PAK4 mediates morphological changes through the regulation of GEF-H1

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Summary
Precise spatial and temporal regulation of Rho GTPases is required in controlling F-actin-based changes in cell morphology. The molecular mechanisms through which microtubules (MTs) modulate the activity of RhoGTPases and regulate the actin cytoskeleton are unclear. Here we show that p21-activated-kinase 4 (PAK4) mediates morphological changes through its association with the Rho-family guanine nucleotide exchange factor (GEF), GEF-H1. We show that this association is dependent upon a novel GEF-H1 interaction domain (GID) within PAK4. Further, we show that PAK4-mediated phosphorylation of Ser810 acts as a switch to block GEF-H1-dependent stress fiber formation while promoting the formation of lamellipodia in NIH-3T3 cells. We found that the endogenous PAK4-GEF-H1 complex associates with MTs and that PAK4 phosphorylation of MT-bound GEF-H1 releases it into the cytoplasm of NIH-3T3 cells, which coincides with the dissolution of stress fibers. Our observations propose a novel role for PAK4 in GEF-H1-dependent crosstalk between MTs and the actin cytoskeleton.

Introduction
The typical cascade of F-actin-based morphological changes depends upon the temporal activation of Cdc42, Rac and Rho leading to the formation of filopodia, lamellipodia and stress fibers, respectively (Ridley and Hall, 1992; Nobes and Hall, 1995). Interplay between the actin cytoskeleton and microtubule (MT) network is important in a variety of cellular processes. In the case of cell motility, the MT network plays a critical role in establishing cell polarity (reviewed in Small et al., 2002). Rho family GTPases also play a role in regulating the MT networks. For example, in addition to its role in filopodia formation, Cdc42 has been linked to the reorientation of the microtubule organizing centre (MTOC) (Palazzo et al., 2001). Rho family GTPase-controlled morphological processes include cell migration, adhesion, morphogenesis, neurite outgrowth, axon guidance, phagocytosis, cell cycle progression and membrane transport (Chimini and Chavrier, 2000; Kaibuchi et al., 1999; Luo, 2000; Van Aelst and D'Souza-Schorey, 1997; Van Aelst and Symons, 2002).

Activation of Rho GTPases relies upon guanine nucleotide exchange factors (GEFs) that stimulate exchange of GDP to GTP, thereby allowing their interaction with effector proteins, including p21-activated protein kinases (PAKs) and GTPase-activating proteins (GAPs) (Bishop and Hall, 2000; Olofsson, 1999).

PAKs are effectors for Rho family GTPases and can mediate some of the morphological changes driven by Cdc42, Rac but not Rho (Abo et al., 1998; Dan et al., 2001; Manser et al., 1997; Obermeier et al., 1998; Sells et al., 1997; Wells et al., 2002; Zhang et al., 2002; Zhao et al., 1998). In the case of PAK4, several downstream effectors have been identified, for example, LIMK and BAD (Dan et al., 2001; Gnesutta et al., 2001). PAK4 is an effector for Cdc42 and has been implicated in tumorigenesis and shown to be required for Ras transformation (Callow et al., 2002; Qu et al., 2001). Some recent data has implicated the PAKs in the control of the MT network (Daub et al., 2001). Activated PAK1 has been found to be associated with MTOCs and to play a role in MT dynamics in neural precursors undergoing mitosis, while overexpression of PAK1 causes mitotic spindle defects in MCF-7 cells (Vadlamudi et al., 2000). One common feature of the PAKs, GEFs and Rho family members is their ability to induce morphological changes, but the mechanism is not fully understood. To address this we explore the possibility that PAK4, through its association and phosphorylation of GEF-H1, is involved in the regulation of these morphological changes as well as the RhoGTPases. Proteins tethered to the MT cytoskeleton are believed to mediate the effect of MTs on Rho GTG activation (Liu et al., 1998; Waterman-Storer et al., 1999). These include MLK2, Lfc, GEF-H1, Vav2 and p190RhoGEF (Fernandez et al., 1999; Glaven et al., 1999; Krendel et al., 2002; Nagata et al., 1998; Ren et al., 1998; van
Horck et al., 2001). For this reason we extended our studies to an MT-bound spliceform of GEF-H1 and observed the effects of phosphorylation on localization and MT integrity.

In this article, we report that PAK4 forms a stable complex with the GTPase regulator GEF-H1 in vitro and in vivo. Our results show that PAK4 phosphorylation of GEF-H1 mediates their ability to relay communication between the actin cytoskeleton and MT network.

Materials and Methods

Interaction screen reagents and cloning of GEF-H1

Plasmid pGEX-4T (Amersham Biosciences Cat. no. 27-4581-01), expressing a polypeptide of the catalytic domain of PAK4 (aa 291-591), was used as a bait in a phage display screen for protein interactors (Zozulya et al., 1999). The phage display library was derived from the breast tumour line MCF-7. Sequences from the phage plasmids were used to query the database at the National Centre for Biotechnology (NCBI) using Basic Local Alignment Search Tool (BLAST). Two of the cDNAs matched >99% identity with the cDNA denoted KIAA0651 (GenBank acc. no. AB014551) and several overlapping expressed sequence tags (ESTs) (note that sequence differences were due to errors in the original deposited sequence).

During the cloning of GEF-H1 we found that the gene had three major splice isoforms, which we refer to as GEF-H1M, GEF-H1S and GEF-H1U. The splice form GEF-H1S (aa 1-921) differs from GEF-H1M splice isoforms, which we refer to as GEF-H1M, GEF-H1S and GEF-H1U. The splice-forms as it contains a mini-exon of 7 amino acids and lacks the exon that encodes for the N-terminal zinc finger domain. The splice-form we refer to as GEF-H1M contains the zinc finger region involved in MT binding and is identical to the full length version (aa 1-985) referred to as GEF-H1 (Krendel et al., 2002). In-frame sequences were subcloned into pGEX-4T for substrate analysis.

DNA plasmids and constructs

To obtain the splice form we call GEF-H1S (GenBank acc. no. AB014551), the oligonucleotides MC1 GCGAGATTCTGTAACAA and MC3b GCAGAATTCTGTAACAA were used to generate a 5′ fragment from pcDNA clones were used for shuttling all GEF-H1 alleles to in frame EcoRI-SalI-digested pEGFP-C (Clontech), respectively. A HindIII-XbaI fragment from pcDNA was used to retrieve the HA-tagged GEF-H1 alleles and subcloned into pSV (Promega). Mutant S810A was introduced by PCR reaction with oligonucleotides MC27 TGTAGATCTCCTCGGCGCCGCTTCTCCCCGCA and MC3b GCAGAATTCTGTAACAA. The mutant insert was introduced to a deletion mutant of GEF-H1S spanning nucleotides 1100-2761 and a wild-type mutant insert was introduced to a deletion mutant of GEF-H1S spanning nucleotides 1100-2761. A 3′ fragment was replaced by a mutant XhoI-XhoI fragment. Oligonucleotides MC32 GCCGCTGCCCTTGGCTTAA and MC57 GCGGCGCACGCG and pBluescript GEF-H1S spanning 1-626 as a template (GenBank acc. no. AB014551). GEF-H1S was created using oligonucleotides MC57 GCCGCTGCCCTTGGCTTAA and MC58 TAAGAGCAAGGCGTGGAGGCGGACCGGAGGTCAGAGA, digested with EcoRI and SacI and joined to the remainder of the gene with a SacI-XhoI fragment from mutant and wild-type alleles. Since GEF-H1M and S proteins differ only in their N-termini, it was generated as a hybrid by joining mutant and wild-type sequences derived from GEF-H1S. The unique 5′ sequence was generated with the oligonucleotides MC61 GCGGAAATTCTGACGGAGCAGATGTCACATCTTCA and MC62 GCAGAATTCTGTAACAA and MC3b GCAGAATTCTGTAACAA. GEF-H1S was constructed in a PCR reaction using IMAGE clone 4157757 (Research Genetics) as a template (GenBank acc. no. BCO20567). pGEX-RBD was amplified from IMAGE clone 3958836 containing the Rho-binding domain (RBD) of Rhotekin with the oligonucleotides MC70 GCGGAATTCCATGCAGGACAGATGTCACATCTTCA and MC71 GCCGCTCCAGGACATCGGCGGCGGAGGTCAGAGA, digested with EcoRI and XhoI and subcloned in-frame in pGEX-4T. pGEX-PBD contains the PKC Cdc42/Rac-binding domain (p21-binding domain) and was a kind gift from Shubha Bagrodia (Pfizer Global Research and Development, San Diego, CA).

Protein production, sequence of peptides and kinase assays

Glutathione-S-transferase (GST)-fused PAK4 (residues 291-591) and GEF-H1 (residues 763-921) were purified by standard procedures and as previously described (Callow et al., 2002). Amino acid sequences spanning GEF-H1b 807-824 include: #1008, RRRSLPGDALpLSFNPP; #1009, RRRpSLPGDALpLSFNPP; #1010, RRRSLPGDALpYSFNPP; and #934 RRRSLPGDALpYSFNPP. In vitro kinase reactions were run as described previously (Callow et al., 2002).

Rules for PAK4 substrate consensus

Peptides derived from the activation loop sequence of PAK4 are high affinity substrates of PAK4 (Callow et al., 2002). By examining the ability of PAK4 to phosphorylate a series of peptides highly related to its activation loop (data not shown), we were able to deduce a rough substrate consensus for PAK4 (RXXSXLG). Both the basic residues and the hydrophobic residues flanking the phospho-acceptor site are important for high affinity substrate recognition by PAK4.

Antibodies and immunoreagents

KLH-linked peptide #1009 (CRRRSRLPGDALYLSFNPP, residues 807-824) synthesized with phosphoserine at position 810 and GST-GEF-H1 peptide #104 (residues 762-921) were used as antigens to raise anti-serum in rabbits. PAK4 antibodies were derived from the antigens #933 (CAGGAGGCCGKAGGSRGFAGHSHEA, residues 122-144) and #80 (CGGHRGGERPKPGSS, residues 148-163). Specificity was analyzed by using western blots of exogenous proteins and dot blots of the following peptides: GEF-H1b peptide residues 807-824 #1008 (RRRSRLPGDALpYSFNPP), #1009 (RRRSLPGDALpYSFNPP), #1010 (RRRSRLPGDALpYSFNPP) and #934 (RRRSRLPGDALpYSFNPP) (supplementary material Fig. S1). Goat anti-mouse and anti-rabbit IgG horseradish peroxidase conjugates were from Roche Molecular. Goat anti-mouse and rabbit antibody linked to fluorophores fluorescein isothiocyanate (FITC) or rhodamine were from Santa Cruz Biotechnology and mouse anti-β-tubulin was from Zymed. Coumarin-phallidin and phallidin-FITC were from Molecular Probes. Mouse anti-Cascade Blue and the fluorescent dyes Texas Red, FITC or Marina Blue were obtained and conjugated to antibodies according to the manufacturer. The solutions were quenched of cross-linking reagent by desalting through G25 Sephadex and mixing with ammonium chloride to a final concentration of 50 mM.
Cell transfection and immunofluorescence
NIH-3T3, 293T, A549, HCT116 and H1299 cells were maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin and streptomycin from Invitrogen (Carlsbad, CA). For transfection, cells were seeded at 10^5 cells ml⁻¹ for NIH-3T3 and 10^4 cells ml⁻¹ for both 293T and H1299 unless otherwise stated. Transfection reagents Lipofectamine 2000 (Invitrogen) or Fugene (Roche Molecular) were used at 3 µl µg⁻¹ of plasmid DNA. Cellular extracts for immunoblotting were prepared in lysis buffer (Callow et al., 2002). For immunoprecipitation experiments lysates were incubated with protein G beads (Pharmacia) linked with the appropriate antibody, glutathione-Sepharose beads (Pharmacia) with the appropriate GST fusion protein, and streptavidin beads (Pierce) linked to the appropriate biotinylated peptides. For immunofluorescence, coverslips were placed in 24-well cell culture vessels and seeded at the densities given above. Transfections took place after 16 hours and, unless otherwise stated, were maintained in Optinum or DMEM with 0.2% FBS for 24 hours until fixation. For endogenous detection in H1299 cells, 1 mM dithiobis succinimidylpropionate (DSP) was used in the in vivo cross-linking experiments followed by quenching in microtubule-stabilizing buffer (MTSB) (Nakamura, 2001). Dodecyltrimethylammonium chloride (DOTMAC) was used instead of Triton X-100 for solubilization. Detailed fixation and solubilization protocols for both exogenous and endogenous proteins are available upon request. Microscopy was performed on a Nikon E800 microscope with 10×60× objective (1.5 NA). Images were captured with a CCD SPOT camera and pseudocolored with SPOT imaging software (Diagnostic instruments).

Quantitation of active GTPases
293T cells were transfected separately with pcDNA-mycRho, pcDNA-HARac or pcDNA-mycCdc42 as reporters and co-transfected with pRSV-HA-GEF-H1 alleles. Extracts were prepared in lysis buffer (Callow et al., 2002) with the addition of 10 mM MgCl₂ and 0.25% deoxycholate. Active Rho, Cdc42 or Rac was calculated by division of the vector (non-GEF) reported or co-transfected with pcDNA-HA-GEF-H1 alleles. Extracts were prepared in lysis buffer (Callow et al., 2002) with the addition of 10 mM MgCl₂ and 0.25% deoxycholate. Active Rho, Cdc42 or Rac was collected from 100 µg 293T extracts, on 20 µl GST-RBD-bound beads or GST-PBD-bound beads (Bagrodia et al., 1998). Purification of the fusion proteins in E. coli is by standard procedures and as previously described (Callow et al., 2002). Glutathione-Sepharose beads were bound with 1 mg/ml purified fusion protein RBD or PBD. Autoradiograms from the western blots of two independent experiments were quantitated by densitometry using ImageJ software (NIH images scan). Signal was normalized for total Rho expression for each loading lane and fold induction was generated value.

Results
PAK4 interaction studies
We employed a phage display screen to identify physiological targets for PAK4 using the kinase domain of PAK4 (aa 291-591) fused to glutathione-S-transferase (GST) as ‘bait’. The phage display hits isolated in the screen corresponded to in-frame fusions derived from several distinct genes. One fragment was derived from the Maguin-2-like gene. Two other hits were derived from the GEF-H1S splice variant of GEF-H1 (we refer to the major splice isoforms as GEF-H1S and GEF-H1M, see Materials and Methods for details). In contrast to GEF-H1M, GEF-H1S is missing the zinc finger region, which is required for MT binding (Krendel et al., 2002).

To confirm the in vitro interaction of GEF-H1S with PAK4, we generated bacterially expressed GST-fusion proteins with the identical sequences isolated by phage display encoding either aa 763-921 of GEF-H1S or aa 385-555 from the Maguin-2-like protein (Fig. 1A). These fusion proteins were bound to glutathione beads and used in binding assays with 293T lysates expressing exogenous PAK4 (Fig. 1A). GST-GEF-H1S (aa 763-921), but not GST or the Maguin-2-like fragment, was able to bind strongly to both the full-length Myc-tagged PAK4 protein and the kinase domain (aa 291-591) (Fig. 1A,C). By contrast, the N-terminal regulatory domain of PAK4 alone (aa 1-309) did not stably interact with GEF-H1S. These results suggest that the interaction is mediated by either a short region N-terminal to the PAK4 kinase domain or by the kinase domain itself.

We used biotinylated peptides derived from the N-terminal

Fig. 1. Lysates were prepared from 293T cells overexpressing (A) Myc-tagged PAK4 alleles or sub domains and incubated with GST, GST-GEF-H1 (787-921) and GST-Maguin-like bound to glutathione beads (1-4 bead designation) or (B) GFP-GEF-H1 and incubated with PAK4 biotinylated peptides (391) and (276-324) linked to streptavidin beads. An equivalent amount of beads and excess lysates (500 µg) were used. Eluates of beads and total lysates were analyzed by SDS-PAGE and immunoblotted with Myc or GEF-H1 antiserum. Panel C represents a schematic outlining the regions of PAK4 used to identify the GEF-H1 interaction domain (GID). (D) Alignment of the GID defined by the minimal binding region of PAK4 with PAK5 and PAK6.
region of PAK4 sequence to map the GEF-H1 binding site (Fig. 1B). A peptide consisting of aa 276-324 showed strong interaction with GEF-H1, while another peptide covering a more C-terminal region, aa 291-355, did not. Together these results demonstrate that the GEF-H1 interaction domain (GID) is not contained within the kinase domain that begins with the highly conserved ‘GxG’ motif at aa 323. Instead, the GID is localized between aa 276 and 324 of PAK4 and probably involves a smaller region beginning around aa 291 of PAK4 (Fig. 1B,C) since, in the context of the kinase domain, aa 291-591 is necessary and sufficient to bind to GEF-H1. Sequence alignment of the PAK4 GID with the corresponding domains of the other PAKs revealed significant homology between group II PAK family members (PAK4, PAK5 and PAK6) in this region (Fig. 1D). By contrast, the GID is not conserved within group-I PAK family members (PAK1, PAK2 and PAK3) (data not shown). Based on the homology within the GID of PAK4 it is possible that PAK5 and PAK6 also interact with GEF-H1 or related proteins.

Endogenous PAK4 and GEF-H1 associate in vivo
To confirm that the in vitro interaction between PAK4 and GEF-H1 is physiologically relevant, we performed co-immunoprecipitation experiments with lysate from H1299 cells with antibodies directed against either PAK4, GEF-H1 or preimmune serum as indicated. The proteins were then analysed by western blotting using GEF-H1-specific antibodies. Endogenous GEF-H1 protein was detected in complexes immunoprecipitated with either PAK4 or GEF-H1 antibodies but not the preimmune serum (Fig. 2A, top panel). The same precipitates were subjected to a kinase reaction, and a band overlapping the immunostaining bands was phosphorylated (Fig. 2A, lower panel).

Earlier studies of overexpressed PAK4 demonstrated its distribution to the Golgi membrane in the presence of activated Cdc42, which results in the induction of filopodia (Abo et al., 1998; Dan et al., 2001). We used immunofluorescence in H1299 cells for this study and, consistent with earlier work, endogenous PAK4 co-localizes with the specific Golgi-marker β-COP but not with the trans-Golgi marker, wheat germ agglutinin. (Fig. 2B and data not shown). To date, no previous studies of PAK4 or GEF-H1 have shown MT localization of the endogenously expressed proteins. We reasoned that overexpressed GEF-H1, which contains the MT-binding zinc finger domain, is chronically attached to MTs owing to a limiting dissociation factor. Using directly labeled specific antibodies for GEF-H1 and PAK4 we found GEF-H1 clearly localizes to the cytoplasm with overlapping staining with PAK4 at Golgi-like structures (Fig. 2C, I,II). We further tested for the presence of phosphorylated GEF-H1 by staining with a phospho-specific S810 antibody (supplementary material Fig. S1A,B). Phosphorylated forms of GEF-
PAK4 phosphorylates GEF-H1S on Ser810 in vitro and in vivo

The interaction of a GEF-H1 phage peptide with a PAK4 kinase domain protein (this study) indicates a direct interaction and infers that GEF-H1 might be a direct substrate of PAK4. We therefore used the recombinant GST polypeptides of the phage-derived cDNAs for GEF-H1, Maguin-2-like and a GST control protein to assay for PAK4 phosphorylation. We found that the GEF-H1 polypeptide (aa 763-921) was a high affinity substrate for PAK4 with a $K_m$ of 1-5 $\mu$M (data not shown). PAK4 fails to phosphorylate the GST control and weakly phosphorylates the GST-Maguin-2-like fusion protein (Fig. 3A).

By examining PAK4 phosphorylation of a series of peptides highly related to its activation loop, we deduced a rough substrate consensus for PAK4 (RRXSLXG) that suggested both basic residues and hydrophobic residues flanking the phospho-acceptor site are important for high affinity substrate recognition by PAK4 (Callow et al., 2002). To further explore the substrate selectivity of PAK4, we compared the sequences of the phage display clones enriched in the PAK4 kinase domain screen with our putative PAK4 substrate consensus (supplementary material Fig. S2A, except for the GEF-H1 N-terminal site that was determined as described below). Most of the sequences, including GEF-H1S, contain a region with high similarity to a high affinity PAK4 substrate (aa 763-921) was a high affinity substrate for PAK4 with a $K_m$ of 1-5 $\mu$M (data not shown). PAK4 fails to phosphorylate the GST control and weakly phosphorylates the GST-Maguin-2-like fusion protein (Fig. 3A).

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also a high affinity substrate for PAK4 with a $K_m$ for PAK4 similar to that of the GST-GEF-H1 (793-921) fusion protein (data not shown). Various phospho-isomers derived from this peptide were synthesized and tested for their ability to be phosphorylated by PAK4 (supplementary material Fig. S2B). Peptides with phosphoserine at position 810 were no longer substrates for PAK4. Furthermore, we confirmed by mutagenesis that Ser810 of GEF-H1S is the bona fide phosphorylation site for PAK4 in vitro (supplementary material Fig. S2C).

Whereas mutation of Ser810 abolished PAK4 phosphorylation of C-terminal fragments of GEF-H1S, an N-terminal fragment (aa 1-386) of GEF-H1S contained additional PAK4 phosphorylation site(s) (supplementary material Fig. S2C). Sequence comparison with the PAK4 substrate consensus pointed to serine residues 57, 66 and 67 as other possible PAK4 phosphorylation sites. By synthesizing peptides corresponding to phospho-isomers of the putative PAK4 phosphorylation sites, we determined that Ser67 is the likely in vitro PAK4 phosphorylation site within the N-terminus of GEF-H1 (supplementary material Fig. S2B). Interestingly, the PAK4 phosphorylation sites within GEF-H1S are potentially conserved with the sites being either a threonine or a glutamic acid in Cdc24, the yeast Cdc42 GEF that is regulated by the PAK-like kinase Cla4, suggesting the potential importance of these sites. The alignment data indicate that the N-terminal glutamic acid 89 and C-terminal threonine 737 correspond to Ser67 and Ser810, respectively, in human GEF-H1S (supplementary material Fig. S3).

To confirm that GEF-H1S phosphorylation by PAK4 occurs at these sites, we performed co-transfection assays to analyze phosphorylation of GEF-H1 by use of a phospho-specific antibody directed against phospho-Ser810 (Fig. 3B). This antibody is specific for GEF-H1 that is phosphorylated on Ser810 (supplementary material Fig. S1). We found that Ser810 is phosphorylated in mammalian cells and that phosphorylation of GEF-H1S on Ser810 is enhanced in the presence of activated PAK4 as well as wild-type PAK4, whereas it is not enhanced in the presence of the kinase-dead mutant (Fig. 3B, lanes 2-4). Expression of the kinase-dead PAK4 is also able to inhibit endogenous PAK4 phosphorylation sites of GEF-H1S on Ser810 (Fig. 3B, compare lane 4 with lane 1). The effect of PAK4 is not dependent on the presence of Cdc42 since overexpression of Cdc42 alone has no effect on GEF-H1 phosphorylation above basal levels (Fig. 3C, lanes 1,2). By contrast overexpression of wild-type PAK4, PAK4 aa 291-591 (kinase domain only construct) and a kinase-active CRIB mutant PAK4 H19,22L induces phosphorylation of GEF-H1 (Fig. 3C, lanes 3-5). In the case of wild-type PAK4, it is possible that endogenous factors combine with this complex to sustain autocrine activity, which belies this phosphorylation of GEF-H1. It is therefore possible that GEF-H1 acts both upstream and downstream of PAK4. The CRIB mutant PAK4 H19,22L appears to be somewhat inhibitory to these endogenous factors compared with the other PAK4 alleles (Fig. 3C, compare lane 5 with lanes 3,4). Similarly, we developed phospho-specific antibodies against Ser67 in GEF-H1S and found that Ser67 of GEF-H1 is phosphorylated in mammalian cells and co-transfection of activated PAK4 with GEF-H1S stimulated phosphorylation of Ser67 (data not shown).

Morphological changes mediated by GEF-H1 alleles
Nucleotide exchange factors of the Rho GTPase family are involved in the regulation of cytoskeletal structures and cell morphology. The conserved Dbl homology (DH) and pleckstrin homology (PH) domains are critical for function of these exchange factors. GEFs have been analysed for their ability to stimulate exchange on Rho GTPase substrates in vitro or by their overexpression in vivo (reviewed by Schmidt and Hall, 2002). In the case of GEF-H1, various groups have shown catalytic activity on Rac and Rho. Transient expression of GEF-H1 in Cos-7 cells and isolated expression of the GEF-H1 DH-PH domains confers specificity towards Rac in vivo and in vitro, respectively (Gao et al., 2001; Ren et al., 1998). Analysis in vitro and in vivo assays in Cos-1 and HeLa cells showed activity of GEF-H1 for Rho (Krendel et al., 2002), which is in agreement with a previous study (Gao et al., 2001).

To investigate whether modification of GEF-H1 plays a role in cell morphology and regulation of Rho proteins we examined the effect of GEF-H1 on cell morphology in fibroblasts using enhanced green fluorescent protein (EGFP) fusion constructs of GEF-H1S and a series of mutant alleles of the PAK4 phosphorylation sites mimicking modified forms of GEF-H1S in NIH3T3 cells. We also constructed a dominant negative allele by mutating the DH domain of GEF-H1 resulting in GEF-H-QR312,313MG. In a p21-binding domain (PBD) assay (Bagrodia et al., 1998) this dominant-negative allele inhibited accumulation of Rac-GTP but not Cdc42-GTP in NIH-3T3 cells (data not shown). The actin cytoskeleton was stained with coumarin-phallicidin to contrast F-actin structures of EGFP fluorescing cells by microscopy (Fig. 4). Wild-type GEF-H1S typically localized to the perinuclear region and cells appeared elongated with occasional stress fibers (Fig. 4B,H). By contrast, overexpression of GEF-H1S-S810A alone led to an accumulation and disorganized array of stress fibers compared with non-transfected cells (Fig. 4C,I). The induction of stress fibers is striking given the relatively modest effects on the actin cytoskeleton upon expression of either wild-type GEF-H1S or PAK4 alleles alone (Fig. 4A,G; supplementary material Fig. S4). The morphology of GEF-H1S-S67A-expressing cells is similar to the morphology of cells expressing the dominant negative GEF-H1S DH mutant (Fig. 4, compare E,K and D,J). Expression of the DH domain mutant GEF-H1S-QR312,313MG, creates a trail of wildly distributed cytoplasmic extensions. The induction of stress fibers in the case of GEF-H1S-S810A is reminiscent of Rho activation, while the disorganized cytoplasmic extensions seen with GEF-H1-Q312R,M313G and GEF-H1S-S67A could be remnants of non-retracted cell tails due to Rho inhibition (Worthylake et al., 2001). The changes in localization and the actin cytoskeleton are especially dramatic with the double mutant of GEF-H1S-(S67A-S810A) (Fig. 4F,L). Wide-arcing lamella devoid of F-actin, as well as cytoplasmic aggregates of F-actin overlapping EGFP-GEF-H1S (S67A-S810A) protein are seen. We excluded the possibility that this was an artifact of accumulated miss-folded proteins since this mutant retained its ability to stimulate Rho (see Discussion). The redistribution of the phosphorylation-site mutant proteins and inappropriate accumulation of cytoplasmic F-actin supports the idea that localization is critical for the ability of GEF-H1 to stimulate stress fibers.
PAK4 phosphorylates GEF-H1 on key regulatory sites

Similarly to previous studies of GEF-H1 activity (Krendel et al., 2002), we were able to show that GEF-H1 mutant proteins retained guanine-nucleotide exchange activity in vivo using an affinity precipitation assay for active Rho and that loss of catalytic activity was achieved only through mutation of the conserved QR of the DH domain, which leads to a decrease in cellular Rho-GTP (active) (Fig. 4M). Although accumulation of RhoA bound to RBD and the level of RhoA expression in whole cells lysates were analyzed by western blotting and detected with anti-Myc antibody, GEF-H1 was detected with anti-HA antibody. The amount of RhoA bound to RBD and the level of RhoA expression in whole cells lysates were analyzed by western blotting and detected with anti-Myc antibody. GEF-H1 was detected with anti-HA antibody. The amount of RhoA bound to RBD and the level of RhoA expression in whole cells lysates were analyzed by western blotting and detected with anti-Myc antibody. GEF-H1 was detected with anti-HA antibody.

Activated PAK4 and GEF-H1S induce lamellipodia formation in NIH-3T3 cells

Induction of F-actin structures in fibroblasts, namely the formation of filopodia, lamellipodia, and stress fibers, is due to signaling by the active forms of Cdc42, Rac and Rho, respectively. Because PAK proteins and specifically PAK4 play a role in regulating F-actin-based morphological structures, we sought to investigate whether PAK4-induced changes in cell morphology were mediated by regulation of GEF-H1S. We co-expressed EGFP-GEF-H1 constructs with various hemagglutinin (HA)-epitope-tagged PAK4 alleles in NIH-3T3 cells. Exogenous PAK4 was detected with an anti-HA antibody (red) and the actin cytoskeleton with coumarin-phallicidin (blue). Although NIH-3T3 cells have low but detectable levels of endogenous PAK4 and GEF-H1 these were not activated under the conditions of the experiment (data not shown). By employing various alleles of PAK4, we were able to confirm which morphological effects were driven through PAK4 phosphorylation of GEF-H1. Co-expressing the PAK4 alleles with the EGFP control produced relatively modest effects on the formation of F-actin-based structures in NIH-3T3 cells (supplementary material Fig. S4). In cells co-expressing wild-type PAK4 together with wild-type GEF-H1S (Fig. 5A), we observed a dramatic increase in filopodia, in contrast to cells expressing either PAK4 or GEF-H1S alone (supplementary material Fig. S4I; Fig. 4B). The filopodial structures seemed to be dependent on the lower activity of the wild-type PAK4 allele and the interaction with GEF-H1S since they were not observed when either protein was expressed in cells alone or in vector co-transfected controls (Fig. 4A; supplementary material Fig. S4I).
activated PAK4-S474E and GEF-H1S, these structures transitioned from filopodia to lamellipodia at the leading edge (Fig. 5B). Neither filopodia nor lamellipodia were observed when GEF-H1S was transfected together with the kinase-inactive PAK4 K350,351A; however, stress fibers were abundantly present (Fig. 5C). Taken together with our analysis of the PAK4 phosphorylation site mutants in GEF-H1S (Fig. 4), this suggests that PAK4 kinase activity is responsible for the transition to lamellipodia formation (Fig. 5A-C).

To confirm whether the formation of these structures is dependent on the ability of PAK4 to phosphorylate GEF-H1S, we used phosphorylation site mutants of GEF-H1S. Co-transfection of a mutant GEF-H1S-S810A allele together with PAK4-S474E shows a profusion of stress fibers (Fig. 5D, asterisks mark cells with stress fiber accumulation). This, together with the result from the kinase-inactive allele (Fig. 5C), led us to hypothesize that phosphorylation of Ser810 is responsible for both the signals that lead to lamellipodia formation and inhibition of stress fiber formation in fibroblasts. Additionally, PAK4-S474E and GEF-H1S-S810A localized to distinct peripheral sites reminiscent of cell adhesion attachment sites that serve as nucleation points for actin stress fibers (Fig. 5D). This type of localization of GEF-H1 has also been seen in HeLa cells (Krendel et al., 2002). Contrasting the effects seen by mutation of S810 and co-expression of mutant GEF-H1S-S67A with those of activated PAK4-S474E, allows GEF-H1 dependent formation of lamellipodia while preventing stress fiber formation (Fig. 5E). Our experiments do not address the qualitative difference between the lamellipodia derived from the non-mutant GEF-H1S and mutant GEF-H1S-S67A (Fig. 5B,E).

Exchange activity of GEF-H1 is achieved through the interaction of the DH domain with Rac or Rho GTPases in their inactive state (Gao et al., 2001; Ren et al., 1998). To determine whether GEF-H1S-mediated effects driven by PAK4 phosphorylation are dependent upon GEF-H1S exchange activity we examined NIH-3T3 cells co-expressing the GEF-H1S dominant negative DH mutant (Q312M,R313G) with various alleles of PAK4 (Fig. 5F and data not shown). We observed wide-arcing lamella lacking actin, in contrast to the actin-rich lamellipodia or stress fibers formed in the presence of active alleles of GEF-H1S and activated PAK4 (Fig. 5B-E). The result confirms that PAK4 signals are relayed directly through the exchange activities of GEF-H1. A previous study of GEF-H1 showed constitutive lamellipodia induction in Cos-7 cells by overexpression of GEF-H1 (Ren et al., 1998). We have shown that, by the addition of an activated PAK4/GEF-H1S complex, we are able to reconstitute this effect in NIH-3T3 cells.

Role of PAK4 and GEF-H1M in NIH-3T3 lamellipodia formation

Residues phosphorylated by PAK4 in GEF-H1S (Ser67 and Ser810) are conserved in the MT-bound form of GEF-H1, which we designate GEF-H1M (Ser143 and Ser885) (supplementary material Fig. S2). GEF-H1M contains a zinc finger domain in its N-terminus that allows it to bind to MTs (Krendel et al., 2002). We investigated what role MT binding plays in the ability of PAK4 to control GEF-H1M activity. This is especially relevant given the fact that the activities of Rac and Rho are also known to coincide with MT growth and disassembly, respectively (Enomoto, 1996; Liu et al., 1998; Waterman-Storer et al., 1999). To address this question, we performed studies with GEF-H1M that allowed simultaneous examination of both MT (through EGFP-GEF-H1M labeling of MTs in vivo) and actin cytoskeleton (through F-actin staining with coumarin-phallicidin). We also studied changes in the MT cytoskeleton by co-staining with β-tubulin antibodies.

In the case of F-actin-based morphological changes, NIH-3T3 cells were transfected with PAK4 and GEF-H1S alleles and examined by fluorescence imaging of EGFP for GEF-H1 alleles, PAK4 constructs were identified by immunostaining using PAK4 antiserum followed by anti-rabbit fluorescent rhodamine conjugate for PAK4 and fluorescent coumarin-phallicidin to label actin. (A-C) GEF-H1S co-transfected with PAK4 alleles WTS,474E and K350,351A. (D-F) GEF-H1S-S810A, GEF-H1S-S67A and GEF-H1S-Q312M,R313G together with PAK4-S474E. Arrow a highlights filopodia; arrow b highlights lamellipodia induction for cells co-expressing PAK4-S474E and GEF-H1S or GEF-H1S-S67A; arrow c marks cells with stress fibers induction. Bar, 10 µm.

Fig. 5. NIH-3T3 cells were transfected with PAK4 and GEF-H1S alleles and examined by fluorescence imaging of EGFP for GEF-H1 alleles, PAK4 constructs were identified by immunostaining using PAK4 antiserum followed by anti-rabbit fluorescent rhodamine conjugate for PAK4 and fluorescent coumarin-phallicidin to label actin. (A-C) GEF-H1S co-transfected with PAK4 alleles WTS,474E and K350,351A. (D-F) GEF-H1S-S810A, GEF-H1S-S67A and GEF-H1S-Q312M,R313G together with PAK4-S474E. Arrow a highlights filopodia; arrow b highlights lamellipodia induction for cells co-expressing PAK4-S474E and GEF-H1S or GEF-H1S-S67A; arrow c marks cells with stress fibers induction. Bar, 10 µm.
the splice isoforms of GEF-H1 show some differences in their effects. Unlike GEF-H1S, which gave occasional distributed stress fibers, the majority of (>80%) cells overexpressing GEF-H1M displayed stress fibers at the cell periphery that gave the appearance of a woven halo of F-actin, which suggests that some Rho stimulating activity is locally restricted to the MT ends (Fig. 6A, I). The absence of filopodial induction in GEF-H1M isoforms co-expressed with wild-type HA-tagged PAK4 distinguishes them from GEF-H1S isoforms (compare Fig. 6A, II with Fig. 5A). Similarly to GEF-H1S, the transition to lamellipodia, however, was observed when GEF-H1M was co-expressed with activated PAK4-S474E (Fig. 6A, III; arrows indicate lamellipodia).

Consistent with reports in the literature, GEF-H1M, like LFC (murine GEF-H1), was stably associated with MTs (Glaven et al., 1999; Ren et al., 1998) (Fig. 6B, I). Overexpression of GEF-H1 is known to stabilize MTs in vivo (Krendel et al., 2002). Stabilization of MTs is apparent by their radial alignment and presence of an apparent MTOC as seen in cells expressing EGFP-GEF-H1M alone or when co-expressed with wild-type and kinase-inactive PAK4 (Fig. 6B, I,III). However, co-expression of activated PAK4 with EGFP-GEF-H1M results in the destabilizing of MTs and the remarkable redistribution of GEF-H1M from MTs to the cytoplasm, while the structure of MTs remained intact (compare Fig. 6A, III with 6B, II). MT polarity is also clearly altered in cells co-expressing GEF-H1M with the different alleles of PAK4 (Fig. 6B, I-III). Soluble GEF-H1S and PAK4 do not colocalize with MTs (supplementary material Fig. S5). Changes in MT polarity are subtle in the presence of GEF-H1S, GEF-H1S-S810A and GEF-H1S-S67A (supplementary material Fig. S5, I-III).

Fig. 6. NIH-3T3 cells were co-transfected with GEF-H1 and PAK4. Expression of GEF-H1 and PAK4 was detected by fluorescence imaging of EGFP for GEF-H1 and PAK4 antiserum (#933) followed with anti-rabbit fluorescent rhodamine for PAK4. Fluorescent coumarin-phallicidin was used to examine the actin cytoskeleton. Anti-β-tubulin followed by anti-mouse Marina Blue conjugate was used to stain for the MT cytoskeleton. (A) GEF-H1M together with PAK4 and the mutant PAK4-S474E. Arrow a highlights an actin halo seen with overexpressed GEF-H1M; arrow b indicates stress fibers organized with actin cables; arrow c highlights actin-rich lamellipodia in cells co-expressing GEF-H1M and the mutant PAK4-S474E. (B) GEF-H1M together with vector and the mutants PAK4-S474E and PAK4 K350,351A. Note the non-polarized partial dissolution of the MT cytoskeleton in the presence of the mutant PAK4-S474E. Arrow d indicates lack of MTOC. Bar, 10 µm.

Discussion

Our results provide insight into the mechanism by which PAK4 can modulate Rho GTPase signaling through its control of GEF-H1. We have discovered the physical interaction between PAK4 and GEF-H1 and identified within PAK4 a novel GID that is conserved between the group II PAKs. Furthermore we found that PAK4 phosphorylates GEF-H1 on key regulatory sites. Our findings support the notion that GEF-H1 signaling specificity is mediated in part through PAK4-dependent recruitment of both Rho family GTPases (such as Cdc42 through the CRIB domain of PAK4) and their guanine exchange factors (such as GEF-H1 through the GID of PAK4). This would help to explain how the multifunctional GEFs are able to relay spatial-temporal signals
to specific Rho family GTPases. Depending upon the signal, this recruitment role and potential feedback phosphorylation of GEF-H1 by PAK4 could allow PAK4 to act both upstream and downstream of the Rho family GTPases. A similar mechanism has been proposed for PAK1- and PIX-dependent activation of Cdc42, which is required for the directional migration of chemotactic leukocytes (Li et al., 2003). In the case of subgroup I PAKs, they interact with the Cool (cloned-out of library)/PIX (PAK-interactive exchange factor) family of tandem DH/PH-containing proteins (Bagrodia et al., 1999). The regulation of PAKs by the Cool/PIX family is complicated, depending upon the splice variant and the family member, the particular isoform/gene of PIX1/2 can act as an inhibitor or an activator, or play a permissive role in stimulating PAK kinase activity (Bagrodia and Cerione, 1999; Daniels et al., 1999; Feng et al., 2002).

An interesting evolutionary parallel exists between the PAK4/GEF-H1 and the Cla4/Cdc24 complex in Saccharomyces cerevisiae, where phosphorylation by the PAK-like Cla4 protein is responsible for the re-localization of Cdc24 and its own activation (Bose et al., 2001) (see also supplementary material Fig. S2 for consensus alignment). Cdc24p acts as both substrate and regulator of Cla4 in a feedback loop (Gulli and Peter, 2001). Although we show positive signals for Rac activation and Rho inhibition through GEF-H1 phosphorylation, we were unable to show such a feedback loop involving Cdc42 in vitro. We and others have shown that the DH domain lacks the necessary specificity to carry out exchange activities on Cdc42, which suggests that the filopodia formation in NIH-3T3 cells seen in our studies requires another unidentified participant in the PAK4/GEF-H1 complex (M.G.C. et al., unpublished) (Guo et al., 2001). PAK4 has been implicated in tumorigenesis and transformation of NIH-3T3 cells (Callow et al., 2002). Kinase-inactive PAK4 can inhibit Ras transformation much like N17Rac1 in the same system (Qiu et al., 1995). GEF-H1 transcript induction has been shown to occur upon Ras transformation of NIH-3T3 cells (Zuber et al., 2000). It is therefore possible that endogenous GEF-H1 underlies some of the events downstream of Ras. Fibroblasts overexpressing GEF-H1 exhibit low constitutive basal levels of Rho activation. The Rho-dependent activity of GEF-H1S is attenuated by PAK4-dependent phosphorylation of GEF-H1S on Ser810, which leads to the formation of actin-rich lamellipodia. Mutation of GEF-H1S at Ser810 blocks the ability of activated PAK4 to induce lamellipodia and leads to the resumption of constitutive induction of Rho, which results in actin stress fiber production (Fig. 7). Indeed, co-expression of kinase-inactive PAK4 leads to a similar blocked effect and accumulation of GEF-H1M from MTs, paralleled by varying degrees of dissolution of the MT network as well as changes in MT polarity.

Evidence exists for both antagonism between Rho family GTPases as well as the control of their activity by phosphorylation. Rac-dependent attenuation of Rho activity has been noted to play a key role in the motility of fibroblasts as well as the control of neurite outgrowth (Kozma et al., 1997; Leeuwen et al., 1997). Furthermore, PAK5 can promote neurite outgrowth in N1E-115 cells presumably by a mechanism involving Rho attenuation (Dan et al., 2002). Our finding of a novel GID that is conserved between PAK4, PAK5 and PAK6 suggests that these group-II-family PAKs can mediate the apparent antagonism between Rac and Rho, in part, by switching the specificity of GEF-H1 from Rac to Rho by either recruitment or modification of its exchange activity. Tyrosine phosphorylation is also known to play a role in the regulation of other Rho-GEF family members. For example, the phosphorylation of Vav by a Src family kinase relieves auto-inhibition imposed on the DH domain (Aghazadeh et al., 2000). This is not the case for GEF-H1. Deletion mutants ΔA49 and Δ122 of GEF-H1 (analogous to those used in the Vav study) failed to modify activity in a serum response element (SRE) transcriptional assay, whereas addition of PAK4 alleles (SRE) transcriptional assay, whereas addition of PAK4 alleles (M.G.C. et al., unpublished) (Guo et al., 2001). Although we do not exclude a role for tyrosine phosphorylation, we concluded that GEF-H1 was not auto-inhibited with respect to Rho, which is in agreement with constitutively active Rho signaling, and that a negative signal results from phosphorylation by PAK4 (Fig. 7).

A recent report has identified GEF-H1 as a target of PAK1 (Zenke et al., 2004). Phosphorylation of GEF-H1M (MT bound) on Ser885 (analogous to Ser810 of GEF-H1S) facilitates the binding of 14-3-3 proteins. Zenke et al. showed that
PAK4 phosphorylates GEF-H1 on key regulatory sites

Our data show unambiguously that, first, PAK4 interacts directly with GEF-H1 through a novel domain, the GID; second, endogenous PAK4 and GEF-H1 exist as a complex in cells; and third, PAK4 and GEF-H1 co-localize in cells and can be detected on Golgi membranes and transiently on MTs. Our results imply that GEF-H1 activity is controlled at the level of its localization to the appropriate complexes containing either substrates Rac-GDP or Rho-GDP. In addition to serving as a scaffold for GEF-H1 and possibly Cdc42, PAK4 phosphorylation of GEF-H1 could control the ability of GEF-H1 to recruit specific GTPases to specific cellular sites. Indeed, given that GEF-H1, like a majority of GEFs, does not seem to show a strong selectivity towards individual Rho GTPases, we favor these two models in the control of GEF-H1 selectivity by PAK4. The feedback regulation of GEF-H1 and complexity of the function of Ser67 and Ser810 phosphorylation by PAK4 comes as a surprise. The clear implication is that the outcome of PAK4 signaling is dependent upon the context of the signaling complex, which at the least involves a GEF, Rho family GTPase, which is further controlled by feedback phosphorylation of the GEF by PAK4. This feedback regulation of GEF-H1 by PAK4 has a recent parallel in the findings of feedback signaling involving the ternary complex of Dbl, Cdc42 and PAK1 (Wang et al., 2004). The in vitro studies by Wang et al. suggest that DH/PH-containing proteins could play a more direct role in cell signaling through their active formation of specific effector complexes (Wang et al., 2004). The idea is an attractive model that would further help to explain the underlying molecular mechanism of feedback between GEF-H1 and PAK4 that we observe in cells. Our observations in this study provide novel insights into upstream regulation of GEF-H1 activity and/or specificity that allows GEF-H1 to play a key role in mediating crosstalk between the actin and MT network, which is crucial for cell morphology and motility.

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