization. Given the multiple genes regulated by cAMP and PKA, other cellular functions may be regulated by p75NTR/cAMP signaling.
NGF/p75NTR signaling has been suggested to enhance local neurogenic inflammation to exacerbate pulmonary disease (Renz et al., 2004). Our study suggests an additional pathway for p75NTR as a regulator of expression of PAI-1 and a mediator of fibrosis. p75NTR in the lung is detected mainly in basal epithelial cells of bronchioles (unpublished data). Similar to p75NTR, PAI-1 is expressed by bronchial epithelial cells (Savov et al., 2003) and its expression results in an antifibrinolytic environment within the airway wall. Fibrin regulates both inflammation and airway remodeling (Idell, 2003; Savov et al., 2003). It is therefore possible that p75NTR-mediated regulation of PAI-1 via PDE4 could influence inflammatory and tissue repair processes in pulmonary disease. Although in chronic obstructive pulmonary disease the PDE4A4 isoform is specifically up-regulated (Houslay et al., 2005), all available inhibitors target the common catalytic domain of all PDE4 isoforms resulting in unwanted side-effects. Our study suggests that the p75NTR/PDE4A4 interaction could be a potential therapeutic target that will achieve the specific inhibition of a single PDE4 isoform and may have therapeutic potential.

Collectively, we have identified a novel cAMP-dependent signaling pathway initiated by p75NTR that specifically regulates plasminogen activity and scar formation after sciatic nerve and lung injury. Though p75NTR is responsible for a variety of cell survival and death decisions (Chao, 2003), our data has revealed an unrecognized property of this receptor to regulate the degradation of cAMP. This property provides a potential mechanism to account for how p75NTR acts at sites of injury to promote ECM remodeling. The impact of high levels of p75NTR expression upon inhibition of extracellular proteolysis indicates that the detrimental effects of p75NTR extend beyond cell growth and axon inhibition. Finally, the dramatic inhibitory effect of p75NTR signaling on plasminogen activation suggests that the p75NTR/PDE4A4 interaction represents a novel target for therapeutic intervention in both neuronal and non-neuronal tissues.

Materials and methods

Animals, sciatric nerve crush, and induction of lung fibrosis

p75NTR−/− mice (Lee et al., 1992) and iPA−/− mice (Carmeliet et al., 1994) were in C57Bl/6 background and purchased from The Jackson Laboratory. Double p75NTR−/−/iPA−/− mice were generated as described previously (Khursigara et al., 1999). Lung fibrosis was induced as described previously (Chen et al., 2004). Mice were killed 4.5 h after LPS or saline administration. For MIP2 assay kits from American Diagnostica and Chemicon, respectively. To elevate supernatants was performed according to the directions of the activity assay kits from American Diagnostica and Chemicon, respectively. To elevate cell culture and transfections

Murine SCs were isolated as described previously (Syroid et al., 2000). NIH3T3 or HEK293 cells were cotransfected either with p75NTR FL, ICD or deletion constructs, and PDE4A5 cDNA using Lipofectamine 2000 (Invitrogen) as described in the Results section. CGNs were isolated from P10 animals (Yamashita and Tohyama, 2003). CGNs were lysed immediately for co-IP, without plating. siRNA directed against p75NTR (Dharmacon) was transfected into SCs and NIH3T3 or p75NTR cells using Dharmafect (Dharmacon).

Immunohistochemistry

Immunohistochemistry was performed as described in Akassoglou et al. (2002). Primary antibodies were sheep anti-human fibrin(ogen) (1:200; US Biologicals), rabbit anti-human iPA (1:300; Molecular Innovations), rabbit anti-p75NTR clone 9651, (1:1,000), goat anti-p75NTR (1:200; Santa Cruz Biotechnology, Inc.), rabbit anti–mouse PAI-1 (1:500; a gift from David Loskutoff, Scripps Research Institute, La Jolla, CA), and mouse anti–S100 (1:200; Neomarkers). For immunofluorescence, secondary antibodies were anti-rabbit FITC and anti–goat Cy3 (1:200; Jackson Immunochromicals). Images were acquired with an Axioplan II epifluorescence microscope (Carl Zeiss Microimaging, Inc.) using dry Plan-Neofluar lenses using 10× 0.3 NA, 20× 0.5 NA, or 40× 0.75 NA objectives equipped with Axiocam HRC digital camera and the Axiovision image analysis system.

Immunoblot

Immunoblot was performed as described previously (Akassoglou et al., 2002). Antibodies used were rabbit anti-p75NTR, clones 9992 and 9651 (1:5,000), mouse anti-fibrin (1:500; Accurate Chemical & Scientific Corp.), rabbit anti-myosin (1:1,000; Sigma-Aldrich), rabbit anti-GAPDH (1:1,000; Abcam) and rabbit anti-PAI-1 (1:5,000; a gift from David Loskutoff). Quantification was performed on the Scion NIH Imaging Software. Fibrin precipitation and quantification from lung tissues was performed exactly as described previously (Lung et al., 2004).

Co-IP

Co-IP was performed as described previously (Khursigara et al., 1999). Cell lysates were prepared in 1% NP-40, 200 mM NaCl, 1 mM EDTA, and 20 mM Tris-HCl, pH 8.0. IP was performed with an anti-p75NTR antibody (9992) and immunoblot with anti-PDE4A4, PDE4B, PDE4C, and PDE4D (Fabgenix). The co-IP buffer using NP-40 has been previously used to examine interactions of p75NTR with other intracellular proteins, such as TRAF-6 (Khursigara et al., 1999) and PKA (Higuchi et al., 2003). For mapping experiments, PDE4A5 cDNA was cotransfected with HA-tagged p75NTR deletion constructs into HEK293 cells. IP was performed with an anti-HA antibody (Cell Signaling). Cell lysates were probed with an anti-PDE4A4 or an anti-p75NTR antibody (9651). For co-IP experiments using recombinant proteins, equimolar amounts (2 μM) of purified recombinant MBP-PDE4A5 (C. Corrêa et al., 1996), MBP-PDE4A4 (McPhee et al., 1999), MBP-PDE4D3 (Yarwood et al., 1999), and GST-p75NTR-ICD (Khursigara et al., 2001) were mixed in binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl2, 1 mM DTT, 0.5% Triton X-100, and 0.5% BSA) and incubated for 1 h at 4°C. Washed glutathione-Sepharose beads were added according to the manufacturer’s instructions for an additional hour. Beads were sedime...
cAMP/PKA assays

10° fibroblasts or 500,000 SCs were lysed in 0.1 N HCl solution and cAMP was measured using a competitive binding cAMP ELISA (Assay Designs). Cells were treated with 100 ng/ml PTX for 16 h. For inhibition of PDE activity, cells were treated for 16 h with 500 μM isobutyl methylxanthine (IBMX; Calbiochem), 18.7 μM 8-methoxy-3H-3-isobutyl-1-methylxanthine (PDE1 inhibitor; Calbiochem), 80 μM erythro-9-(2-Hydroxy-3-nonyl)ladenine (PDE2 inhibitor; Calbiochem), 100 nM trequinsin (PDE1 inhibitor; Calbiochem), and 10 μM rolipram (PDE4 inhibitor; Calbiochem). Cells were treated with forskolin in the presence of the inhibitors for 1 h. Because these inhibitors specifically inhibit a PDE isoform and have no effect on the other PDE iso-enzymes (Beavo and Reifsnnyder, 1990), they are extensively used for the identification of the specific PDE isoform that is involved in different cellular functions. Induction with neurotransphins was performed using 100 ng/ml NGF or 50 ng/ml BDNF, 750 ng/ml of FcTrkB, or 1.35 μg/ml of Fce755(2)/40-nm filter for CFP and 535/25-nm emission filter for YFP.

In situ zymography

In situ zymographies were performed as described previously (Akassoglou et al., 2000). Quantification of in situ zymographies was performed by measuring the area of the lytic zone surrounding each nerve, and dividing that value by the area of the nerve. Images were collected after 8 h of incubation for the sciatic nerve and 4 h of incubation for the sciatic nerve. For cell zymographies, cultures were washed four times with PBS/BSA and overlaid with 200 μl of Dulbecco’s minimum essential medium containing 1% LMP agarose, 2.5% boiled nonfat milk, and 25 μg/ml human plasminogen. The overlay was allowed to harden, and plates were incubated in a cell culture incubator at 37°C. Pictures of lytic zones were taken using an inverted microscope under dark field (Carl Zeiss Microimaging, Inc.).

Construction of pm-AKAR2.2 and PDE4A4,CT

For the construction of pm-AKAR2.2 we used the previously described cytoplasmic plasmid sensor, AKAR2 (Zhang et al., 2005). pm-AKAR2.2 consists of a cDNA containing a FRET pair, monomeric enhanced cyan fluorescent protein (ECFP), and monomeric citrine (an optimized version of YFP), fused to forhead associated domain 1 (FHA1) (Rad53p 22–162), and the PKA sub-strate sequence LRRATLVD via linkers. A206K mutations were incorporated for the chimaeric sequence from K-Ras K K K K K K S K T K C V I M was added to target the construct to ECFP and Citrine by the QuikChange method (Stratagene). The C-terminal forkhead associated domain 1 (FHA1) (Rad53p 22–162), and the PKA sub-

Peptide array mapping

Peptide libraries were synthesized by automatic SPOT synthesis (Frank, 2002). Synthetic overlapping peptides (25 amino acids in length) were spotted on Whatman 50 cellulose membranes according to standard pro-tocols by using Fmoc-chemistry with the AutoSpot Robot ASS 222 (Intavis Bioanalytical Instruments AG). Membranes were overlaid with 10 μg/ml recombinant GST-p75NTR (Bound recombinant GST-p75NTR IC (Khursi- et al., 2001) was detected using rabbit anti-GST (1:2,000; GE Healthcare) followed by secondary anti-rabbit horseradish peroxidase antibody (1:2,500; Dianova). Alkaline washing was performed as described previously (Bolger et al., 2006).

Online supplemental material

Fig. 51 shows that genetic loss of p75NTR increases tPA mRNA levels and proteolytic activity in the cerebellum. Fig. 52 demonstrates that treatment with neurotrophins has no effect on the tPA levels in NIH3T3 cells. Fig. 53 shows endogenous co-munnoprecipitations of PDE4A5 and p75NTR from injured sciatic nerve and from primary CGNs. Fig. S4 shows results from transient transfections of NIH3T3 cells with p75NTR and the PKA activity reporters, AKAR3 and pm-AKAR3. Fig. S3 shows that inhibition of PDE4s with rolipram decreases fibrin deposition both in LPS-induced lung fibrosis and sciatic nerve crush injury. The online version of this article is available at http://www.jcb.org/cgi/content/full/jcb.200701040/D1C1.

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