

# Ligand-induced recruitment of Na<sup>+</sup>/H<sup>+</sup>-exchanger regulatory factor to the PDGF (platelet-derived growth factor) receptor regulates actin cytoskeleton reorganization by PDGF

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Proteins interacting with the human PDGF (platelet-derived growth factor)  $\beta$ -receptor were isolated using immobilized peptides derived from the receptor C-terminus as a bait. We identified two PDZ domain proteins, namely NHERF (Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor, also called EBP50) and NHERF2 (E3KARP, SIP-1, TKA-1), which have been shown previously to associate with the murine PDGF receptor [Maudsley, Zamah, Rahman, Blitzer, Luttrell, Lefkowitz and Hall (2000) *Mol. Cell. Biol.* **20**, 8352–8363]. In porcine aortic endothelial cells and in fibroblasts, NHERF recruitment was induced by PDGF treatment, but the receptor kinase activity was not required for the formation of the complex, suggesting that NHERF was not recruited in a phosphotyrosine-dependent manner. Instead, the interaction was abolished by mutation of the consensus C-terminal PDZ-interacting domain of the receptor (Leu-1106 to Ala), or truncation of the last

75 amino acid residues of the receptor. Disruption of NHERF binding to the receptor enhanced actin filament reorganization, but did not affect PDGF-induced mitogenicity and chemotaxis. Although NHERF was initially characterized as a factor required for intracellular pH regulation by  $\beta$ 2-adrenergic receptors, we observed that it was not involved in pH regulation by PDGF. Collectively, these results suggest that the ligand-induced association of NHERF PDZ domain with the PDGF receptor tyrosine kinase controls the extent of cytoskeleton reorganization in response to PDGF.

**Key words:** chemotaxis, cytoskeleton, ezrin-binding protein 50 (EBP50), intracellular pH, Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor (NHERF), platelet-derived growth factor (PDGF).

## INTRODUCTION

Platelet-derived growth factor (PDGF) isoforms play key roles in embryonic development by acting on cells of mesenchymal origin, such as mesangial cells, fibroblasts and vascular-smooth-muscle cells [1]. They induce reorganization of the cytoskeleton, increase in intracellular pH and calcium concentration, massive changes in gene expression and, ultimately, cell migration and proliferation [2]. Four different PDGF chains assemble into five isoforms, namely PDGF-AA, -BB, -AB, -CC and -DD, which bind with different affinities to two structurally similar PDGF receptor (PDGFR) tyrosine kinases, PDGFR $\alpha$  and PDGFR $\beta$  [2–4]. Signalling by PDGFRs involves the following steps: (i) activation of the receptor tyrosine kinase activity on ligand-induced dimerization; (ii) phosphorylation of the receptor on multiple tyrosine residues; and (iii) docking of signalling proteins containing SH2 domains that interact with phosphorylated tyrosines and surrounding amino acid residues [2,5]. Well-characterized signal transduction proteins that are recruited to the PDGFRs include PI3K (phosphoinositide 3-kinase), PLC $\gamma$ , Src, Grb2 (growth-factor-receptor-bound protein 2) and SHP-2 (SH2 domain-containing phosphatase 2), which are responsible for activation of downstream kinases such as Akt/protein kinase B, protein kinase C and ERK (extracellular-signal-regulated kinase).

Recently, the murine PDGFR was shown to recruit NHERF (Na<sup>+</sup>/H<sup>+</sup>-exchanger regulatory factor), also called ezrin-binding protein 50 (EBP50), which lacks SH2 domains [6]. In cells over-

expressing both the receptor and NHERF, this interaction was constitutive and depended on the binding of the N-terminal PDZ domain of NHERF to the C-terminus of the PDGFR. NHERF targets proteins for apical localization in epithelial cells, mediates intracellular pH regulation by the  $\beta$ 2-adrenergic receptor and regulates signalling in response to parathyroid hormone [7–10]. Maudsley et al. [6] suggested that NHERF binding enhanced the PDGFR tyrosine kinase activity, thereby potentiating the cellular effects of PDGF.

In the present study, we show that in PAE (porcine aortic endothelial) cells expressing normal levels of PDGFRs, NHERF recruitment was induced by PDGF stimulation, independent of the tyrosine phosphorylation of the receptor. A thorough investigation of the possible involvement of NHERF in the cellular activities of PDGF revealed a role of NHERF in PDGF-induced reorganization of the cytoskeleton.

## EXPERIMENTAL

### Cell culture and reagents

PAE cells were cultured in Ham's F-12 medium supplemented with 10% foetal calf serum. HEK-293T (human embryonic kidney 293T) cells and AG01518 fibroblasts were cultured in Dulbecco's modified Eagle's medium with 10% foetal calf serum. Anti-phospho-Akt (Ser-473) and anti-phospho-ERK antibodies

Abbreviations used: BCECF, 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein; CCD, charge-coupled-device; ERK, extracellular-signal-regulated kinase; HEK-293T cells, human embryonic kidney 293T cells; NHE, Na<sup>+</sup>/H<sup>+</sup> exchanger; NHERF, NHE regulatory factor; PAE, porcine aortic endothelial (cells); PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PI3K, phosphoinositide 3-kinase; PY99, anti-phosphotyrosine; WGA, wheatgerm agglutinin.

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were purchased from Cell Signaling (Beverly, MA, U.S.A.). Anti-phosphotyrosine (PY99), anti-PDGFR $\beta$  (958) and anti-Akt1/2 were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, U.S.A.). Anti-NHERF antibodies were raised against a synthetic peptide corresponding to amino acid residues 342–355 of human NHERF (QMDWSKKNELFSNL) and purified as described in [11], using the same peptide coupled with Sulfolink beads (Pierce, Rockford, IL, U.S.A.). Anti-ERK2 antiserum EET has been described previously [11]. Peptides, synthesized on an Applied Biosystems 433A instrument using Fmoc chemistry, were purified by preparative reversed-phase chromatography.

### Mutagenesis and transfection

Site-directed mutagenesis was performed on a cDNA encoding the full-length PDGFR $\beta$  inserted into the pcDNA3 cloning vector (Invitrogen, Carlsbad, CA, U.S.A.), using QuickChange (Stratagene, La Jolla, CA, U.S.A.). The mutations were confirmed by DNA sequencing. PAE and HEK-293 cells were transfected using LIPOFECTAMINE<sup>TM</sup> Plus as recommended by the manufacturer (Invitrogen). PAE clones were selected for G418 resistance, and tested for homogeneous PDGFR $\beta$  expression by flow cytometry using a monoclonal antibody directed against the extracellular part of the receptor. At least two independent clones of each type were used in each experiment.

### Immunoprecipitation and Western-blot analysis

Subconfluent HEK-293T or PAE cells cultured in 10 cm dishes were washed, starved overnight in a medium containing 0.1 % BSA and then stimulated with PDGF-BB (50 ng/ml) for 10 min, unless otherwise stated. Cells were washed in ice-cold PBS, lysed and processed for immunoprecipitation with anti-NHERF antibodies and Protein-A–Sepharose. One-fifth of the lysate was incubated with WGA (wheatgerm agglutinin)–Sepharose (Amersham Biosciences). After boiling in Laemmli reducing buffer, samples were separated by SDS/PAGE, transferred on to an Immobilon membrane, blocked with fat-free 5 % (w/v) dry milk in PBS for 1 h and probed with PY99 (Santa Cruz Biotechnologies), anti-PDGFR $\beta$  or anti-NHERF antibodies (1  $\mu$ g/ml). Blots were visualized by chemiluminescence using a FUJI LAS2000 cooled CCD (charge-coupled-device) camera. AG01518 cells were processed similarly, except that lysate from two 15 cm dishes was used for each immunoprecipitation. The protein content of each sample was measured using BCA (bicinchoninic acid assay; Pierce).

### Intracellular pH measurements

PAE cells were allowed to attach to coverslips for a few hours in culture medium containing 10 % foetal calf serum, washed and starved overnight in a medium supplemented with 0.1 % BSA. Cells were washed with Hanks buffer (2.5 mM Hepes, pH 7.4/140 mM NaCl/5 mM KCl/2 mM CaCl<sub>2</sub>/1 mM Na<sub>2</sub>HPO<sub>4</sub>/25 mM glucose/0.05 % BSA) and loaded with 2  $\mu$ M BCECF [2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein] acetoxymethyl ester (Molecular Probes, Eugene, OR, U.S.A.) and 0.1 % pluronic acid for 30 min in Hanks buffer [12]. Coverslips with the BCECF-loaded cells were rinsed, and used as exchangeable bottoms of an open superfusion chamber thermostatically regulated at 37 °C. Cannulas fixed to this chamber were connected to a peristaltic pump allowing steady superfusion of a 2.5 mm Hanks buffer layer at the rate of 1 ml/min. Intracellular pH was measured with a dual-wavelength microfluorometric system (Deltascan,

Photon Technology International, Princeton, NJ, U.S.A.) [13]. The excitation light was alternatively directed to two monochromators by a chopper mirror spinning at 50 Hz. The monochromator outputs were connected via a bifurcated optical fibre to the epifluorescence attachment of an inverted microscope (Nikon Diaphot) equipped with a  $\times$ 100 objective. The pH-dependent fluorescence of 3–5 cells was recorded at 530 nm with a photomultiplier using a 25 nm half-bandwidth interference filter. The background-subtracted signals, obtained by excitation at 440 and 490 nm, were recorded at 2 Hz using FeliX software (Photon Technology International). PDGF-BB (20 ng/ml) was added to the superfusion medium 5 min after stabilization of the baseline, and fluorescence was followed for another 30 min. Finally, we monitored cell fluorescence in the presence of K<sup>+</sup>-rich calibration buffers (125 mM KCl/1 mM CaCl<sub>2</sub>/1 mM MgCl<sub>2</sub>/25 mM Mops, pH 6.80–7.40) containing 10  $\mu$ g/ml nigericin (Sigma). This calibration was used to convert corrected 490 nm/440 nm excitation ratios to intracellular pH values [12].

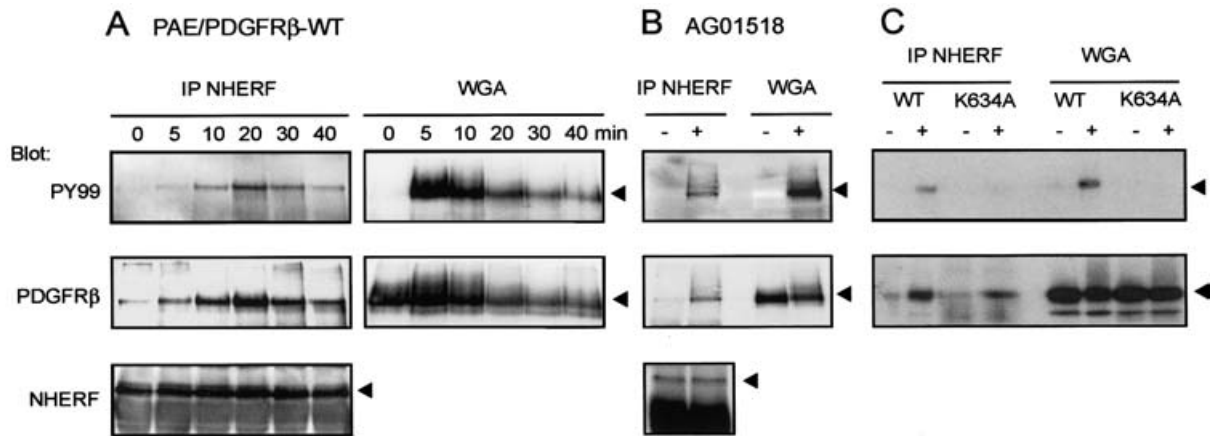
### Chemotaxis, mitogenicity and actin reorganization

PAE cells were washed, starved overnight as described above, trypsinized quickly and washed in the presence of 1 % Trasylol (Bayer, Leverkusen, Germany) to inactivate trypsin. Chemotaxis was measured using ChemoTx microplates as recommended by the manufacturer (Neuro Probes, Gaithersburg, MD, U.S.A.). Polycarbonate filters (8  $\mu$ m pore size) were coated with purified collagen (100  $\mu$ g/ml; Cohesion, Palo Alto, CA, U.S.A.) or human fibronectin (50  $\mu$ g/ml; Becton Dickinson, Bedford, MA, U.S.A.) on both sides. Cells were resuspended at  $2 \times 10^6$  cells/ml in Phenol Red-free medium, containing 0.1 % BSA, deposited in 30  $\mu$ l drops on the upper side of the filter and allowed to migrate towards PDGF-BB (1–100 ng/ml) for 4 h at 37 °C. The filter was removed from the chamber, washed with PBS to remove cells that did not migrate and stained with a Giemsa solution. The number of cells that have migrated through the filter was estimated using a cooled CCD camera (Fuji) and the AIDA software. Similar results were obtained using a modified Boyden chamber (results not shown). Mitogenicity and actin reorganization were measured as described previously [14].

## RESULTS

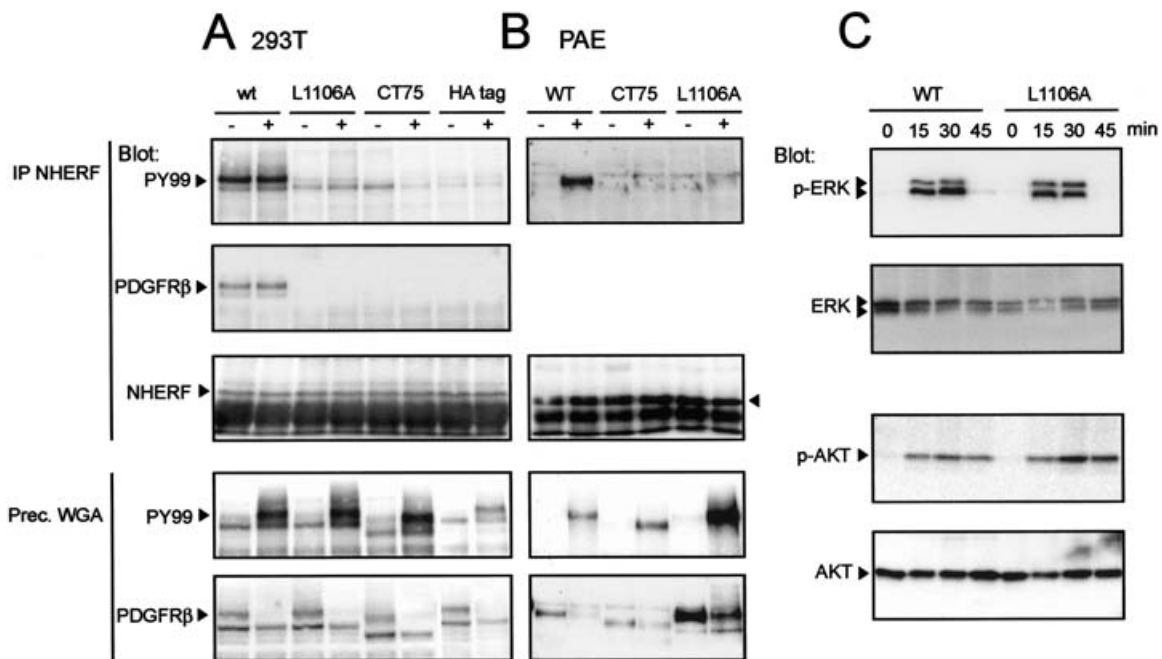
### NHERF interacts with PDGFR $\beta$ in a ligand-dependent manner

Using an immobilized peptide corresponding to the last 13 amino acid residues of the human PDGFR $\beta$  as a bait, we isolated two proteins, NHERF (also called EBP50) and NHERF2 that were identified by MS and Western-blot analysis. NHERF contains two PDZ domains that have been shown to interact with the DSFL C-terminus in murine PDGFRs *in vitro* and in transfected cells [6]. To study further the interaction between NHERF and PDGFR $\beta$ , we performed immunoprecipitations of NHERF from PAE cells expressing PDGFR $\beta$  at physiological level. In unstimulated cells, the association was weak and not consistent, but it was dramatically enhanced by treatment with PDGF-BB (Figure 1A). It reached a maximum 20 min after ligand addition. At that time point, most of the PDGFR $\beta$  had been internalized and a significant part degraded, as shown from the amount of receptor isolated with WGA–Sepharose, which binds glycosylated proteins, such as mature PDGFR $\beta$ , with high affinity. Similar results were obtained in PAE cells transfected with PDGFR $\alpha$  (not shown). Next, we immunoprecipitated NHERF from AG01518 normal human foreskin fibroblasts, which naturally express PDGFR $\beta$ .



**Figure 1** Co-precipitation of NHERF with PDGFR $\beta$  in a ligand-dependent manner

(A) PAE cells were starved overnight, stimulated with PDGF-BB for the indicated periods of time and lysed. For immunoprecipitation with anti-NHERF antibodies, 80% of the lysate was used. The remaining proteins were incubated with WGA-Sepharose to enrich PDGFRs. Samples were analysed by Western blotting with the PY99 anti-phosphotyrosine antibody, anti-PDGFR $\beta$  antiserum or anti-NHERF antibodies. After 20 min, PDGF treatment increased PDGFR $\beta$  co-precipitation with NHERF at least 10-fold (three independent experiments). (B) AG01518 cells were starved for 16 h, stimulated with PDGF-BB (+) for 15 min and processed as above. (C) Same experiment as in (A) with PAE cells expressing wild-type or K634A (kinase dead) mutant PDGFR $\beta$ . Components specifically recognized by the different antibodies are indicated by arrowheads.



**Figure 2** Characterization of PDGFR $\beta$  mutants that do not bind to NHERF

Transiently transfected HEK-293T cells (A), or stably transfected PAE cells (B) expressing wild-type or mutant PDGFR $\beta$  constructs, were serum-deprived for 16 h, stimulated for 20 min with PDGF-BB and processed as mentioned in Figure 1. Specifically recognized components are indicated by arrowheads. (C) PAE/PDGFR $\beta$ -WT or -L1106A cells were starved overnight, stimulated for the indicated periods of time with PDGF-BB and lysed. Equal amounts of protein were subjected to Western-blot analysis using anti-phospho-ERK (Thr-202/Tyr-204), anti-ERK, anti-phospho-Akt (Ser-473) or anti-Akt1/2 antibodies. ERK phosphorylation was not affected by PDGFR $\beta$  mutation, whereas Akt phosphorylation was increased  $1.9 \pm 0.1$ -fold (average of three independent experiments).

The blot presented in Figure 1(B) shows that PDGF stimulation also increased PDGFR $\beta$  co-precipitation in AG01518 cells, in line with the results obtained in PAE cells.

We next studied whether the tyrosine kinase activity of the receptor was required for NHERF binding, using the kinase dead PDGFR $\beta$ -K634A (Lys<sup>134</sup>  $\rightarrow$  Ala) mutant expressed in PAE cells [15]. As expected, no tyrosine phosphorylation of that mutant could be detected (Figure 1C). NHERF co-precipitated with PDGFR $\beta$ -K634A to the same extent as with the wild-type

receptor, suggesting that neither the kinase activity of the receptor nor its tyrosine phosphorylation was required.

#### Disruption of NHERF binding to PDGFR $\beta$ affects phosphorylation of Akt but not of ERK

To study the importance of the interaction of NHERF with the C-terminal end of the receptor, we constructed three PDGFR $\beta$

mutants. First, the last amino acid residue, a leucine, which plays an essential role in the NHERF-binding motif, was changed to alanine (L1106A) [6,8,16]. We also deleted the last 75 amino acids (CT75), creating a DPKP C-terminal end which is unlikely to bind to NHERF [8]. This mutant retains all the phosphorylated tyrosines of the receptor. Finally, a haemagglutinin tag (GYPDVDPDYA) was added to the C-terminal end after the -EDSFL sequence. These mutant receptors were first transiently transfected in HEK-293T cells. As shown in Figure 2(A), all three modifications abolished binding of endogenous NHERF. Similarly, neither the L1106A mutant nor the CT75 mutant interacted with NHERF in stably transfected PAE cells (Figure 2B). Taken together, these results confirmed that NHERF interacts with the C-terminal end of the receptor.

Interestingly, we found that the association was constitutive in transfected HEK-293 cells (Figure 2A), consistent with observations by Maudsley et al. [6]. In their report, they suggested that in cells overexpressing both PDGFR $\beta$  and NHERF, NHERF binding to the receptor enhanced the receptor activity and ERK activation. However, we found that mutants that were unable to bind to endogenous NHERF were phosphorylated to a very similar level compared with wild-type receptor, both in HEK-293 and in PAE cells (Figure 2C). In line with this result, ERK phosphorylation was not affected by the L1106A mutation. In contrast, Akt phosphorylation was reproducibly increased approx. 2-fold (Figure 2C).

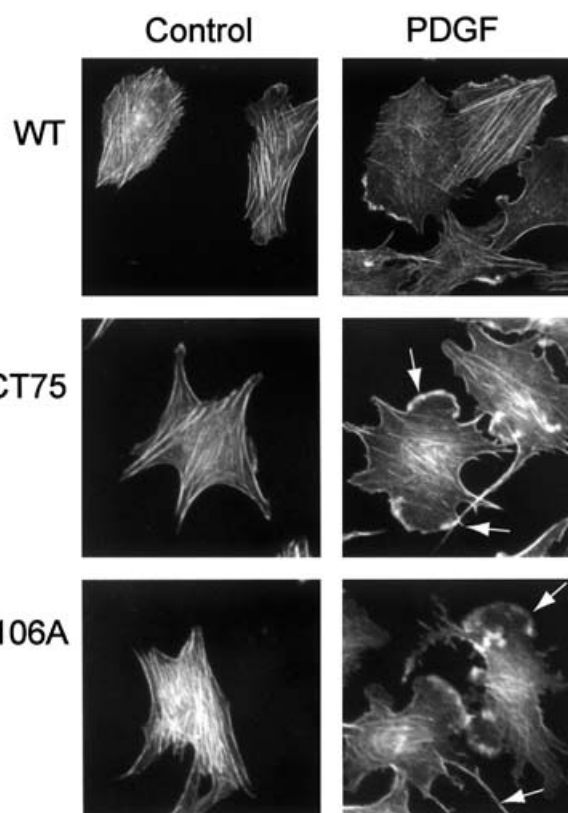
#### NHERF is involved in PDGF-induced cytoskeletal rearrangement, but not mitogenicity or chemotaxis

The activities of PDGF in PAE cells have been extensively characterized, and include stimulation of mitogenicity, chemotaxis and cytoskeletal reorganization [14,17]. As shown in Figure 3, reorganization of the actin cytoskeleton upon PDGF treatment was much more pronounced in PAE cells expressing PDGFR $\beta$ -L1106A or -CT75 compared with wild-type receptor. In PAE/PDGFR $\beta$ -WT, only a fraction of the cells presented ruffling edges and stress fibre reorganization, whereas most of PAE/PDGFR $\beta$ -L1106A and -CT75 did. In addition, cells expressing any of the mutant receptors showed much more dramatic changes, with more pronounced loss of stress fibres, intense edge ruffling and even some filopodia, which are rarely seen in PAE/PDGFR $\beta$ -WT treated with PDGF. Unstimulated cells with wild-type or mutant receptors showed a similar pattern of cytoskeletal organization, consistent with the idea that NHERF plays a specific role downstream of PDGFR.

In contrast, disruption of NHERF binding to the PDGFR had no effect on mitogenicity, measured as [<sup>3</sup>H]thymidine incorporation (Figure 4A). Chemotaxis driven by a PDGF gradient on collagen- or fibronectin-coated filters was not affected either (Figure 4B). These results were reproduced with two independent cell clones for each type of mutant receptor. In conclusion, our results suggest that NHERF specifically regulates actin cytoskeleton reorganization in response to PDGF.

#### NHERF is not involved in the regulation of intracellular pH by PDGF

NHERF is a protein required for intracellular pH regulation by the  $\beta$ 2-adrenergic receptor in epithelial cells of the kidney proximal tubule [8]. We tested whether NHERF could also play a role in the stimulation of Na<sup>+</sup>/H<sup>+</sup> exchange by PDGF. PAE/PDGFR $\beta$ -WT cells were loaded with BCECF, whose fluorescence is sensitive to intracellular pH, and cell fluorescence was quantified in real time [12]. Stimulation with PDGF-BB induced a quick increase of approx. 0.2 pH unit (Figure 5). Cells expressing L1106A or



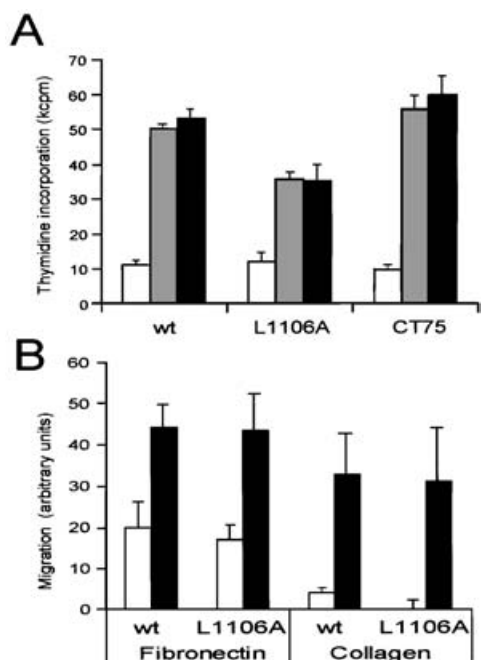
**Figure 3** Regulation of PDGF-induced actin cytoskeletal reorganization by NHERF recruitment

PAE cells expressing wild-type or mutant PDGFR $\beta$  were cultured on glass coverslips for 24 h, serum-deprived overnight, and then stimulated for 20 min with PDGF-BB or left untreated as a control. Cells were fixed in the presence of paraformaldehyde, incubated with TRITC-phalloidin for 30 min, washed and mounted on slides with Fluoromount-G. Pictures were taken at a  $\times$  600 magnification using an Axioplan2 fluorescence microscope (Carl Zeiss, Jena, Germany) coupled with a Hamamatsu digital camera controlled by the QED software. Arrows indicate enhanced ruffling activity and filopodia formation in PDGF-stimulated cells expressing CT75 or L1106A PDGFR $\beta$  mutants.

CT75 showed very similar responses, suggesting that association of NHERF to PDGFR $\beta$  is not important for the pH increase in response to PDGF-BB (Figure 5B).

#### DISCUSSION

In the present study, we showed that the interaction of the human PDGFR C-terminal end with NHERF is ligand-dependent in transfected PAE cells, which express normal amounts of these proteins, and in normal human fibroblasts. This result was surprising since it contrasted with previously reported observations showing that NHERF constitutively binds to PDGFR $\beta$  with a relatively high affinity [6]. A probable explanation for the discrepancy is that PDGFR $\beta$  competes with many other cellular proteins that bind to the same PDZ domain of NHERF [7,9,18,19]. Therefore recruitment of NHERF, which forms oligomers, may require receptor dimerization, increasing the avidity of the receptor for NHERF. When PDGFR $\beta$  is expressed at a high level, such as in transfected HEK-293T cells (this study), or in the models used by Maudsley et al. [6], a fraction of the receptors is constitutively activated and clustered, which probably induces the formation of a constitutive NHERF-receptor complex. In addition, increasing the concentration of PDGFR $\beta$  should favour



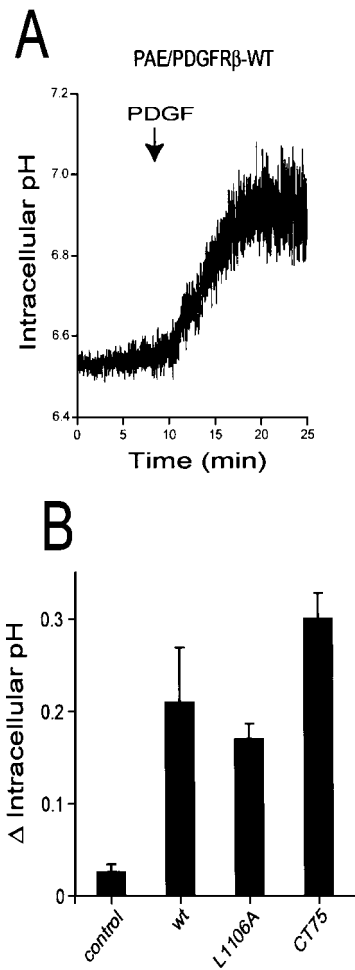
**Figure 4 Mitogenicity and chemotaxis in response to PDGF without NHERF recruitment**

(A) PAE cells were starved for 24 h and treated with PDGF-BB (grey bars, 5 ng/ml; black bars, 50 ng/ml), or with control medium (white bars) in the presence of [<sup>3</sup>H]thymidine for 24 h. Thymidine incorporation was measured in trichloroacetic acid precipitates. All cell types responded similarly to stimulation with 10% fetal calf serum (results not shown). One representative experiment out of three is shown with S.D. calculated from triplicate cultures. (B) PAE/PDGFR $\beta$ -WT or -L1106A were starved overnight, trypsinized, applied on a filter coated with fibronectin or collagen in a Neuroprobe chamber, and allowed to migrate towards PDGF-BB (10 ng/ml, filled bars) or control medium (open bars) for 4 h. Cells that had migrated to the other side of the filter were stained with Giemsa and quantified using a cooled CCD camera. One representative experiment with standard deviations calculated from quadruplicate experiments is shown.

the formation of the PDGFR $\beta$ -NHERF complex over the complex of NHERF with other proteins.

To the best of our knowledge, this is the first example of a ligand-induced association between a receptor and a PDZ domain-containing protein. NHERF binding to  $\beta$ 2-adrenergic receptor, cystic fibrosis transmembrane conductance regulator, P2Y1 purinergic receptor and parathyroid hormone 1 receptor is constitutive, as well as binding of IIP-1 to the insulin-like growth factor-1 receptor [8–10,20]. However, NHERF binding can be regulated under certain conditions. One example is the phosphorylation of the serine in the -DSLL motif at the C-terminus of a  $\beta$ 2-adrenergic receptor by G-protein-coupled-receptor kinase 5, which inhibits NHERF recruitment [21]. Future studies will address whether a similar regulatory mechanism occurs for the PDGFR $\beta$ .

Disruption of NHERF binding had no effect on tyrosine phosphorylation of the receptor in HEK-293T and PAE cells. This finding thus provides no support for the notion that NHERF potentiates the receptor kinase activity, in contrast with what was suggested previously [6]. In line with our observations, signal transductions by PDGFR $\beta$ -WT, -CT75 and -L1106A were comparable in terms of phosphorylation of ERK, as well as mitogenicity and chemotaxis in response to PDGF. However, we cannot exclude the possibility that receptor activation is enhanced by NHERF when it is expressed at a high level, e.g. ectopically or after treatment with oestrogens, for instance [6,22].



**Figure 5 Ineffectiveness of NHERF recruitment in intracellular pH increase**

(A) Serum-starved PAE/PDGFR $\beta$ -WT were loaded with BCECF for 30 min and analysed using a fluorescence microscope coupled with a photomultiplier and a detector as described in the Experimental section. After 10 min (baseline), PDGF-BB was added in the perfusion medium (20 ng/ml). At the end of each experiment, a calibration was performed using K<sup>+</sup>-rich buffered solutions containing nigericin. (B) PAE/PDGFR $\beta$ -WT, -L1106A, -CT75 or untransfected PAE cells (control) were stimulated with PDGF-BB as above. The average increase in intracellular pH on PDGF stimulation in three independent experiments is shown with S.E.M.

NHERF is required for the inhibition of NHE3 by protein kinase A downstream of  $\beta$ 2-adrenergic receptors [8]. This increased the possibility that NHERF could interfere with the activation of NHE and the increase in intracellular pH elicited by PDGF. In PAE cells, however, we failed to detect any difference between wild-type and mutant receptors. Growth factors might act mainly on NHE1 [23], which does not interact with NHERF or NHERF-2. NHE regulation by the  $\beta$ 2-adrenergic receptors may also require the recruitment of other proteins that are not associated with PDGFR.

Our results suggest that NHERF plays a negative role in actin cytoskeletal reorganization by PDGF, since PAE/PDGFR $\beta$ -L1106A and -CT75 showed a significantly increased response. The signalling cascade responsible for inducing these changes has been extensively studied, and involves PI3K, and small GTPases such as Rho, Rac and Cdc42 [5]. The observation that Akt, another effector of PI3K, is more phosphorylated in PAE/PDGFR $\beta$ -L1106A suggests that this pathway is hyperactive in these cells. NHERF could also interfere with Rho family

GTPase activation. The fact that NHERF interacts with merlin, ezrin, radixin and moesin is of interest in this context; indeed, several reports suggest that these proteins might inhibit small GTPases [7]. Recently, moesin was shown to function as an antagonist of the Rho pathway in *Drosophila melanogaster* [24]. In addition, merlin, a protein encoded by the nuclear factor-2 tumour suppressor gene, inhibits Rac signalling [25]. Interestingly, merlin-deficient fibroblasts showed an increased protrusive ruffling in the presence of PDGF, which is consistent with an involvement of this pathway in the effects of NHERF on PDGF-induced actin rearrangements [25]. However, we failed to detect any activation of Rho family proteins, probably because of its transient and local character in PAE cells treated with PDGF. Future studies will have to assess whether recruitment of ezrin or radixin or moesin to the PDGFR complex by NHERF can contribute to the termination of small GTPase signals.

In conclusion, our experiments suggest that NHERF can be recruited by the PDGFR $\beta$  in a ligand-dependent manner, and has a specific role in cytoskeleton reorganization on PDGF stimulation.

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## REFERENCES

- Bethsholtz, C., Karlsson, L. and Lindahl, P. (2001) Developmental roles of platelet-derived growth factors. *Bioessays* **23**, 494–507
- Heldin, C. H. and Westermark, B. (1999) Mechanism of action and *in vivo* role of platelet-derived growth factor. *Physiol. Rev.* **79**, 1283–1316
- Bergsten, E., Uutela, M., Li, X., Pietras, K., Östman, A., Heldin, C. H., Alitalo, K. and Eriksson, U. (2001) PDGF-D is a specific, protease-activated ligand for the PDGF $\beta$  receptor. *Nat. Cell Biol.* **3**, 512–516
- Li, X., Ponten, A., Aase, K., Karlsson, L., Abramsson, A., Uutela, M., Bäckström, G., Hellström, M., Bostrom, H., Li, H. et al. (2000) PDGF-C is a new protease-activated ligand for the PDGF  $\alpha$ -receptor. *Nat. Cell Biol.* **2**, 302–309
- Heldin, C. H., Östman, A. and Rönstrand, L. (1998) Signal transduction via platelet-derived growth factor receptors. *Biochim. Biophys. Acta* **1378**, F79–F113
- Maudsley, S., Zamah, A. M., Rahman, N., Blitzer, J. T., Luttrell, L. M., Lefkowitz, R. J. and Hall, R. A. (2000) Platelet-derived growth factor receptor association with Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor potentiates receptor activity. *Mol. Cell. Biol.* **20**, 8352–8363
- Bretscher, A., Chambers, D., Nguyen, R. and Reczek, D. (2000) ERM-merlin and EBP50 protein families in plasma membrane organization and function. *Annu. Rev. Cell Dev. Biol.* **16**, 113–143
- Hall, R. A., Premont, R. T., Chow, C. W., Blitzer, J. T., Pitcher, J. A., Claing, A., Stoffel, R. H., Barak, L. S., Shenolikar, S., Weinman, E. J. et al. (1998) The  $\beta$ 2-adrenergic receptor interacts with the Na<sup>+</sup>/H<sup>+</sup>-exchanger regulatory factor to control Na<sup>+</sup>/H<sup>+</sup> exchange. *Nature (London)* **392**, 626–630
- Shenolikar, S. and Weinman, E. J. (2001) NHERF: targeting and trafficking membrane proteins. *Am. J. Physiol. Renal. Physiol.* **280**, F389–F395
- Mahon, M. J., Donowitz, M., Yun, C. C. and Segre, G. V. (2002) Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor 2 directs parathyroid hormone 1 receptor signalling. *Nature (London)* **417**, 858–861
- Lennartsson, J., Blume-Jensen, P., Hermanson, M., Ponten, E., Carlberg, M. and Rönstrand, L. (1999) Phosphorylation of Shc by Src family kinases is necessary for stem cell factor receptor/c-kit mediated activation of the Ras/MAP kinase pathway and c-fos induction. *Oncogene* **18**, 5546–5553
- Grapengiesser, E., Gylfe, E. and Hellman, B. (1989) Regulation of pH in individual pancreatic  $\beta$ -cells as evaluated by fluorescence ratio microscopy. *Biochim. Biophys. Acta* **1014**, 219–224
- Tengholm, A., Hellman, B. and Gylfe, E. (2001) The endoplasmic reticulum is a glucose-modulated high-affinity sink for Ca<sup>2+</sup> in mouse pancreatic  $\beta$ -cells. *J. Physiol. (Cambridge, U.K.)* **530**, 533–540
- Ruusala, A., Sundberg, C., Arvidsson, A. K., Rupp-Thureson, E., Heldin, C. H. and Claesson-Welsh, L. (1998) Platelet-derived growth factor (PDGF)-induced actin rearrangement is deregulated in cells expressing a mutant Y778F PDGF  $\beta$ -receptor. *J. Cell Sci.* **111**, 111–120
- Westermark, B., Siegbahn, A., Heldin, C. H. and Claesson-Welsh, L. (1990) B-type receptor for platelet-derived growth factor mediates a chemotactic response by means of ligand-induced activation of the receptor protein-tyrosine kinase. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 128–132
- Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J. and Lefkowitz, R. J. (1998) A C-terminal motif found in the  $\beta$ 2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor family of PDZ proteins. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 8496–8501
- Hansen, K., Johnell, M., Siegbahn, A., Rorsman, C., Engstrom, U., Wernstedt, C., Heldin, C. H. and Rönstrand, L. (1996) Mutation of a Src phosphorylation site in the PDGF  $\beta$ -receptor leads to increased PDGF-stimulated chemotaxis but decreased mitogenesis. *Embo J.* **15**, 5299–5313
- Brdickova, N., Brdicka, T., Andera, L., Spicka, J., Angelisova, P., Milgram, S. L. and Horejsi, V. (2001) Interaction between two adapter proteins, PAG and EBP50: a possible link between membrane rafts and actin cytoskeleton. *FEBS Lett.* **507**, 133–136
- Tang, Y., Tang, J., Chen, Z., Trost, C., Flockenzi, V., Li, M., Ramesh, V. and Zhu, M. X. (2000) Association of mammalian trp4 and phospholipase C isozymes with a PDZ domain-containing protein, NHERF. *J. Biol. Chem.* **275**, 37559–37564
- Ligensa, T., Krauss, S., Demuth, D., Schumacher, R., Camonis, J., Jaques, G. and Weidner, K. M. (2001) A PDZ domain protein interacts with the C-terminal tail of the insulin-like growth factor-1 receptor but not with the insulin receptor. *J. Biol. Chem.* **276**, 33419–33427
- Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A. and von Zastrow, M. (1999) A kinase-regulated PDZ-domain interaction controls endocytic sorting of the  $\beta$ 2-adrenergic receptor. *Nature (London)* **401**, 286–290
- Ediger, T. R., Kraus, W. L., Weinman, E. J. and Katzenellenbogen, B. S. (1999) Estrogen receptor regulation of the Na<sup>+</sup>/H<sup>+</sup> exchange regulatory factor. *Endocrinology* **140**, 2976–2982
- Counillon, L. and Pouyssegur, J. (1995) Structure–function studies and molecular regulation of the growth factor activatable sodium–hydrogen exchanger (NHE-1). *Cardiovasc. Res.* **29**, 147–154
- Speck, O., Hughes, S. C., Noren, N. K., Kulikauskas, R. M. and Fehon, R. G. (2003) Moesin functions antagonistically to the Rho pathway to maintain epithelial integrity. *Nature (London)* **421**, 83–87
- Shaw, R. J., Paez, J. G., Curto, M., Yaktine, A., Pruitt, W. M., Saotome, I., O'Bryan, J. P., Gupta, V., Ratner, N., Der, C. J. et al. (2001) The Nf2 tumor suppressor, merlin, functions in Rac-dependent signaling. *Dev. Cell* **1**, 63–72

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