A NHERF binding site links the βPDGFR to the cytoskeleton and regulates cell spreading and migration

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Summary

The Na^+/H^+ exchanger regulatory factor, NHERF, is a multifunctional adapter protein involved in a wide range of physiological activities. NHERF associates with merlin and the ezrin/radixin/moesin (MERM) family of membrane-actin cytoskeletal linker proteins through its C-terminus and is capable of interacting via its PDZ1 domain to the βPDGF receptor (βPDGFR). Thus, NHERF, potentially links the βPDGFR to the actin cytoskeleton through its interaction with MERM proteins. In the present study, we have examined whether abolishing the interaction of βPDGFR with NHERF results in actin cytoskeletal rearrangements. We have stably expressed a wild-type βPDGFR, a mutant βPDGFR (L1106A) that is incapable of interacting with NHERF, as well as a kinase defective mutant receptor (K634R), in PDGFR-deficient mouse embryonic fibroblasts. Our observations indicate that cells expressing βPDGFR (L1106A) were impaired in their ability to spread and migrate on fibronectin compared with wild-type and K634R cells. L1106A mutant cells also revealed an increased number of focal adhesions, a condensed F-actin ring at the cell periphery and a decrease in total focal adhesion kinase (FAK) tyrosine phosphorylation. Further, we show that NHERF and MERM proteins could act as intermediary bridging proteins between βPDGFR and FAK. Thus, the interaction of βPDGFR with NHERF may provide an essential link between the cell membrane and the cortical actin cytoskeleton independent of receptor activity.

Key words: βPDGFR, NHERF, Cell spreading, Migration, Merlin, ERM

Introduction

The Na^+/H^+ exchanger regulatory factor (NHERF), also known as EBP50 (ERM-binding phosphoprotein 50) is a multifunctional adapter protein that possesses two related PDZ (PSD-95/Dlg/ZO-1) domains. Although initially isolated as an essential cofactor for cyclic AMP-mediated inhibition of the rabbit kidney Na^+/H^+ exchanger (NHE3) (Weinman et al., 1993; Weinman et al., 1995), NHERF, through its C-terminal tail, was shown to bind merlin and the ERM (MERM) family of membrane-cytoskeletal linker proteins (Murthy et al., 1998; Reczek et al., 1997; Reczek and Bretscher, 1998). Subsequently, NHERF was shown to interact via its first PDZ domain with specific C-terminal residues in a variety of target proteins. The PDZ1 domain of NHERF was initially shown to bind the β_2-adrenergic receptor (β_2-AR) (Hall et al., 1998b). Mutagenesis of the β_2-AR tail showed that the optimal C-terminal motif for binding the PDZ1 of NHERF and its related family member NHERF2, is D[S/T]xL, a motif distinct from those recognized by other PDZ domains (Hall et al., 1998a). Based on this consensus sequence it was predicted that other receptors, including the α and β platelet-derived growth factor receptors (PDGFRs) and many ion transporters, including the cystic fibrosis transmembrane conductance regulator (CFTR), can potentially bind NHERF (Hall et al., 1998a). Subsequently, NHERF was shown to be an important regulator and integrator of multiple signaling pathways by virtue of its ability to bind a diverse array of proteins such as membrane receptors, ion channels, non-receptor cytosolic kinases and ERM proteins (Bretscher et al., 2000; Minkoff et al., 1999; Voltz et al., 2001).

The C-terminal tail of βPDGFR, through its binding motif DSFL, was shown to associate with the PDZ1 of NHERF, and this association was suggested to potentiate receptor activity (Maudsley et al., 2000). PDGFR autophosphorylation, in response to ligand-induced receptor dimerization, results in its association with a variety of SH2-domain containing signal transduction molecules that mediate various effects on cell growth, chemotaxis, actin reorganization and anti-apoptosis (Heldin et al., 1998; Heldin and Westermark, 1999). PDGF-induced actin cytoskeletal reorganization and cell migration are mediated through phosphoinositide 3-kinase (PI3K) (Fruman et al., 1998; Hawkins et al., 1995; Vanhaesebroeck et al., 1997; Wennstrom et al., 1994), phospholipase C-γ (PLCγ) (Kamat and Carpenter, 1997; Kanazawa et al., 2002), protein kinase C (Derman et al., 1997) and the small G proteins of the Rho

family, Rac and Cdc42 (Hall, 1998; Heldin and Westermark, 1999). The formation of cortical actin-enriched lamellipodia, membrane ruffles and filopodia, downstream of RacGTPase activation, are cellular features characteristic of PDGF receptor stimulation (Hall, 1998; Nobes et al., 1995). In addition, activated Rac and Cdc42 induce formation of peripheral focal complexes critical for proper cell adhesion and spreading (Nobes and Hall, 1995; Ridley and Hall, 1992; Ridley et al., 1992).

ERM proteins, together with the related neurofibromatosis 2 (NF2) tumor suppressor protein merlin, provide a regulated link between the membrane and the cortical actin cytoskeleton, and also participate in Rho/Rac mediated signal-transduction pathways (Bretscher et al., 2002). Functional inactivation and expression studies of ERM proteins indicate that they play essential roles in many fundamental processes including determination of cell shape and surface structures, cell-matrix and cell-cell adhesion, cell spreading and motility (Mangeat et al., 1999). Although merlin shares a high degree of structural similarity with ERM proteins, it clearly possesses functions that do not overlap with the ERMs, given its ability to suppress tumor cell growth (Gusella et al., 1999; Sun et al., 2002). Furthermore, the binding sites for NHERF are thought to be masked in the dormant ERM monomers, due to the intramolecular association of their N- and C-terminal domains, whereas merlin monomers are thought to be less tightly regulated, and thus capable of constitutively binding NHERF (Gonzalez-Agosti et al., 1999; Nguyen et al., 2001; Reczek and Bretscher, 1998).

The ability of NHERF to bind βPDGFR via its PDZ1 domain and MERM proteins through its C-terminal tail raises the question whether βPDGFR is functionally linked to the actin cytoskeleton through this association. In the present study, we have investigated whether βPDGFR-NHERF binding plays a role in PDGF-dependent and/or independent actin cytoskeletal reorganization by comparing these processes in cells expressing either wild-type βPDGFR or a mutant version of βPDGFR, that is no longer capable of interacting with NHERF family proteins. A βPDGFR C-terminal point mutation that abolishes NHERF binding displays defects in integrin-mediated spreading and migration when compared to cells expressing the wild-type receptor. Our findings suggest that the βPDGFR, through its association with NHERF and MERM proteins, plays an essential role in actin-based cellular processes independent of receptor activation.

Materials and Methods

Reagents and antibodies

Recombinant human PDGF-BB, fibronectin and soybean trypsin inhibitor were purchased from Sigma (St Louis, MO). Rabbit polyclonal antisera raised against the C-terminus of human βPDGFR (30A) was described previously (Kazlauskas et al., 1991). Rabbit polyclonal antibodies for βPDGFR, sc-432 and focal adhesion kinase (FAK) were purchased from from Santa Cruz Biotechnology (Santa Cruz, CA). Merlin monoclonal antibody 1C4 and merlin rabbit polyclonal antibody MP8 recognize both merlin isoforms and were described previously (Gonzalez-Agosti et al., 1996). The moesin polyclonal antibody was a generous gift from F. Solomon (MIT, Cambridge, MA) and was described previously (Winckler et al., 1994). A rabbit polyclonal antibody, IC270, was raised against GST-NHERF fusion protein (aa 270-358) and was characterized elsewhere (Gonzalez-Agosti et al., 1999). Affinity-eluted IC270 was biotinylated to detect the ~50 kDa NHERF protein in immunoprecipitates using EZ-Link Sulfo-NHS-LC-Biotin (Pierce Chemical Co., Rockford, IL) according to manufacturer's instructions. Streptavidin-HRP conjugate (Amersham Pharmacia Biotechnology, Piscataway, NJ) at the dilution of 1:1000 was used to detect the biotinylated IC270. Monoclonal antibody to phosphotyrosine (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal antibodies for FAK, pY397 and paxillin were purchased from Transduction Laboratories/BD Biosciences.

Construction of βPDGFR mutants

Subcloning of human wild-type βPDGFR and kinase-inactive βPDGFR, K634R, in the pLXSN retroviral expression vector (Miller and Rosman, 1989) was described previously (Vaillancourt et al., 1996; Valius and Kazlauskas, 1993). To obtain the βPDGFR L1106A mutant, site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). PCR primers, mutPDGFR-forward (CAGAGGATAGCTTCGCGTAGGGGGCTGGC) and mutPDGFR-reverse (GCCAGCCCCCTACGCGAAGTCATCCTCTG) were used to introduce the change leucine1106 to alanine. The PCR product was transformed under ampicillin selection and the DNA prepared and sequenced for verification of mutant L1106A incorporation.

Cell lines

The F cells are simian virus 40 (SV40) large T antigen-immortalized mouse embryonic fibroblasts (MEFs) established from embryonic stage 9 (E9) αPDGFR- and βPDGFR-double-knockout embryos, kindly provided by M. Tallquist and P. Soriano (Andrews et al., 1999). F cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco/Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1× penicillin-streptomycin-L-glutamine (GIBCO/Invitrogen). Human wild-type βPDGFR (WT), βPDGFR with the mutations L1106A and K634R in pLXSN, and pLXSN alone, were stably expressed in F cells using the 293T system (DeMali et al., 1999). Briefly, subconfluent 293T cells were transfected with the pLXSN retroviral vector and amphotropic packaging plasmid using Lipofectamine 2000 (GIBCO/Invitrogen). After 48 hours, virus containing the mutant and WT βPDGFRs were obtained and used to infect F cells. Both, isolated clones and pooled populations of infected F cells, were selected in the presence of G418 and assayed for βPDGFR expression levels similar to comparable WT MEFs. Consistent results were obtained in initial experiments using either the individual clone-derived or pooled populations of clones from each cell line. For subsequent studies, two different pooled F cell populations from each cell line were selected. Glioma 238 cells and MGF1092 adult human primary fibroblast cells were maintained in DMEM and Eagle's minimal essential medium (EMEM) (BioWhittaker, Walkersville, MD), supplemented with 10% FBS, respectively,.

Preparation of recombinant NHERF and merlin proteins

Human full-length NHERF [amino acids (aa) 1-358] and truncated fusion proteins containing PDZ1 (aa 11-97), PDZ2 (aa 149-236), PDZ1 and PDZ2 (aa 11-236), PDZ2 and the C-terminus IC149 (aa 149-358) were amplified by PCR and subcloned into the *BamH*I-*Not*I sites of pGEX4T (Amersham Pharmacia Biotechnology). GST-NHERF and GST-merlin fusion proteins were expressed and purified in *Escherichia coli* using gluthathione-Sepharose 4B (Amersham Biosciences) (Gonzalez-Agosti et al., 1996).

Affinity precipitation assays

To examine NHERF protein interactons, confluent F cells or glioma

238 cells were washed twice in cold phosphate-buffered saline (PBS) and lysed in RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet p-40 (NP-40), 0.5% deoxycholate acid, 0.1% sodium dodecyl sulfate (SDS), 2 mM EDTA] containing phosphate inhibitors (2 mM sodium orthovanadate and 50 mM sodium fluoroide) and a 1× complete protease inhibitor cocktail (Roche Molecular Biochemicals, IN). Lysates were incubated with 600 pmol of GST-NHERF fusion proteins or GST alone (control), immobilized on glutathione (GSH) Sepharose 4B beads (Amersham Pharmacia Biotechnology) and rocked at 4°C for 16 hours. The beads were washed extensively with PBS containing 4 mM of the serine protease inhibitor Pefabloc (Roche) and resuspended in 1× sample buffer (33% glycerol, 6.7% SDS, 330 mM dithiothreitol). In some experiments, 200 pmol GST-merlin full-length isoforms and GST-merlin truncated fusion proteins, immobilized on GSH beads, were incubated with MGF1092 fibroblast lysates that were prepared using 1% NP-40 lysis buffer (20 mM HEPES pH 7.8, 50 mM KCl, 1 mM EDTA and 1% NP-40) with protease and phosphatase inhibitors as described above. Beads were washed 5× in lysis buffer with Pefabloc. Proteins bound to beads were resolved by SDS-PAGE, transferred electrophoretically to nitrocellulose membranes (Bio-Rad, Hercules, CA) and subjected to immunoblot analysis using the appropriate antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotechnology) detection systems.

Co-immunoprecipitation of NHERF and βPDGF receptor

Confluent F cells were washed twice in cold PBS and lysed in RIPA buffer containing protease and phosphatase inhibitors as described above. Lysates were clarified by centrifugation (13,000 ***g***) and incubated with normal rabbit serum and protein A-agarose beads (Roche Molecular Biochemicals) for 1 hour at 4°C. Precleared lysates were immunoprecipitated with NHERF (1C270) antiserum, rocked for 2 hours at 4°C, followed by incubation with protein A-agarose beads for 1.5 hours. The precipitated protein complexes were washed 5× in RIPA buffer with 4 mM Pefabloc. Beads were resuspended in 1× sample buffer before direct analysis by SDS-PAGE and immunoblotting as described above.

Cell spreading on fibronectin

F cells were examined for spreading properties as described previously (Inagaki et al., 2000; Yu et al., 1998). Briefly, F cells were serum-deprived for 16 hours in DMEM supplemented with 0.2% FBS. Cells were detached from culture dishes with 0.025% trypsin and 0.27 mM EDTA and washed with DMEM containing 0.25 mg/ml soybean trypsin inhibitor. Cells (4×10^5) were washed twice and resuspended in DMEM before replating overnight at 4°C on 35 mm tissue culture dishes that had been precoated with fibronectin (FN) (10 μg/ml). Cells were allowed to spread for 20, 40 and 90 minutes and visualized with an inverted Nikon phase contrast-2 microscope. Random fields were photographed at the indicated times using a charge-coupled device (CCD) camera (Hamamatsu Photonics, Hamamatsu City, Japan) and IP Lab imaging software (Scanalytics, Fairfax, VA). Spread cells were defined as cells that had attained a flattened morphology and were phase-dark, whereas cells that possessed a rounded morphology and were phase-bright were scored as nonspread. Data represent the mean percentages of spread cells±s.d. for a minimum of three experiments.

Similarly, F cells were plated onto FN-coated glass coverslips for specific times and fixed with 4% paraformaldehyde in PBS for immunocytochemical analysis of F-actin and focal adhesion complex formation.

In some spreading experiments, wild-type βPDGFR F cells were cotransfected with a hemagglutinin (HA) epitope-tagged NHERF C-terminal fragment (aa 270-358) in pcDNA3 (Invitrogen) and pEGFP (enhanced green fluorescent protein) (Clontech) or pEGFP alone using Lipofectamine 2000. After 24 hours, cells were serum-deprived in DMEM supplemented with 0.2% FBS for another 24 hours. Cells were trypsinized, treated with 0.25 mg/ml soybean trypsin inhibitor, washed in DMEM and allowed to recover at 37°C for 20 minutes before replating on FN-coated glass coverslips (10 μg/ml). Cells were allowed to spread for 90 minutes, and were then fixed and stained for F-actin.

Cell migration assay

Cell migration was assessed in wound healing assays essentially as described previously (Hauck et al., 2000). Confluent F cells, plated on tissue culture dishes precoated with or without FN (10 μg/ml), were wounded by manual scratching with a 200-μl pipette tip, washed with PBS and incubated at 37°C in complete media or reduced-serum supplemented media (10% or 0.2% FBS, respectively). At the indicated times, phase contrast images at specific wound sites were captured.

PDGF-induced actin cytoskeletal reorganization

F cells were plated subconfluently on glass coverslips at a density of approximately 40,000 cells per 35 mm dish in complete growth medium. The cells were washed in PBS before serum-deprivation for 16 hours with DMEM containing 0.2% FBS. Medium was removed and cells were either treated with 5 ng/ml PDGF-BB in DMEM for 10 minutes at 37°C or not treated. Cells were fixed in 4% paraformaldehyde in PBS for 30 minutes at 37°C followed by several PBS washes.

Immunofluorescent staining of F-actin and focal adhesion complexes

Immunocytochemistry was described previously (Gonzalez-Agosti et al., 1996). Essentially, fixed cells were permeabilized with 0.1% NP-40 in PBS for 15 minutes, blocked in 10% normal goat serum in PBS and incubated with a paxillin monoclonal antibody (1:50 in 0.1% BSA in PBS) for 1 hour at 37°C. After several PBS washes, focal adhesions complexes were detected by staining with goat anti-mouse IgG conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR) for 30 minutes at 37°C. Similarly, F-actin was visualized by staining with Alexa Fluor 594-conjugated phalloidin. For double staining, detection of F-actin organization was used simultaneously with secondary antibody application. Coverslips were mounted on slides using the ProLong Antifade Kit (Molecular Probes). Images were obtained using LSM 5 Pascal software (Zeiss Physiology Software) coupled to a Zeiss LSM Pascal Vario 2 RGB confocal system.

Fibronectin-mediated focal adhesion kinase (FAK) tyrosine phosphorylation

FN-induced phosphorylation of FAK was assessed as described previously (Hagel et al., 2002). Mutant L1106A and WT F cells (80 to 90% confluent) were serum-deprived in DMEM containing 0.2% FBS for 16 hours and detached using 0.025% trypsin, 0.27 mM EDTA in PBS. Soybean trypsin inhibitor in serum-free (SF) DMEM with 5 mM EDTA was added to cell suspensions, followed by two washes with SF-DMEM. Cells were resuspended and maintained in SF-DMEM at 37°C for 30-40 minutes before replating on precoated cell culture dishes; the cell culture dishes were precoated with 5 μg/ml FN overnight at 4°C and blocked with 1% BSA in PBS for 1 hour at 37°C. At the indicated times, total cell lysates were harvested using RIPA buffer (detailed above) and total protein was quantified using the Bio-Rad DC protein assay kit. Equal quantities of F cell lysates from suspended and adherent cell cultures were resolved by SDS-PAGE. Tyrosine phosphorylated FAK and total FAK protein were detected with appropriate antibodies by immunoblot analysis. The relative

FAK tyrosine phosphorylation values were obtained by scanning autoradiographs with a laser scanning densitometer and normalizing values to total FAK protein. The results are representative of three independent assays.

Results

A point mutation in the C-terminal tail of the βPDGFR abolishes NHERF association

The C-terminal tail of the PDGF receptor contains the DSFL motif for binding the first PDZ domain of NHERF and has been shown to bind directly to NHERF (Maudsley et al., 2000). We and others have shown previously that NHERF, through its extreme C-terminal region, associates with the MERM family of actin binding proteins (Murthy et al., 1998; Reczek et al., 1997) (Fig. 1A). To determine whether βPDGFR is functionally linked to the actin cytoskeleton via its association with NHERF, we developed a cell-based model system to compare the cytoskeletal architecture of cells expressing wild-type βPDGFR or a mutant form of the receptor that is no longer capable of interacting with NHERF.

Employing affinity precipitation assays, we demonstrate that the PDZ1 domain of NHERF (Fig. 1A**)** is capable of binding the βPDGF receptor. GST-NHERF fusion proteins encompassing PDZ1 but not PDZ2, precipitated endogenous βPDGFR in glioma cell lysates (Fig. 1B). Further analysis demonstrated that full-length NHERF, as well as a deletion construct containing PDZ1+2, precipitated βPDGFR, but that a C-terminal NHERF construct IC149 (aa 149-358) containing PDZ2 could not (Fig. 1B). These data are consistent with the findings (shown by blot overlay) that aa 1-151 of NHERF bound the βPDGFR C-terminus (Maudsley et al., 2000). Interestingly, merlin bound to C-terminal and full-length NHERF as expected, whereas another ERM family member, moesin, failed to bind under these experimental conditions (Fig. 1B).

To assess whether βPDGFR-NHERF binding participates in PDGF-induced actin cytoskeletal dynamics, we generated a point-mutation in βPDGFR: the extreme C-terminal leucine of the receptor, which is essential for PDZ1 binding, was replaced with an alanine (L1106A). Immortalized MEFs, established from E9 PDGFR double-knockout embryos ($\alpha PDGFR^{-/-}$, $\beta PDGFR^{-/-}$) and referred to as F cells (DeMali et al., 1999), were retrovirally (and stably) infected with either the wild-type βPDGFR or the L1106A mutant. Also created were F cells expressing vector only or a kinase-defective βPDGFR. The kinase-defective βPDGFR cannot autophosphorylate because of a single-point mutation (K634R) in the catalytic domain which renders the receptor kinase-inactive (Kelly et al., 1991; Vaillancourt et al., 1996). Pooled G418-resistant populations of clones expressing similar amounts of WT and mutant receptors were selected for further study. GST-NHERF affinity-precipitation assays were conducted on lysates derived from F cells expressing vector only, WT, or the mutants L1106A and K634R. As expected, interactions with NHERF

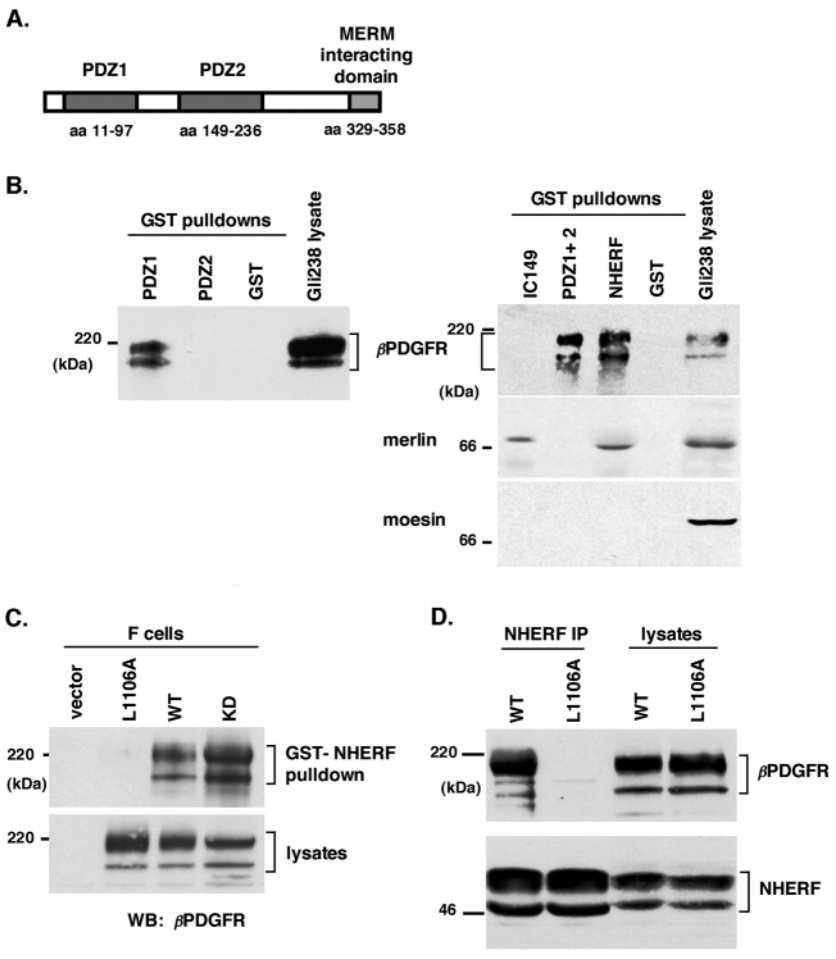

Fig. 1. NHERF interacts with βPDGFR in vitro and in vivo in a PDGF-independent manner. (A) Schematic representation of NHERF binding domains. NHERF contains two PSD-95/Dlg/Zo-1 homology (PDZ) domains, protein-protein interaction domains known to associate with specific C-terminal motifs on target proteins. Merlin and ERM (MERM) proteins interact with the 30 amino acids of the C-terminal end of NHERF. (B) Affinity precipitation assays. GST-NHERF fusion proteins encompassing domains PDZ1 (aa 11-97), PDZ1+2 (aa 11-236) and the full-length protein (aa 1-358), affinity precipitate βPDGFR in glioma 238 cells, whereas PDZ2 alone (aa 149-236) or the C-terminus containing PDZ2 (IC149; aa 149-358), does not. Merlin, but not moesin, is detected in GST-IC149 and full-length NHERF affinity precipitates. (C) The mutant L1106A βPDGFR does not bind NHERF. Pooled populations of G418-resistant F cells stably expressing the retroviral pLXSN vector (vector), mutant L1106A, wild type (WT) or the kinase-defective mutant K634R, were lysed and assayed for their ability to affinity precipitate with GST-NHERF. (D) Endogenous NHERF immunoprecipitates together with WT but not mutant L1106A βPDGFR in F cell cultures that were maintained in complete growth medium.

were only detected in F cells expressing either WT or mutant K634R, but not in cells expressing vector only or mutant L1106A (Fig. 1C). Furthermore, co-immunoprecipitations performed with an NHERF antibody revealed that βPDGFR immunoprecipitates together with endogenous NHERF in WT βPDGFR-expressing F cells, but not in cells expressing the mutant L1106A (Fig. 1D).

PDGF-dependent actin cytoskeletal changes do not require a βPDGFR-NHERF association

The formation of edge ruffling, loss of actin stress fibers and the formation of circular ruffles on the dorsal surface of cells are characteristic actin cytoskeletal features that are induced by PDGF and transduced by βPDGFR activation (Heldin and Westermark, 1999). We examined the formation of PDGF-induced actin cytoskeletal structures in F cells expressing WT or the mutant L1106A to evaluate whether βPDGFR-NHERF binding contributes to their production. As shown in Fig. 2, both F cells expressing WT or the mutant L1106A βPDGFR demonstrated a distribution of F-actin at the cell periphery, the formation of filopodia, circular dorsal ruffles and diminished actin stress fibers in response to PDGF stimulation. These results suggest that βPDGFR association with NHERF is not required for PDGF-dependent actin cytoskeleton changes.

Mutation of the NHERF binding site in the βPDGFR results in delayed cell spreading

Although no apparent distinction in PDGF-induced actin cytoskeletal rearrangement was discerned, we did notice variability in cell spreading in F cells expressing WT or mutant L1106A βPDGFR during routine cell culturing. To assess cell spreading more efficiently, serum-deprived F cells were plated onto FN-coated dishes and examined by phase-contrast microscopy at various time-points. As shown in Fig. 3A, F cells expressing the mutant L1106A were impaired in their ability to spread compared with WT cells. Twenty minutes after plating, F cells expressing the mutant L1106A appeared predominately phase-bright and more round than WT F cells expressing the βPDGFR, which had attained a much more flattened morphology. Although a greater proportion of F cells expressing the mutant L1106A had obtained a more flattened phenotype by 90 minutes (as evidenced by the appearance of phase-dark cells) they were still predominantly round with very few extensions compared with WT, K634R and vector only F cells. Further, the spreading defects resulting from the expression of mutant L1106A were observed in cells plated in serum-supplemented media, although they were less pronounced. The differences in spreading and morphology of F cells expressing the mutant L1106A βPDGFR persisted beyond 90 minutes (Fig. 3B) but were much less apparent by four hours. Interestingly, the K634R-expressing F cells appeared to spread similarly to WT F cells, indicating that defective spreading observed in mutant cells is independent of receptor activity.

Since defects in the ability of cells to adhere to fibronectin or other matrices can impair proper cell spreading, we examined whether the capacity of F cells expressing WT or the mutant L1106A βPDGFR to adhere to fibronectin influenced their spreading capability. F cells seeded in fibronectin-coated 96-well dishes were allowed to adhere for various times before washing away nonadherent cells. Attached cells were quantified by staining with Crystal Violet followed by colorimetric measurements. No significant differences were observed in F-cell-adhesion to fibronectin, indicating that reduced cell spreading did not result from defective cell adherence (data not shown).

F cells expressing L1106A display reduced cell migration

Effects of L1106A expression on cell motility were addressed in wound healing assays. Confluent F cell monolayers expressing vector, WT βPDGFR, or the mutants L1106A or K634R were wounded, and the rate of wound closure was examined by phase-contrast imaging at various time intervals. As shown in Fig. 4, mutant L1106A expression impaired F cell migration. These cells were markedly delayed in wound closure when compared to the F cells expressing WT, K634R or vector only. By 32 hours, the F cells expressing WT βPDGFR had advanced into the unoccupied area of the plate, again establishing a confluent cell layer, whereas F cells expressing mutant L1106A βPDGFR exhibited a delayed migration, moving at a much-reduced rate. Moreover, these observations were consistent in assays conducted with cells maintained in serum-supplemented media. These data establish that the L1106A mutant βPDGFR expressing cells display defects in cell motility.

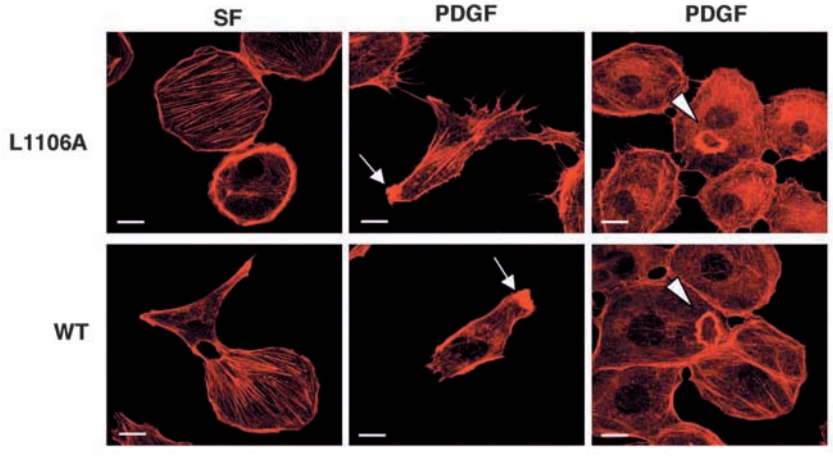

Fig. 2. NHERF binding to βPDGFR is not essential for PDGF-mediated actin cytoskeletal reorganization. PDGF-BB stimulation (5 ng/ml for 10 minutes) of F cells expressing WT or the mutant L1106A results in the aggregation of F-actin at the cell periphery (arrows), dorsal ruffles (arrowheads), filopodia formation and diminution of actin stress fibers. Scale bars, 10 μm.

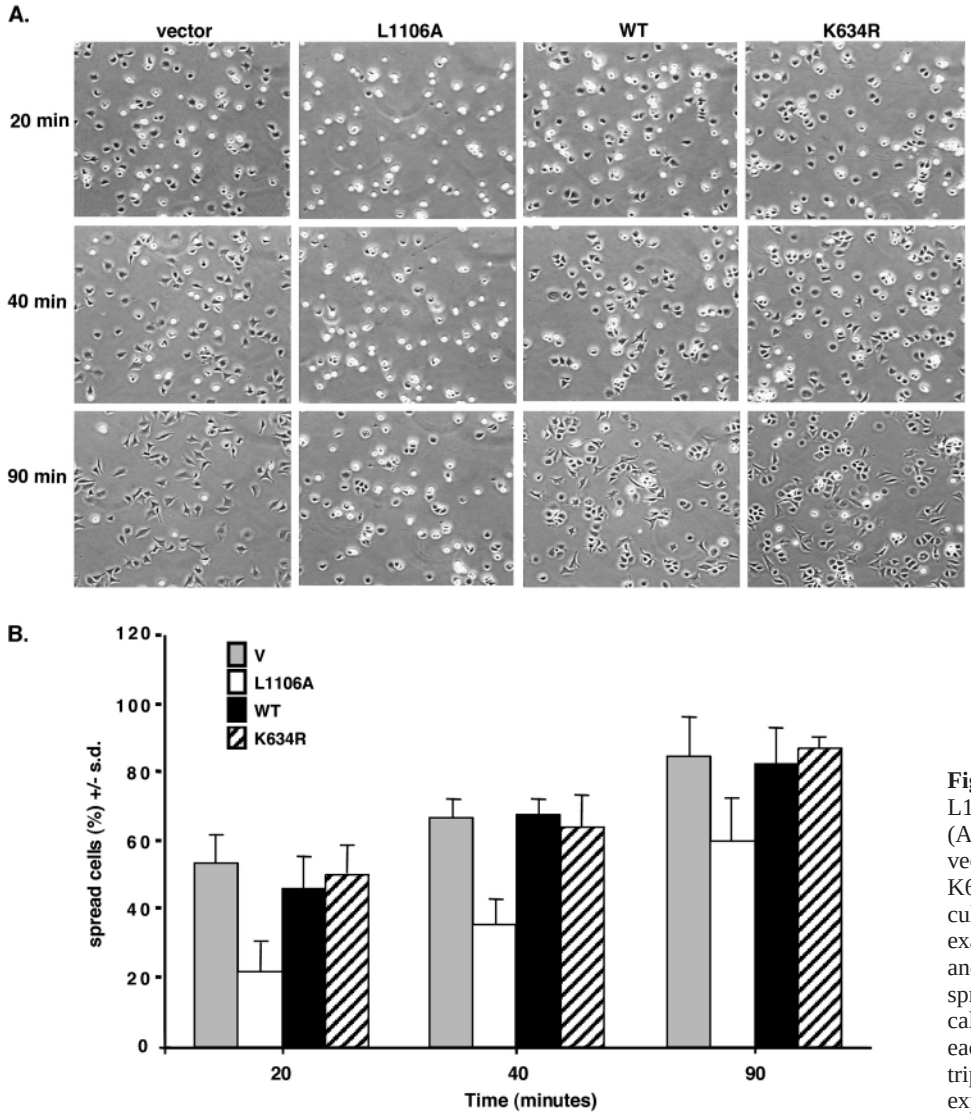

Fig. 3. F cells expressing the mutant L1106A exhibit delayed spreading on FN. (A) Serum-deprived F cells expressing vector only, WT, or the mutants L1106A or K634R were plated on FN-coated tissue culture plates in serum-free media and examined for their ability to spread 20, 40 and 90 minutes after plating. (B) F cell spreading-efficiency was quantified by calculating the percentage of spread cells at each time point. Shown is the mean±s.d. of triplicates from at least three independent experiments.

Altered F-actin rearrangement and focal adhesion formation in F cells that express the mutant L1106A

Next we examined the actin cytoskeletal architecture and focal adhesion/contact formation by immunocytochemistry. Serum-deprived F cells plated on fibronectin-coated coverslips were fixed at various time-points and stained with Alexa Fluor 594-conjugated phalloidin to examine F-actin. Focal adhesions were visualized by staining for paxillin. At 90 minutes post-plating, a preponderance of F cells expressing mutant L1106A βPDGFR maintained a round appearance and displayed a dense ring of cortical actin. This increase in F-actin staining at the cell periphery appeared to colocalize with an elevated number of short, dense focal adhesions (Fig. 5A). In addition, actin stress fibers appeared scattered throughout the cell, terminating at focal adhesions on the cell surface. By contrast, the cell cortex of F cells expressing the WT βPDGFR stained lightly for F-actin and was characterized by the appearance of fine focal adhesion complexes predominately at the termini of cell extensions.

Overexpression of MERM binding region of NHERF hinders cell spreading

To assess whether the association of NHERF and MERM proteins is a critical component to efficient cell spreading, the NHERF C-terminal MERM binding domain (aa 270-358) was transiently overexpressed to block NHERF-MERM binding in F cells expressing WT βPDGFR. Cells co-expressing the C-terminus of NHERF and GFP, compared with cells expressing GFP alone, demonstrate a more rounded, and less spread phenotype (Fig. 5B). This further supports the idea that NHERF-MERM binding might contribute to actin cytoskeletal regulation via a βPDGFR association.

Diminished FAK phosphorylation in L1106A mutant F cells

F cells expressing mutant L1106A have a reduced efficiency to spread and migrate compared with F cells expressing WT βPDGFR. We therefore investigated whether the activity of proteins crucial for the regulation of these processes, such as

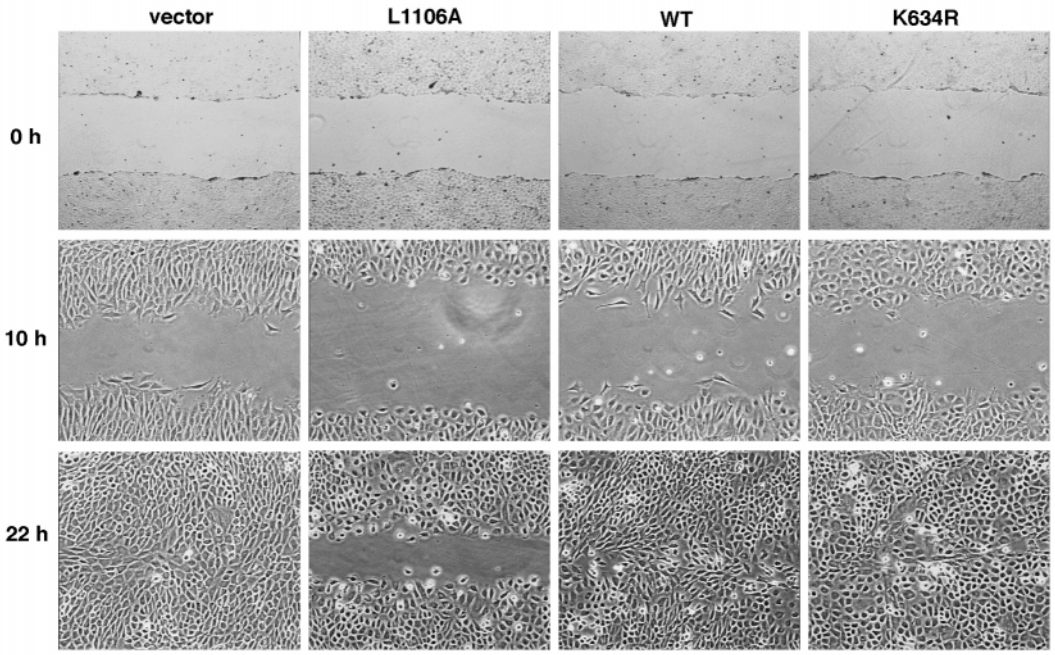

Fig. 4. F cells expressing the mutant L1106A migrate slower in wound healing assays. L1106A expression inhibits F cell migration compared with cells that express vector, WT or the mutant K634R F in wound healing assays. Upon wounding, F cell monolayers were incubated in DMEM supplemented with 0.2% FBS and analyzed for their ability to repopulate the wounded area. Phase-contrast images were taken at 10 and 22 hours after wounding. Assays were conducted at least three times and yielded similar results.

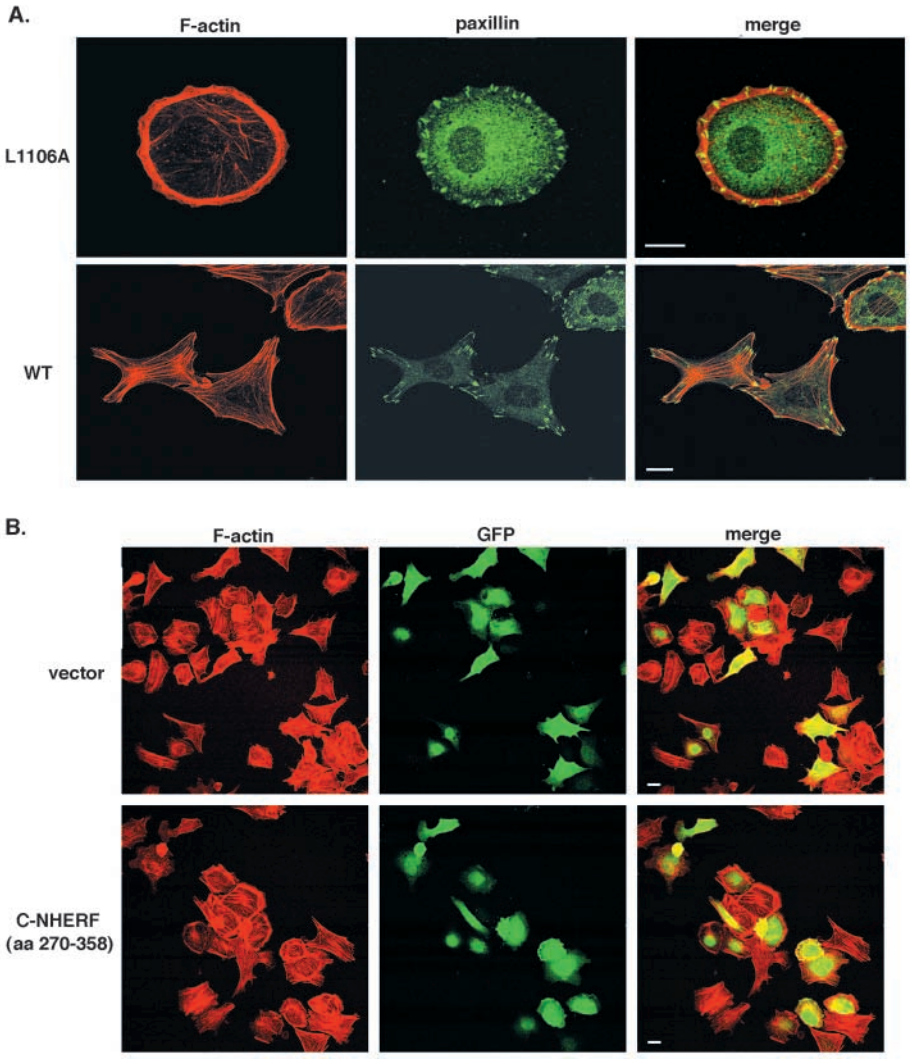

Fig 5. Altered F-actin rearrangement in F cells. (A) F cells expressing the mutant L1106A exhibit altered F-actin rearrangement and focal adhesion formation. F cells, expressing WT βPDGFR or the L1106A mutant in serum-free medium, were plated on FN-coated coverslips for 90 minutes and fixed. Cells were then stained with anti-paxillin (secondary antibody: Alexa Fluor 488-conjugated goat anti-mouse IgG) to examine focal adhesion formation and with Alexa Fluor 594-conjugated phalloidin to examine the actin cytoskeleton. F cells expressing the mutant L1106A exhibit a nonpolar spreading-morphology, a dense ring of cortical F-actin and an increased number and density of focal adhesions. Scale bars, 10 μm. (B) F cells expressing WT βPDGFR and the C-terminus of NHERF display delayed spreading on FN. Serum-deprived F cells expressingWT βPDGFR together with a HA-tagged C-terminal fragment of NHERF (aa 270-358), which encompasses the MERM binding domain and EGFP, or F cells expressing WT βPDGFR and EGFP alone (vector), were plated on FN-coated coverslips. After 90 minutes, the cells were fixed and stained for F-actin with Alexa Fluor 594-conjugated phalloidin. GFP expression identifies transfected cells. Scale bars, 20 μm.

focal adhesion kinase (FAK), paxillin or p130Cas, was altered. FAK expression and tyrosine phosphorylation were examined by immunoblot analysis of cell lysates derived from serum-starved F cells which were kept in suspension or plated onto fibronectin-coated dishes at specified times. F cells expressing mutant L1106A exhibited a decrease in total FAK tyrosine phosphorylation (detected by 4G10) compared with F cells expressing WT βPDGFR (Fig. 6A). However, FAK phosphorylation at Y397, the major autophosphorylation site, was not significantly diminished in mutant L1106A cells compared with F cells expressing WT βPDGFR (Fig. 6B). These data are consistent with studies demonstrating that reduced cell spreading and motility correlate with reduced FAK tyrosine phosphorylation. Protein expression and tyrosine-phosphorylation analyses of p130Cas and paxillin in response to cell adherence did not show obvious differences between mutant L1106A- and WT-expressing F cells (data not shown).

FAK associates with merlin and NHERF

Earlier work indicated that the ERM family member ezrin directly interacts with FAK (Poullet et al., 2001). We therefore examined the interaction of merlin with FAK and show that, similar to ezrin, the N-terminal domain but not the C-terminal domain of merlin can retain FAK from fibroblast lysates in GST pull-down assays (Fig. 7A). However, unlike full-length ezrin, where the FAK binding site is cryptic, full-length merlin isoforms are able to bind FAK (Fig. 7B). Furthermore, in pull-down assays performed with full-length NHERF, FAK was retained along with merlin, suggesting that FAK might indeed be in a complex with merlin and NHERF (Fig. 7C).

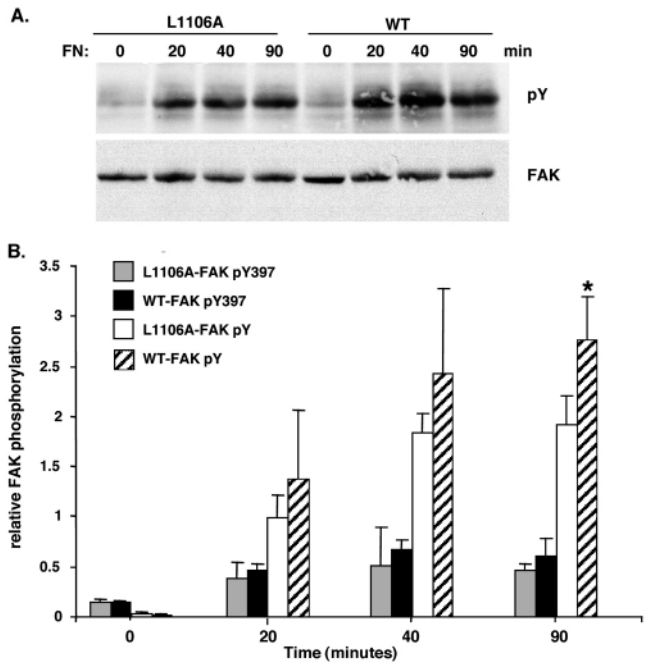

Fig. 6. FN-mediated FAK tyrosine phosphorylation. (A) Serum-deprived F cells expressing WT or mutant L1106A βPDGFR were plated on FN-coated cell culture plates for the indicated times. Cell lysates were prepared and analyzed for either FAK Y397 phosphorylation or total FAK tyrosine phosphorylation (4G10). The same blots were detected with a polyclonal FAK antibody to assess FAK levels. An immunoblot representating three independent experiments shows decreased total tyrosine phosphorylation of FAK in cells expressing the mutant L1106A. (B) Quantitative comparison of FAK Y397 and total tyrosine phosphorylation from F cells expressing WT or the mutant L1106A βPDGFR. Values were obtained from laser scanning densitometry and indicate the relative mean±s.d. from band intensities normalized to total FAK protein of three independent experiments. The asterisk indicates statistical significance (P<0.05).

Discussion

The first PDZ domain of NHERF and the related NHERF2 proteins, specifically bind to target proteins that have a D[S/T]xL motif at the C-terminal end. The motif recognized by NHERF is quite distinct from other PDZ proteins in that leucine is preferred at the terminal position and aspartate is preferred at the –3 position (Hall et al., 1998a). Initial studies revealed NHERF association with the C-terminal tail-domain of the β2-adrenergic receptor (β2-AR), the purinergic P2Y1 receptor, and the cystic fibrosis transmembrane conductance regulator (CFTR), which terminate in DSLL, DTSL and DTRL, respectively. Subsequently, a plethora of proteins including ion channels, ion transporters, co-transporters, the β1 subunit of the vacuolar proton pump, and the α and β PDGFRs, with this particular PDZ motif at their C-terminal tail were shown to associate with the PDZ1 domain of NHERF (Voltz et al., 2001). Furthermore, the crystal structure of human NHERF PDZ1 has been determined recently, which reveals the molecular mechanism of the C-terminal leucine recognition by this PDZ domain and provides insights into the specificity of the NHERF interaction with the C-termini of several membrane receptors and ion channels including the β2-AR, the PDGFR and the CFTR (Karthikeyan et al., 2001a; Karthikeyan et al., 2001b). Thus, at present, no other PDZ domain displays exactly the same preferences as the first PDZ domain of NHERF and NHERF2.

The N-terminal NHERF domain, spanning amino acids 1 to 151, directly associates with the C-terminal tail of the βPDGFR (Maudsley et al., 2000). This association was shown to potentiate PDGFR activity, apparently by stabilizing the formation of active PDGFR complexes. It was also demonstrated that, PDGF-stimulated receptor autophosphorylation and signaling to the ERK-MAPK cascade is enhanced by NHERF binding to the βPDGFR. However, studies aimed at defining the possible role of this interaction in actin cytoskeletal reorganization were not performed. In this study, we investigated whether βPDGFR is functionally linked to the actin cytoskeleton via its association with NHERF. Employing affinity pull-down assays, we first confirmed the association between NHERF and βPDGFR. We and others have previously shown that NHERF, through its C-terminus, binds to the MERM family of actin binding proteins (Murthy et al., 1998; Reczek et al., 1997; Reczek and Bretscher, 1998). Thus, NHERF functions as a key adapter protein, potentially linking many ion channels and receptors to the actin cytoskeleton. However, because of a tight association between the N- and the C-terminal domains of the ERM proteins, their association with NHERF is normally masked (Nguyen et al., 2001). Unlike the ERM proteins, the association of merlin with NHERF is not completely blocked in affinity pull-down assays performed with either full-length

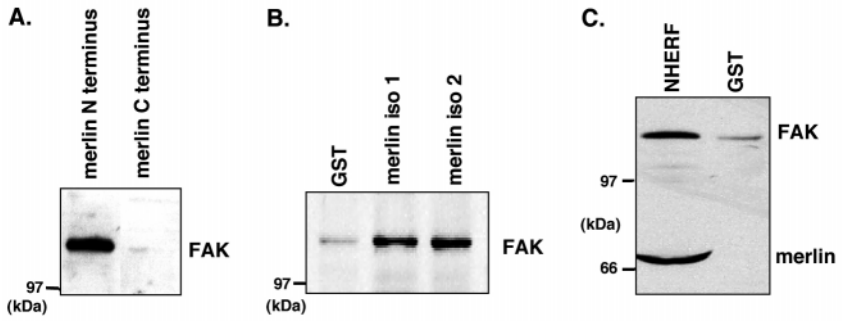

Fig 7. Affinity precipitation of FAK with merlin and NHERF. (A) GST merlin N-terminus (aa 1-345), but not merlin C-terminus (aa340-590), affinity precipitates FAK. (B) Merlin full-length isoform 1 (aa 1-595) and isoform 2 (aa 1-590) GST fusion proteins, which differ by 16 C-terminal amino acids, associate with FAK in MGF1092 cells. (C) Full-length NHERF-GST fusion protein associates with merlin and FAK in glioma 238 cell lysates.

or the C-terminal segment of NHERF, which is consistent with the presence of merlin but not moesin (Fig. 1B). Thus, the complex of βPDGFR, NHERF and MERM in vivo could be regulated by the concentration, as well as the activation states of merlin and ERM proteins.

It is well established that PDGF activates Rac, leading to actin polymerization at the cell periphery and resulting in lamellipodial extensions and membrane ruffling (Mackay and Hall, 1998). To determine whether βPDGFR association with NHERF plays a role in actin cytoskeletal reorganization, we mutated the leucine in the C-terminal tail of βPDGFR to an alanine (L1106A) and show that this point mutation in the PDZ1 binding motif of the βPDGFR abolishes the interaction with NHERF. This mutant, when expressed in F cells that lack both the α and the βPDGFR, has no effect on PDGF-mediated edge ruffles or dorsal ruffles. However, when expressed in F cells, it displays deficiencies in integrin-mediated cell spreading and migration that are not seen in F cells lacking PDGF receptors or expressing either the WT or the kinase-defective K634R mutant. It is therefore possible that, βPDGFR binding to NHERF regulates spreading and migration through a mechanism that is independent of the receptor activity. Notice, that the plasma membrane ion exchanger NHE1 acts as an anchor for actin filaments through a structural link between NHE1 and ERM proteins that is independent of its function as an ion exchanger (Denker et al., 2000). Furthermore, recent data presented for CFTR, another NHERF binding protein, shows that CFTR modulates RANTES gene expression through the C-terminal PDZ1-interacting domain and is independent of chloride channel activity of CFTR. NHERF is shown to be involved in CFTR-dependent RANTES expression (Estell et al., 2003). These findings support our notion that the C-terminal tail-domain of βPDGFR, through its binding to NHERF, might regulate cellular activities that are independent of receptor activity.

Our results show that total FAK phosphorylation is decreased in F cells expressing the L1106A mutant receptor when compared with the wild type receptor. However, whether this alone contributes to the changes in cell spreading that we have observed in L1106A mutant cells is unclear. We also demonstrate here that FAK potentially exists in a complex with merlin and NHERF. The FERM domain in merlin or the ERM proteins mediates the interaction with FAK, and thus it is possible that NHERF associates with FAK through MERM proteins. Whether FAK, similar to MERM proteins, binds directly to NHERF through its own N-terminal FERM domain remains to be determined. FAK plays an essential role in cell spreading and cell migration (Parsons, 2003) and functions as an important receptor-proximal link, integrating growth-factor and integrin signaling to promote cell migration (Sieg et al., 2000) (Fig. 8). Furthermore, the FAK N-terminal domain was shown to be associated with activated complexes of the epidermal growth factor receptor (EGFR) and the PDGFR, which both can be disrupted by pretreatment of cells with cytochalasin D, which prevents actin polymerization but not tyrosine phosphorylation of PDGFR or EGFR (Sieg et al., 2000). Notice that, even in the absence of PDGF, a weak association between FAK and PDGFR was seen, whereas in the absence of EGF, no association was seen between EGFR and FAK (Sieg et al., 2000). Because no direct interaction between FAK N-terminal domain and the PDGFR or EGFR was established, it was suggested that the association of FAK with these receptors is mediated by one or more intermediary bridging proteins (Fig. 8) (adapted from Sieg et al., 2000). Our results imply that NHERF and MERM proteins could be the bridging proteins that mediate the association of FAK with the βPDGFR (Fig. 8). Based on current knowledge, the L1106A mutation in βPDGFR should not influence its interaction with any other partners besides NHERF and its related family member NHERF2. Our results therefore suggest that, the

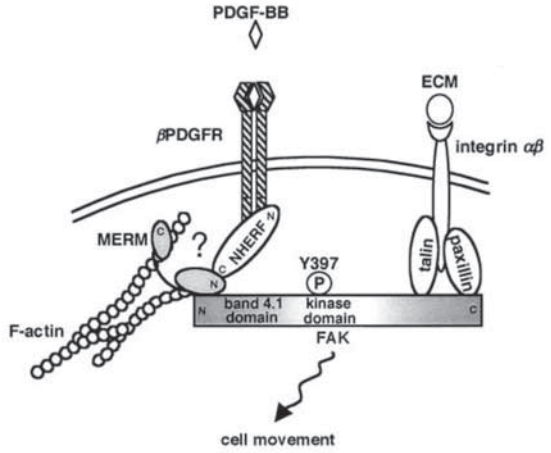

Fig 8. Model for βPDGFR-NHERF linking growth factor receptor and integrin signaling (adapted from Sieg et al., 2000). FAK is shown to form a complex with βPDGFR-EGFR and suggested to be an important link between growth factor receptor and integrin signaling pathways (Sieg et al., 2000). As a direct interaction between FAK and βPDFR–EGFR could not be demonstrated, it was suggested that one or more intermediary bridging proteins mediate this association. Our results suggest that NHERF and MERM proteins could be the intermediary bridging proteins.

L1106A mutation in βPDGFR disrupts a complex connection of βPDGFR, NHERF, MERM and FAK to the actin cytoskeleton. Furthermore, overexpression of the NHERF C-terminal domain results in defects of spreading, possibly through blocking the association of NHERF with MERM proteins. However, it cannot be ruled out that the L1106A mutation has a distinct 'gain of function' that might play a role in cell spreading and migration, either by interacting with other protein(s) or by altering the expression of protein(s). Further studies are essential to define the factor(s) that might mediate spreading defects observed in the mutant cells.

In summary, the results presented here demonstrate a novel function for the PDGFR as an effective structural component in a macromolecular complex to the actin cytoskeleton, regulating actin-based cellular processes independent of its ligand-induced receptor-activity. Specifically, our data indicate that the interaction of βPDGFR with NHERF provides a link between the cell membrane and the cortical actin cytoskeleton via MERM binding, which might promote proper cell spreading and motility in F cells.

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