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Adhesion Stimulates Direct PAK1/ERK2 Association and Leads to ERK-dependent PAK1 Thr[212] Phosphorylation*

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Liisa J. Sundberg-Smith‡, Jason T. Doherty, Christopher P. Mack and Joan M. Taylor§

From the Department of Pathology, University of North Carolina, Chapel Hill, North Carolina 27599

The Rac1/Cdc42 effector p21-activated kinase (PAK) is activated by various signaling cascades including receptor-tyrosine kinases and integrins and regulates a number of processes such as cell proliferation and motility. PAK activity has been shown to be required for maximal activation of the canonical Ras/Raf/MEK/ERK Map kinase signaling cascade, likely because of PAK co-activation of Raf and MEK. Herein, we found that adhesion signaling also stimulates an association between PAK1 and ERK1/2. PAK1 and ERK1/2 co-immunoprecipitated from rat aortic smooth muscle cells (SMC) plated on fibronectin, and the two proteins colocalized in membrane ruffles and adhesion complexes following PDGF-BB or sphingosine 1-phosphate treatment, respectively. Far Western analysis demonstrated a direct association between the two proteins, and peptide mapping identified an ERK2 binding site within the autoinhibitory domain of PAK1. Interestingly, deletion of a major ERK binding site in PAK attenuates activation of an ERK-dependent serum-responsive element (SRE)-luciferase reporter gene, indicating that association between PAK and ERK is required to facilitate ERK signaling. We also show that ERK2 phosphorylates PAK1 on Thr[212] *in vitro* and that Thr[212] is phosphorylated in smooth muscle cells following PDGF-BB treatment in an adhesion- and MEK/ERK-dependent fashion. Expression of a phosphomimic variant, PAK-T212E, does not alter ERK association, but markedly attenuates downstream ERK signaling. Taken together, these data suggest that PAK1 may facilitate ERK signaling by serving as a scaffold to recruit Raf, MEK, and ERK to adhesion complexes, and that subsequent growth factor-stimulated phosphorylation of PAK-Thr[212] by ERK may serve to provide a negative feedback signal to control coordinate activation of ERK by growth factor- and matrix-induced signals.

The Ras superfamily of small GTPases including H-Ras, R-Ras, and Rho family members (Rac, Rho, and Cdc42) among others are activated by numerous transmembrane receptors such as receptor-tyrosine kinases, G-protein-coupled receptors, and integrins and regulate a variety of cellular processes including proliferation, differentiation, and migration (1). These GTPases are molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state (2, 3). In the GTP-bound form, the Ras-related GTPases interact with and activate a number of effector molecules that have been implicated in regulating cell cycle progression and/or cell migration such as the serine/threonine protein kinases, lipid kinases, and actin-binding/scaffolding proteins (3, 4).

Ras and Rac regulate MAP[1] kinase signaling, and a variety of agonists use Ras-dependent activation of ERK and/or Rac-dependent activation of c-Jun N-terminal kinase as dominant mitogenic signaling pathways (5). MAPK activation typically occurs by highly conserved multilevel kinase cascades (*i.e.* Ste20/Ste11/Ste7/Fus3 in yeast and Raf/MEK/ERK in mammalian cells) eventually leading to MAPK nuclear translocation and transcription factor activation (6, 7). Although the mammalian MAPK signaling pathways were initially thought to be independent and parallel, recent studies indicate that there is significant cross-talk between them.

Several lines of evidence indicate that integrins and growth factors promote coordinated activation of the ERK signaling cascade. Integrin signaling has been shown to be required for maximal activation of either growth factor-stimulated Raf or MEK activation an event likely dependent on the specific integrin receptors engaged in the particular cell type used (8–10). Studies indicate that the Rac effector, p21-activated protein kinase (PAK), a homologue to the yeast MAP4K, Ste20, may serve as a convergence point between growth factor- and integrin-mediated Ras/ERK signaling. GTP-bound Rac and Cdc42 activate PAK1 in an adhesion-dependent fashion by binding to the p21-binding domain (PBD or CRIB) localized within the N-terminal autoinhibitory domain of PAKs 1–3 (11, 12). This interaction exposes the PAK C-terminal kinase domain permitting activation, autophosphorylation, and downstream signaling (13, 14). Notably, plating cells on the extracellular matrix fibronectin enhances the ability of Rac to activate PAK, and β_1 integrins have been shown to transmit signals downstream to c-Jun N-terminal kinase through the PI 3-kinase/Rac/PAK cascade (15, 16).

Interestingly, Howe *et al.* (17) demonstrated that inhibition of PAK attenuated integrin-dependent ERK activation, and subsequent studies showed that this was likely because of the ability of PAK to enhance adhesion-dependent Raf and/or MEK activation. Activated PAK associates with Raf and phosphoryl-

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§ To whom correspondence should be addressed: Dept. of Pathology and Lab Medicine, 420 Brinkhous-Bullitt Blvd. CB 7525, University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-843-5512; Fax: 919-966-6718; E-mail: jmt3x@med.unc.edu.

[1] The abbreviations used are: MAP, mitogen-activated protein; PDGF, platelet-derived growth factor; PBS, phosphate-buffered saline; SMC, smooth muscle cell; GFP, green fluorescent protein; MEK, extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; PAK, p21-activated kinase; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; FAK, focal adhesion kinase; pThr, phosphorylated threonine; FRNK, FAK-related non-kinase.

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ates Ser[338] and Ser[339] within the catalytic domain. Although several phosphorylation sites have been reported to be necessary for Raf activation, phosphorylation of these serines has been shown to be essential for maximal Raf activation by Ras in response to integrin-mediated PAK1 activation (14, 18). However, the convergence of integrin signaling at the level of Raf is controversial, and at least one study refutes the necessity of PAK3 for Ser[338] phosphorylation in response to epidermal growth factor (19). Recent studies indicate that PAK also phosphorylates MEK on a site (Ser[298]) that is necessary for maximal MEK activation by Raf and that adhesion-dependent PAK signaling enhances association between MEK and ERK (5, 20–22). Thus, convergence of these two well established pathways likely occurs at the level of PAK, which can effect ERK signaling at multiple levels including co-activation of Raf and MEK. Whether the pathways leading from PAK to ERK are differentially regulated in an integrin-dependent cell type-specific fashion are important questions that remain to be addressed.

Herein, we report that adhesion signaling induces a direct association between ERK and PAK1. Taken together with previous data, our results indicate that PAK may coordinate signaling between Raf, MEK, and ERK by acting as a scaffold for these proteins. We also present evidence that ERK2 phosphorylates PAK1 on Thr[212], a site just downstream of the ERK binding site on PAK, and that this event may provide negative feedback inhibition of ERK signaling. These data highlight yet another level whereby adhesion signaling may regulate synergy between Ras and Rho family proteins to dynamically regulate the activation state of ERK at distinct regions within the cell.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The phosphospecific ERK1/2 antibody and the anti-PAK1 antibody were purchased from Cell Signaling. The phospho-Tyr[15] Cdc2 antibody was purchased from Santa Cruz Biotechnology. The phosphospecific PAK-Thr[212] antibody, anti-FLAG (M5), anti-acetylated tubulin and anti-vinculin antibodies, alsterpaullone and fibronectin were purchased from Sigma. PDGF-BB and UO126 were purchased from Calbiochem. The purified active ERK2 and anti-ERK2 antibody were purchased from UBI. The anti-paxillin antibody was purchased from Transduction Laboratories, and Texas-Red phalloidin was purchased from Molecular Probes.

Expression Constructs—Rat His-PAK1 and His-ERK2 (wild-type and kinase-defective) constructs were generous gifts from Leslie Parise (UNC) and Melanie Cobb (UTSW), respectively. GFP-PAK1 was made by inserting full-length wild-type human PAK1 (generous gift from Alan Howe, UVT) into EGFP-C1 vector (Clontech) cut with BglI+ EcoR1. GST-PAK1 (amino acids 1–290) was constructed as described previously (23). S212E/A and S223A variants were generated by PCR site-directed mutagenesis of GST- or GFP-tagged PAK1 constructs. Forward and reverse complementary primers corresponding to the following sequence were used: for PAK1-S212A, 5′: GAACCACTTCCTGTCGCTCCAACTCGGGACGTGG, for S212E, 5′: GTGATTGAACCACTTCCTGTCGAACCAACTCGGGACGTGGC, for PAK1-S223A, 5′: GGCTACATCTCCCATTGCACCTACTGAAAATAACACC. Mutations were confirmed by direct sequencing.

Cell Culture and Agonist Treatment—Rat aortic smooth muscle cells (SMC) were obtained from rat thoracic aortas by enzymatic digestion as described previously (24). Cells were used from passages 7–21 and were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 plus 10% fetal bovine serum and 1% penicillin-streptomycin. In some experiments, cells were serum-starved for 4 h before treatment with PDGF-BB (20 ng/ml) for the times indicated. For adhesion suspension experiments, cells were trypsinized, neutralized in soybean trypsin inhibitor (1 mg/ml in PBS), collected by centrifugation, and resuspended in serum-free DMEM:F12 plus 1% penicillin-streptomycin. Cells were held in suspension or plated on fibronectin-coated (40 μg/ml) dishes for the indicated times. A7R5 (ATCC) smooth muscle cells were maintained in DMEM plus 10% fetal bovine serum and 1% penicillin-streptomycin.

Protein Purification—His-ERK2 (wild-type and kinase-defective) and His-PAK1 were purified from bacterial lysates using Qiagen nickel-NTA (nickel-nitriloacetic acid) -agarose according to the manufacturer's protocol (The QIAexpressionist[tm] 5th Ed.). Briefly, cleared lysates in a buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0 plus 1 mg/ml lysozyme, 1 mM Na_3VO_4, 40 mM NaF, 100 μM leupeptin, 1 mM AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride), and 0.05 TIU/ml aprotinin were combined with 1 ml of nickel-NTA-agarose, rotated for 1 h at 4 °C, and then transferred to a chromatography column. The His-ERK nickel-NTA-agarose complexes were washed three times with 50 mM imidazole wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazole, pH 8.0) and eluted with 250 mM imidazole elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The His-PAK1 nickel-NTA-agarose complex was washed three times with 30 mM imidazole wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 30 mM imidazole, pH 8.0) and one time with 40 mM imidazole wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 40 mM imidazole, pH 8.0). 25 μl of each elution fraction was analyzed by SDS-PAGE to determine the appropriate fractions to pool. Proteins were dialyzed (50 mM Hepes, 150 mM NaCl, and 10% glycerol) overnight at 4 °C and then aliquoted and stored at −80 °C for subsequent experiments.

GST Pull-down Assay—GST-PAK (amino acids 1–290 of PAK1, J. Chernoff) was purified from bacterial lysates using glutathione-agarose beads (Amersham Biosciences) as described previously (23). SMC were incubated in serum-free medium (DMEM:F12 plus 1% penicillin-streptomycin) and treated with PDGF-BB (20 ng/ml) as described above. Cells were lysed in Buffer A (50 mM Tris, pH 7.6, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 0.5 mM $MgCl_2$, plus protease inhibitors), and 500 μg of protein were combined with 30 μg of GST-PAK1 fusion protein and rotated for 30–60 min at 4 °C. The beads were then washed twice with Buffer B (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5 mM $MgCl_2$, plus protease inhibitors) and once with Tris-buffered saline (TBS, 0.2 M NaCl, 50 mM Tris-HCl, pH 7.4). The beads were resuspended in SDS-PAGE sample buffer, boiled for 5 min, electrophoresed on a 12% SDS-polyacrylamide gel, and analyzed by Western blotting using either an anti-pERK1/2 or anti-ERK2 antibodies (1:1,000).

In Vitro Kinase Assay—To detect ERK phosphorylation, purified His-ERK2 (kinase-dead, 0.1–1 μg), and His-PAK1 (0.1–0.5 μg) were incubated with 10 μCi of [γ-^{32}P]ATP in kinase buffer (50 mM Hepes pH 7.3, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM NaF, 0.25% Triton 100-X) for 10 min at 30 °C. To detect phosphorylation of PAK1, freshly purified GST, GST-PAK1-(1–290), or GST-PAK1 variants (5 μg) were incubated with 10 ng of active ERK2 (UBI) in kinase buffer in the presence of 50 μM ATP with or without [γ-P^{32}]ATP (10 μCi)) for 10 min at 30 °C. Samples were resolved by SDS-PAGE. The radioactive gels were fixed in 50% methanol, 10% acetic acid, and 20% glycerol for 20 min and then rehydrated for 90 min in 7% acetic acid 5% methanol, and 20% glycerol. The gel was dried for 2 h at 80 °C and then was exposed to Kodak XAR autoradiograph film. The gel was then rehydrated in ddH_2O and then stained with 1% Coomassie Blue. For non-radioactive kinase assays, the gel was transferred to nitrocellulose, and analyzed by Western blotting using the phospho-Thr[212] antibody.

Western Blotting—Western blots were performed using the appropriate antibodies at a 1:1000 dilution. Blots were washed in TBST (TBS plus 0.05% Triton-X), followed by incubation with either horseradish peroxidase-conjugated rabbit anti-mouse antibody or -protein A-Sepharose (Amersham Biosciences) at a 1:2000 dilution. Blots were visualized after incubation with chemiluminescence reagents (ECL, Amersham Biosciences).

Far Western—Purified GST and GST-PAK1 beads were electrophoresed by SDS-PAGE (12%) and transferred to nitrocellulose. The blot was incubated in 5% powdered milk/TBST + 0.2% sodium azide for 48 h at 4 °C (to block the membrane and renature proteins). The blot was then incubated with purified His-ERK2 (8 μg/ml in 5% powdered milk/TBST) overnight at 4 °C. The blot was washed three times (10 min) with TBST and then incubated with India HisProbe-HRP (1:5,000 Pierce) for 1 h at room temperature. The blot was washed with TBST overnight at 4 °C before detection with SuperSignal West Pico (Pierce) chemiluminescence substrate.

PAK1 Spot Blot—A membrane containing 12-mer overlapping peptides derived from the PAK1 sequence was prepared by SPOT synthesis (ABIMED, generously provided by Leslie Parise). The PAK1 spot blot was blocked with 10% powdered milk in TBS-T, followed by incubation with 2 μg/ml purified His-ERK2 in binding buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 1% glycerol, 5% bovine serum albumin, and 0.05% Tween 20), overnight at 4 °C. The blot was washed three times (10 min) with TBST. The blot was then incubated with anti-ERK2 antibody, followed by incubation with horseradish peroxidase-conjugated protein A-Sepharose at a 1:2000 dilution, and visualized by chemiluminescence (ECL).

Luciferase Assay—A7R5 cells were transfected with 0.75 μg of SRE-

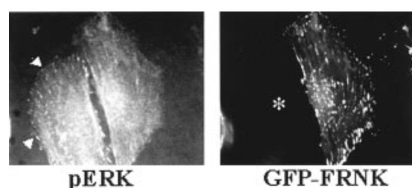

FIG. 1. **GFP-FRNK expression attenuates ERK activity in focal adhesions.** SMC were transfected with GFP-FRNK and plated on fibronectin-coated chamber slides (10 μg/ml) for 90 min in the presence of serum. Cells were fixed as described under "Experimental Procedures" and stained with an antiphospho-ERK antibody (1:200) followed by a Texas Red-conjugated donkey anti-rabbit antibody (2 μg/ml). GFP-FRNK was visualized by direct fluorescence.

luciferase reporter construct and 0.25 μg of the GFP construct variant per well using Superfect (Stratagene) following the manufacturer's protocol. All transfections were done in quadruplicate. Luciferase assays were performed 48-h post-transfection using Steady-Glo Luciferase Assay kit (Promega) following the manufacturer's protocol. Relative promoter activity was expressed as the mean ± S.E. relative to total protein.

Immunocytochemistry—A7R5 cells were transfected with the desired GFP-tagged construct using Superfect (Qiagen). After 48 h, the cells were trypsinized, rinsed in soybean trypsin inhibitor (1 mg/ml), centrifuged, washed twice in PBS, and resuspended in serum-free DMEM plus 1% penicillin-streptomycin. Cells were counted and plated on fibronectin-coated (10 μg/ml) slides (Lab-Tek) for 20–90 min. Staining procedure was followed as previously published (25). In brief, cells were fixed with 4% paraformaldehyde, permeabilized with 4% Triton X-100 in PBS, incubated with specified primary antibody for 1 h at the following concentrations: anti-perk 1:200, anti-vinculin 1:50, anti-paxillin 1:250, anti-acetylated tubulin (1:1000). After washing with PBS, slides were incubated for 1 h with either Texas Red-conjugated donkey anti-rabbit or donkey anti-mouse antibodies (2 μg/ml) or Texas Red-conjugated phalloidin to detect filamentous actin.

Mass Spectral Analysis—GST-PAK1 (amino acids 1–290, 10 μg) was electrophoresed on a 14% precast Tris-glycine SDS-PAGE gel (Invitrogen). The gel was fixed by soaking in 25% isopropyl alcohol/10% acetic acid for 20 min, stained with 0.01% Coomassie Blue in 10% acetic acid overnight, followed by destaining in 10% acetic acid. Appropriate bands were excised, trypsinized, and peptides were eluted and subjected to MALDI-TOF/TOF (ABI 4700 Proteomics Analyzer) and LC-MS (Micromass Q/TOF API-US LC/MS/MS) to define the cleavage site as described previously (26).

RESULTS

We reported previously that inhibition of FAK in SMC by ectopic overexpression of its dominant-interfering form FAK-related non-kinase (FRNK) did not attenuate the magnitude or duration of total cellular ERK activity induced by PDGF-BB, angiotensin II, or fibronectin. However, recent studies indicate that active ERK is localized in several different compartments within the cell including focal adhesions (21, 27–29). To determine whether adhesion-dependent FAK signaling might regulate activation of ERK at distinct sites within the cell, we plated GFP-FRNK-transfected SMC on fibronectin for 60 min and stained cells with an antibody that recognizes active (phosphorylated) ERK1/2. As shown in Fig. 1, ectopic expression of FRNK does dramatically reduce adhesion-stimulated ERK activation in focal adhesions.

In light of our previous data revealing that FRNK expression markedly attenuated integrin-stimulated Rac1 activity, and mounting evidence that the Rac1 effector, PAK regulates ERK activity *in vivo* in an adhesion-dependent fashion, we examined whether PAK might be involved in regulating ERK activation in focal adhesions. As shown in Fig. 2*A*, ectopically expressed GFP-PAK and phospho-ERK1/2 co-localize in membrane ruffles following PDGF-BB stimulation and co-localize within nascent focal adhesions following sphingosine 1-phosphate treatment when SMC are plated on fibronectin. In addition, immunoprecipitation experiments revealed an adhesion-

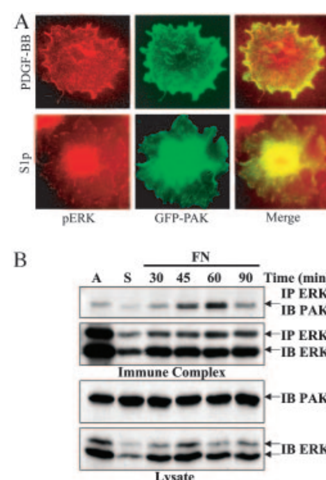

FIG. 2. **ERK1/2 and GFP-PAK1 co-localize, and endogenous ERK1/2 and PAK1 associate in SMC plated on fibronectin.** *A*, A7R5 cells were transfected with GFP-PAK1 and plated on fibronectin-coated slides in serum-free media. After 45 min, cells were treated with PDGF-BB (20 ng/ml) or sphingosine 1-phosphate (*S1p*; 1 μM) for and additional 45 min. Endogenous pERK and ectopically expressed GFP-PAK1 co-localize in membrane ruffles and focal adhesions, respectively as indicated by *arrowheads*. *B*, rat aortic SMC were either left attached (*A*) or trypsinized and held in suspension for 90 min (*S*) or plated on 40 μg/ml fibronectin (*FN*) for indicated times. Cells were lysed, and endogenous ERK was immunoprecipitated (*IP*) using an anti-pERK1/2 antibody as outlined under "Experimental Procedures." Western blotting (*IB*) was performed with anti-PAK1 and anti-ERK antibodies. *Bottom panels* show a 5% lysate loading control for PAK1 and ERK1/2. Data in Fig. 2 are representative of three separate experiments.

dependent association of endogenous ERK1/2 and PAK1 in SMC. SMC were either continuously grown in serum (denoted *A*, for attached) or trypsinized and held in suspension (*S*) in serum-free medium and/or plated on fibronectin (*FN*)-coated plates for the times indicated. As shown in Fig. 2*B*, association between PAK and ERK appears to peak after 60 min and surpasses the amount co-immunoprecipitated in stably adherent cells. Very little of the PAK1-ERK complex was formed in non-adherent cells, as might be expected, because activation of PAK is dependent on adhesion signaling (30). Taken together, these data support the hypothesis that PAK1 and ERK1/2 associate in intact cells.

Interestingly, we showed that a GST-PAK1 fusion protein containing the N-terminal 290 amino acids of PAK1 could efficiently precipitate phosphorylated ERK1/2 from SMC lysates, indicating that the N terminus of PAK is sufficient for ERK binding (Fig. 3*A*). Subsequent experiments, in which ERK2 was precipitated from serum-starved SMC or SMC treated with 20 ng/ml PDGF-BB for 10 min (to maximally activate ERK), revealed that phosphorylated (active) and unphosphorylated (inactive) ERK2 are precipitated equally well with the PAK1 fusion protein (Fig. 3*B*), indicating that preactivation of ERK was not required for PAK1 binding.

Previous studies have shown that Raf also binds to PAK; however, the binding site for Raf is located within the C terminus of PAK (in a region not included in our GST-PAK1 construct). Thus it was unlikely that ERK was precipitated by an indirect association with Raf and its binding partner MEK. Nonetheless, a number of proteins have been shown to associate with the N terminus of PAK, including the small GTPases Rac and Cdc42 and the adapter proteins NCK, GRB2, and PKL (31–33). Therefore to further rule out the possibility of an indirect ERK-PAK1 association, we used a Far Western approach. We probed a membrane containing increasing amounts of GST-PAK1 fusion protein with purified His-ERK2 protein and processed the membrane by Western blotting with an

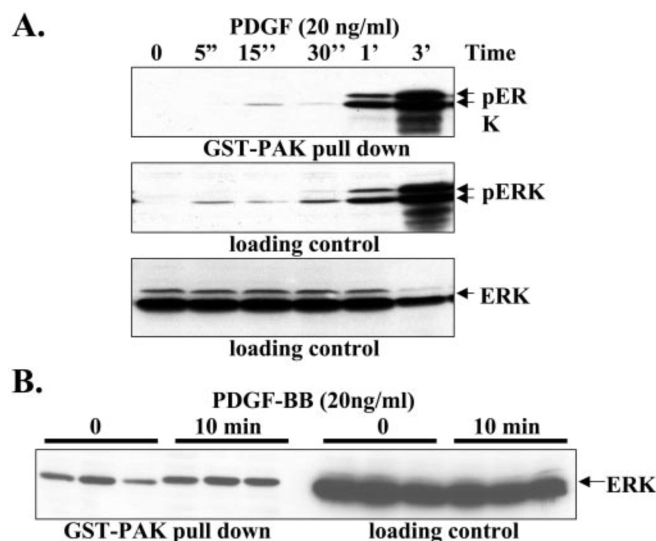

FIG. 3. **ERK precipitates from SMC cell lysate with GST-PAK1 fusion protein.** SMC were serum-starved for 4 h before treatment with PDGF-BB (20 ng/ml) for the times indicated. Lysates were incubated with 30 μg of purified GST-PAK1 (amino acids 1–290) for 30 min, and complexes were precipitated and analyzed by SDS-PAGE as described under "Experimental Procedures." *A*, Western blotting was performed using an antiphospho-ERK1/2 antibody (pERK, *top panel*). *Middle* and *bottom panels* represent a 10% lysate loading control for phospho-ERK1/2 and total ERK2, respectively. *B*, pull-downs were performed in triplicate for each time point. Western blotting was performed with an antibody recognizing total ERK1/2. Data are representative of three to five separate experiments.

anti-His antibody. As shown in Fig. 4*A*, purified His-ERK2 binds directly to GST-PAK1 in a concentration-dependent fashion. In separate experiments, the binding of His-ERK2 to GST-PAK1 was also detected using ERK-specific antibodies (data not shown). It should be noted that in most preparations of GST-PAK1–290, we observe the full-length product as well as one major putative cleavage product (Fig. 4*B*). Interestingly, His-ERK2 also readily associated with the presumptive cleavage product, but did not associate with GST alone (Fig. 4*C*). In order to identify the nature of the breakdown product, we isolated the full-length band (denoted P1) and the cleavage product (denoted P2) from a Coomassie Blue-stained polyacrylamide gel and submitted the bands for mass spectral analysis. A combination of MALDI-TOF/TOF and LC-MS data revealed that P1 corresponded to GST fused to N-terminal amino acids 1–290 of PAK1 (as expected) while P2 corresponded to GST fused to PAK1 amino acids 1–55. The PAK sequence from 1–55 appeared to be sufficient for ERK2 binding, but the observation that ERK2 bound the larger 1–290 fragment slightly better (compare amounts precipitated with 0.5 μg of fusion protein in Fig. 4, *A versus C*) indicates that additional sites within the 56–290 region may also be important.

In order to further map the interaction site on PAK1 for ERK2, a membrane containing immobilized 12-mer peptides derived from the amino acid sequence of PAK1 was incubated with purified His-ERK2 and subsequently probed with an anti-ERK2 antibody. As shown in Fig. 4*D*, interactions were detected in three distinct regions corresponding to amino acids 40–54 (site A), 86–94 (site B), and 124–138 (site C) of PAK1. All three binding sites are within the autoinhibitory domain of PAK. Site B is within the CRIB domain that is responsible for binding to Cdc42 and Rac while sites A and C flank this region. Coupled with the result from the previous experiment, these data indicate that amino residues 40–54 within PAK1 are sufficient for ERK2 binding, but that additional interactions within the CRIB domain and autoinhibitory domain may be required for a high affinity interaction. Interestingly, crystal structure analysis of the autoinhibitory domain of PAK1 (amino acids 70–149) revealed that sites B and C, which form a β-sheet and α-helix, respectively, are in close proximity, indicating that this region may provide a single docking site for ERK (34).

Consistent with the idea that ERK makes multiple contacts within the PAK1 N terminus, a GST-PAK fusion protein with a deletion of amino acids 40–54 (GST-PAKdA) greatly reduces, but does not completely block PAK-ERK association (Fig. 5*A*). GST-PAKdA and GST-PAK-(1–290) did however precipitate comparable amounts of Rac1 from SMC lysates, indicating that the tertiary structure of the CRIB domain in this deletion construct is likely intact (Fig. 5*A*, *bottom panel*). To determine the effect of the PAK-ERK interaction on ERK signaling, we generated a similar deletion (delta amino acids 40–54) in the context of full-length GFP-PAK (GFP-PAKdA). This mutation did not affect PAK localization to focal adhesions (data not shown). We then co-transfected either GFP-PAK or GFP-PAKdA along with a serum-responsive element (SRE)-luciferase reporter construct and measured the activity of this ERK-dependent transgene. In the presence of serum, GFP-PAK expression significantly enhanced SRE-LUC activity, whereas GFP-PAKdA expression did not (Fig. 5*B*). These results indicate that PAK/ERK association is important for PAK-dependent ERK signaling.

Because we mapped the binding site of ERK2 to the autoinhibitory domain of PAK1, we reasoned that ERK binding to PAK might relieve autoinhibition and activate PAK by a mechanism similar to that observed for Rac or Cdc42. To address this question, we incubated purified kinase-defective ERK2 (KD-ERK2) with purified His-PAK1 and performed an *in vitro* kinase assay. As shown in Fig. 6, purified His-PAK1 autophosphorylates in a concentration-dependent fashion; however, titration of KD-ERK2 (up to a 15-fold molar excess over PAK) into the reaction does not enhance PAK1 autophosphorylation. Actually, KD-ERK2 appeared to attenuate PAK activity at the higher concentrations (0.25–1 μg), but the significance of this inhibition is unclear. This experiment also revealed that PAK1 does not phosphorylate ERK2 *in vitro* as evident by the lack of radioactivity incorporated into KD-ERK2.

In terms of the reciprocal phosphorylation event, PAK1 contains two consensus sites for ERK phosphorylation, PVTP (Thr^{212}) and PISP (Ser^{223}) just downstream of the defined ERK2 binding site. To determine whether ERK2 phosphorylates PAK1 directly, we performed an *in vitro* kinase assay in which a GST-PAK1 fusion protein containing the two putative phosphorylation sites (amino acids 1–290) was incubated with purified active ERK2 and [γ-^{32}P]ATP. Fig. 7*A* shows that the N-terminal GST-PAK1 fusion protein, but not GST alone, was efficiently phosphorylated by ERK2 *in vitro*. Based on this observation, we mutated each of the consensus amino acids to alanine individually and in combination to determine if these sites were phosphorylated by ERK2 *in vitro*. As shown in Fig. 7*B*, the T212A mutation dramatically reduced ERK-mediated phosphorylation. The S223A mutation had only a slight effect on its own or in combination with T212A. To confirm these data, ERK2-mediated phosphorylation of Thr^{212} was measured using a phosphothreonine 212-specific antibody. Results shown in Fig. 7*C*, provide further evidence that PAK-Thr^{212} is a major target for ERK phosphorylation.

To determine whether Thr^{212} is phosphorylated by ERK in cells, we treated SMC with PDGF-BB for various times and examined the phosphorylation of PAK1-Thr^{212} (pThr^{212}) by Western analysis. As shown in Fig. 8*A*, PDGF-BB stimulated a time-dependent increase in pThr^{212} that lagged slightly behind

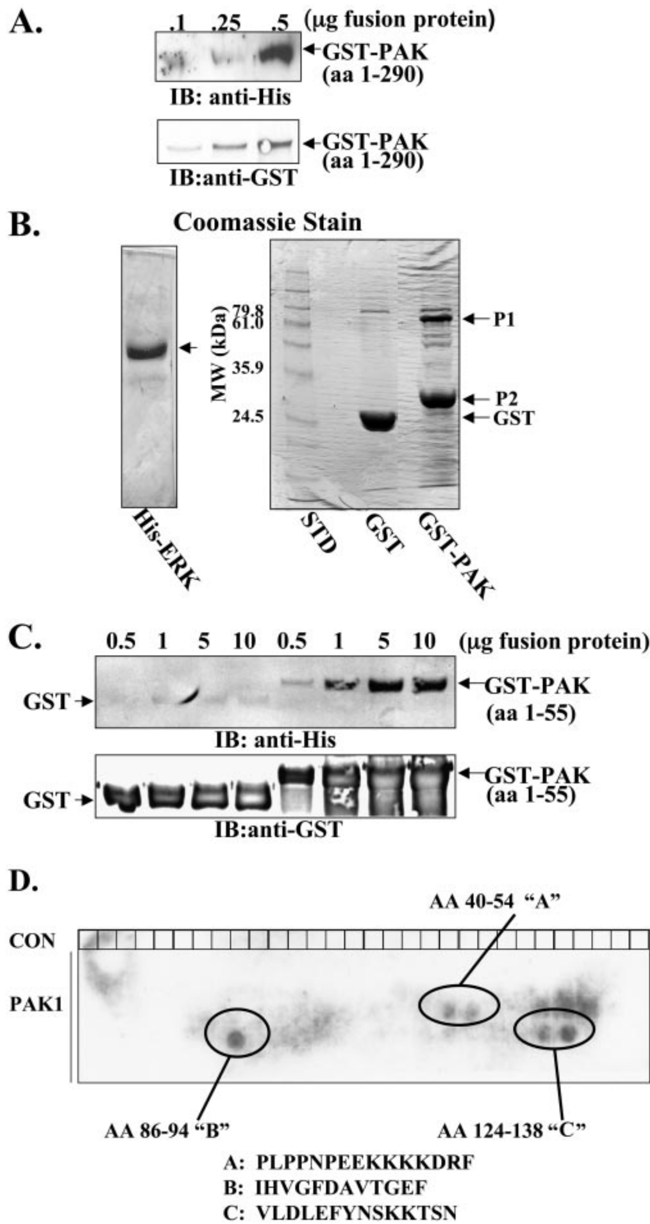

FIG. 4. **ERK2 binds directly to N terminus of PAK1.** *A*, indicated concentrations of immobilized GST-PAK1 (amino acids 1–290) was electrophoresed, transferred to nitrocellulose, incubated with purified His-ERK2 protein, then probed with anti-His antibody as described under "Experimental Procedures" (*top*). Western blot was stripped and reprobed with an anti-GST antibody to reveal the amount of GST-PAK1 loaded at the indicated concentrations (*bottom*). *B*, His-ERK2 (*left*), GST, and GST-PAK1 (*right*) proteins were purified as described under "Experimental Procedures." Proteins (5 μg) were analyzed on a 12% SDS-acrylamide gel and stained with Coomassie Blue. Mass spectral analysis revealed that the higher molecular mass band in the GST-PAK1 lane (denoted *P1*) contains GST and amino acids 1–290 of PAK1 and the lower molecular mass band (denoted P2) contains GST and amino acids 1–55 of PAK1. *C*, Far Western was performed as described above using GST or GST-PAK1-(1–55) as bait and His-ERK2 as a probe. The membrane was probed with anti-His antibody (*top*) and then stripped and reprobed with an anti-GST antibody to reveal the amount of GST and GST-PAK1 (amino acids 1–55) loaded at the indicated concentrations (*bottom*). *D*, PAK1 SPOT peptide membrane was blocked and incubated with purified His-ERK2 protein as described under "Experimental Procedures." The membrane was probed with an anti-ERK antibody and binding was detected by chemiluminescence. Three binding sites were identified by chemiluminescence and labeled *site A*, amino acids 40–54, *B*, amino acids 86–94; and *C*, amino acids 124–138. The *first row* of the grid contains 12-mer random control peptides and the remaining *square* contains overlapping 12-mers derived from the PAK1 sequence. Data in *panels A–C* are representative of at least three separate experiments.

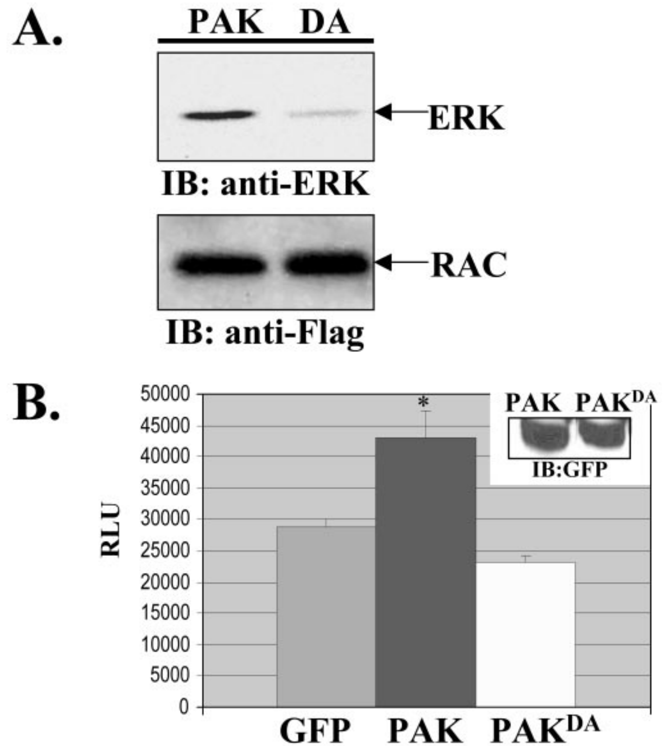

FIG. 5. *A*, equal amounts of pooled SMC lysate (500 μg) were incubated with either GST-PAK (25 μg) or a GST-PAK variant lacking amino acids 40–54 (GST-DA, 25 μg), and complexes were precipitated and analyzed by SDS-PAGE and Western blotting as described above (*top panel*). A7R5 cells were transfected with FLAG-L61 Rac, cells were lysed 48-h post-transfection, lysates were pooled, and 500 μg of protein were incubated with 25 μg of GST-PAK or GST-DA as described above. Western blotting was performed with an anti-FLAG antibody (*bottom panel*). *B*, A7R5 cells were transfected with 0.75 μg of SRE-luciferase reporter plasmid along with 0.25 μg of either GFP-C1, GFP-PAK, or GFP-PAK^{DA}. 48-h post-transfection, cells were lysed and analyzed for luciferase activity. The *inset* shows equivalent expression levels of the GFP-PAK1 and GFP-PAK^{DA} constructs. Data were normalized to total cellular protein. The graph represents mean ± S.E. of four separate experiments. The *single asterisk* indicates significant increases from control GFP-transfected cells ($p < 0.05$).

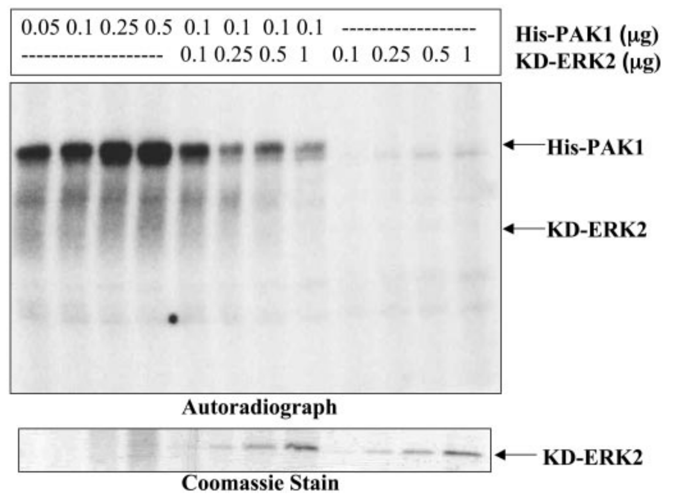

FIG. 6. **PAK1 does not phosphorylate ERK2 *in vitro*.** An *in vitro* kinase assay was performed with purified His-PAK1 (*lanes 1–4*), kinase-defective ERK2 (*KD-ERK*; *lanes 9–12*), or both (*lanes 5–8*) in the presence of [γ-^{32}P]ATP for 10 min at 30 °C as described under "Experimental Procedures." Samples were examined by SDS-PAGE, and the gel was either dried and exposed to Kodak XAR imaging film for 2 h (*top*), or rehydrated and stained with Coomassie Blue R-250 to reveal KD-ERK loading (*bottom*). Data are representative of two separate experiments.

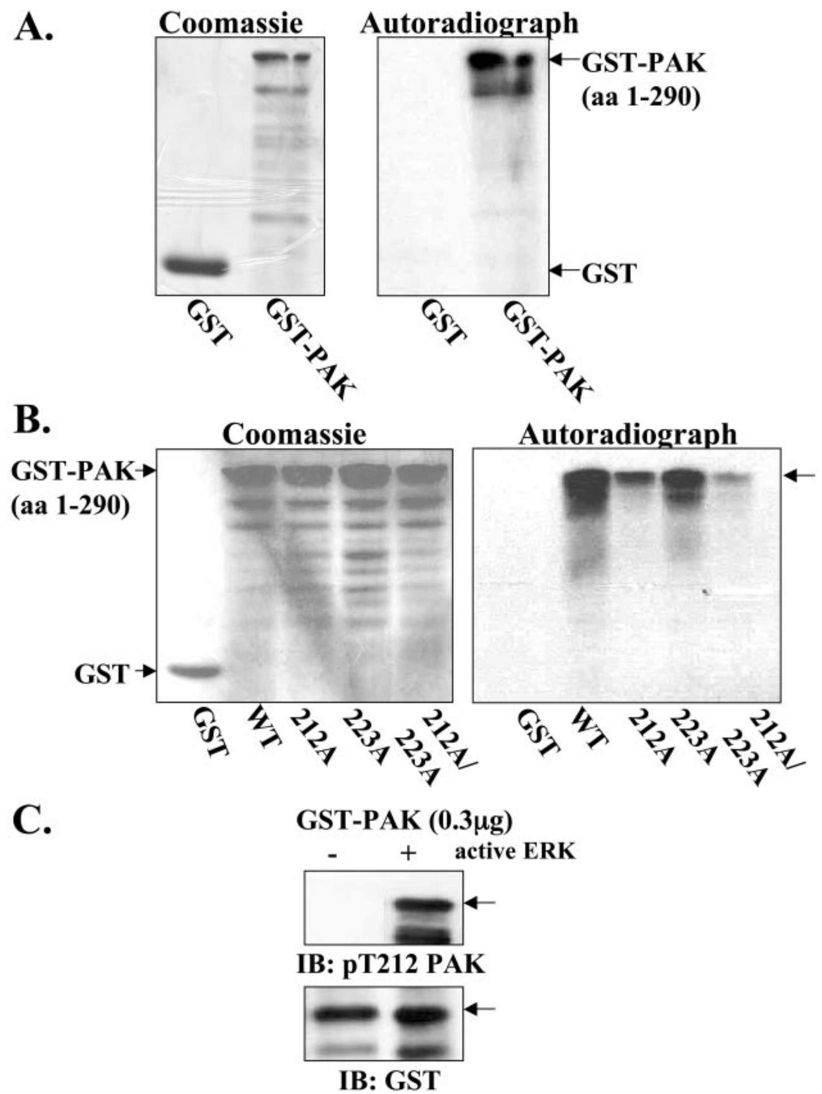

FIG. 7. **ERK2 phosphorylates PAK1 on Thr^{212} *in vitro.*** *A*, equal amounts of GST and GST-PAK1 (5 μg) were incubated with purified active ERK2 (10 ng) and [γ-^{32}P]ATP for 10 min at 30 °C as described under "Experimental Procedures." Samples were analyzed by SDS-PAGE, and the gel was either stained with Coomassie Blue R-250 (*left*), or dried and exposed to Kodak XAR film for 20 min (*right*). *B*, *in vitro* kinase assay was performed as described above with equal amounts of GST, GST-PAK1 (*WT*), GST-PAK1T212A (*212A*), GST-PAK1S223A (*223A*), or GST-PAK1T212A/S223A (*212A/223A*). Coomassie-stained gel shows equal loading for each variant (*left*), and the autoradiograph reveals level of ERK2-stimulated phosphorylation (*right*). *C*, 0.3 μg of GST-PAK1 was incubated with (+) or without (−) active ERK2 (10 ng) and cold ATP (50 μM) for 10 min at 30 °C as described under "Experimental Procedures." Samples were analyzed by SDS-PAGE, the gel was transferred to nitrocellulose, and a Western blot was performed using an anti-pThr^{212} PAK1 antibody (*top*). The membrane was stripped and reprobed using an anti-GST antibody (*bottom*) to reveal equal loading. Data are representative of at least three separate experiments.

activation of ERK, with an observed peak in phosphorylation around 10 min (Fig. 8*A*). Pretreatment of SMC with the MEK inhibitor, UO126, almost completely blocked PDGF-BB-stimulated PAK1 phosphorylation, indicating that this response is mediated by ERK signaling. Interestingly, although adhesion signaling can stimulate association between ERK and PAK, plating on fibronectin alone for 30–90 min is not sufficient to induce pThr^{212} (Fig. 7*B*). Nonetheless, adhesion is required for PDGF-BB stimulated pThr^{212}, since PDGF-BB treatment of SMC held in suspension did not alter pThr^{212} levels (Fig. 8*C*).

Recent reports demonstrated that Cdc2/Cdk5 can phosphorylate PAK1-Thr^{212} in a cell cycle-dependent manner (35–38). To rule out the possibility that these cyclin-dependent kinases are involved in PDGF-BB-stimulated Thr^{212} phosphorylation, we exposed SMC to the Cdk1 inhibitor alsterpaullone prior to PDGF-BB stimulation and examined the level of Thr^{212} phosphorylation. As shown in Fig. 9*A*, the cyclin-dependent kinase inhibitor did not diminish Thr^{212} phosphorylation. Interestingly, PDGF-BB inhibited Cdc2/Cdk5 activity in SMC as measured by the inhibitory phosphorylation of Cdc2-Tyr^{15} (Fig. 9*B*) further suggesting that adhesion-dependent agonist-stimulated phosphorylation of PAK1-Thr^{212} in SMC is dependent upon ERK and not Cdc2/Cdk5.

Irrespective of the kinases involved, phosphorylation of PAK1-Thr^{212} has been implicated in the regulation of post-mitotic cell spreading and microtubule organization (35–37). In addition, phospho-ERK1/2 and -PAK1 co-localize in focal adhesion structures in SMC, so we hypothesized that PAK-Thr^{212} phosphorylation might regulate focal adhesion formation. To this end, we transfected GFP-tagged variants of PAK1 including a non-phosphorylatable Thr^{212} (T212A) and one that mimics phosphorylation, T212E, into A7R5 SMC that were then plated onto fibronectin for 20–90 min. No change in the rate of cell spreading was observed between GFP-PAK1-, GFP-PAKT212A-, or GFP-PAKT212E-expressing cells (data not shown). Furthermore, each of the variants localized in focal adhesions and had no effect on the organization of nascent or mature focal adhesions as assessed by paxillin and vinculin staining, respectively. Organization of actin microfilaments and acetylated tubulin were also indistinguishable between wild-type GFP-PAK1 and the phosphorylation variants (Fig. 10).

Because PAK potentiates ERK activation, we sought to determine whether PAK-Thr^{212} phosphorylation might regulate ERK-PAK association and/or ERK-dependent signaling. GST-PAK, GST-PAK pretreated with purified active ERK2, and [γ-^{32}P]ATP, and GST-PAKT212E each precipitated similar amounts of ERK from SMC lysates, indicating that pThr^{212} does not attenuate ERK-PAK association (data not shown). To determine the possible effects of Thr^{212} phosphorylation on downstream signaling, we examined the effect of GFP-PAK and GFP-PAKT212E expression on transactivation of the ERK-dependent SRE-luciferase reporter gene. As shown in Fig. 10, in contrast to GFP-PAK, which stimulated serum-induced lucif-

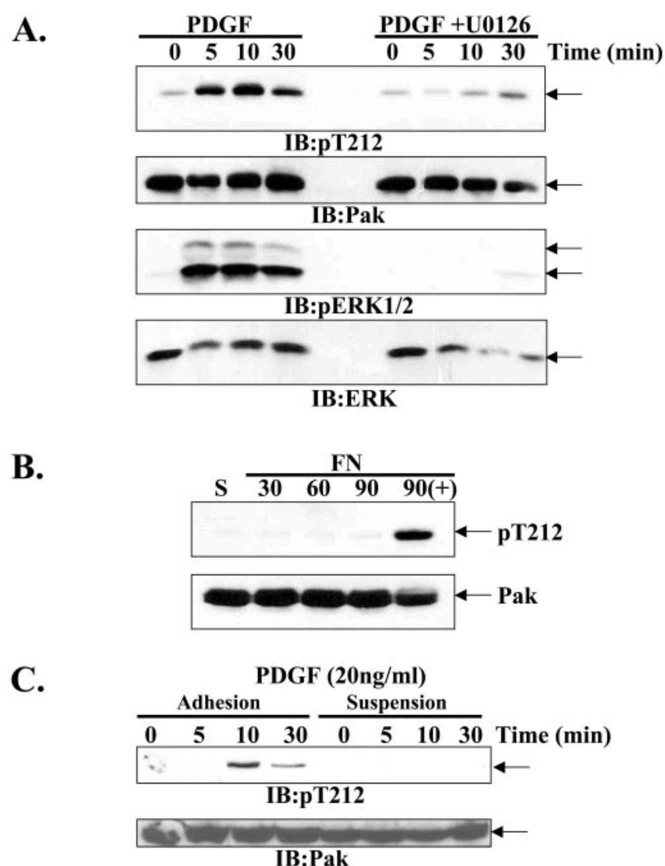

FIG. 8. **PDGF-mediated Thr^{212} PAK1 phosphorylation in SMC is dependent on both ERK and adhesion signaling.** *A*, SMC were serum-starved and treated with PDGF-BB (20 ng/ml) for times indicated with or without a prior 30-min pretreatment with U0126 (10 μM). Cell extracts were prepared, and samples were examined by Western blotting using anti-pThr^{212} PAK1, anti-PAK1, anti-pERK1/2, and anti-ERK2 antibodies. *B*, SMC were either held in suspension (*S*) or plated on fibronectin for the indicated times or treated with 20 ng of PDGF-BB for 10 min after plating (+). Western blotting was performed using anti-pT212 PAK1 and anti-PAK1 antibodies. *C*, SMC were either held in suspension or plated on fibronectin for 90 min. Cells were treated with PDGF-BB (20 ng/ml) for the times indicated. Western blotting was performed using anti-pThr^{212} PAK1 and anti-PAK1 antibodies.

erase activity, GFP-PAKT212E expression markedly attenuated this response. Based on the observations presented in this report, we hypothesize that Rac-dependent adhesion signaling activates PAK and induces an association between PAK and components of the ERK signaling cascade to facilitate ERK signaling, and that subsequent growth factor stimulated phosphorylation of PAK-Thr^{212} by ERK may serve to provide a negative feedback signal to control coordinate activation of ERK by growth factor- and matrix-induced signals (see Fig. 11).

DISCUSSION

PAK kinases regulate various cellular processes such as proliferation, migration, contraction, and apoptosis. PAK1 is a Rac/Cdc42 effector that coordinates actin-based cellular protrusions, an important step in persistent directional migration (39, 40). Recent studies reveal that PAK1 activity is also essential for maximal activation of the mitogenic Raf/MEK/ERK cascade (40). Indeed, PAK can synergize with Ras to activate Raf and can synergize with MEK to activate ERK (5, 14, 17, 18, 20–22). Herein we report that ERK2 associates with PAK1 in an adhesion-dependent manner through sites mapped within the N-terminal autoinhibitory domain of PAK. In addition, we show that a PAK deletion variant that does not readily associate with ERK, has a reduced capacity to enhance ERK-dependent transactivation of an SRE-reporter construct. These data are consistent with previous findings that adhesion-dependent Rac activation is required for efficient accumulation of active ERK in the nucleus (41, 42). The ability of PAK to bind (and phosphorylate) Raf, phosphorylate MEK and, as demonstrated in this study, bind to ERK, indicates that PAK may serve as a scaffold to recruit members of the Raf·MEK·ERK complex to focal adhesions and subsequently facilitate signaling through the ERK pathway (Fig. 12).

In yeast, Ste5, which regulates activation of the Fus3 MAP kinase mating pathway is a classic example of a high fidelity signaling scaffold, because it interacts with each member of the cascade (Ste20, Ste11, Ste7, and Fus3) (43, 44). To date, a protein that acts in an analogous fashion to Fus3 in the ERK cascade has not been identified. However, kinase suppressor of Ras (KSR) and MEK-binding partner 1 (MP-1) are putative ERK scaffolding molecules, because they bind directly to both MEK and ERK (specifically MEK1 and ERK1 in the case of MP-1) (45, 46). Herein, we have shown that PAK (the Ste 20 homologue or MAP4K of the ERK pathway) interacts with ERK2 through a region within the N-terminal autoinhibitory domain. Because PAK has also been shown to interact with Raf within sequences in the C terminus, it may serve as an additional potential scaffold for the ERK cascade. Even though there is no evidence of a direct PAK-MEK interaction, it has been shown that upon Raf binding to the C terminus of PAK, MEK becomes phosphorylated at Ser^{298}, a site required for maximal MEK activation (14, 18). Furthermore, Slack-Davis *et al.* (21) showed that adhesion induces PAK-dependent phosphorylation of MEK-Ser^{298}, and Elben *et al.* (22) have shown that PAK activation was required for adhesion-dependent association of MEK1 with ERK. Based on these reports, it is possible that PAK1 could function to target Raf, MEK, and ERK to nascent focal adhesions to impart restricted activation of the ERK cascade at the leading edge of the cell during migration.

We identified three distinct ERK2 binding sites that mapped within the N-terminal autoinhibitory domain of PAK1: amino acids 40–54, 86–94, and 124–138 (denoted A, B, and C respectively). Although the reported crystal structure of the complex between the N-terminal autoregulatory fragment (amino acids 70–149) and the C-terminal kinase domain (amino acids 249–545) of PAK1 does not contain the most proximal A binding site, the structure reveals that the β-sheet comprising the "B" binding site packs tightly against the α-helix comprising the "C" binding site, indicating that the binding sites for ERK2 that we defined may indeed constitute a single binding pocket within PAK1. Interestingly, the "A" binding site in PAK1, which we have shown is sufficient for ERK binding does contain a cluster of basic residues similar to the "D-domain" found in other ERK binding partners including MEK, MAPK phosphatase, and ribosomal S6 kinase (47, 48). This motif has been shown to function independently (47, 48), corroborating our Far Western data revealing that PAK amino acids 1–55 were sufficient for directing the ERK2-PAK1 interaction, and our findings that a GST-PAK fusion protein with a deletion of amino acids 40–54 had a markedly reduced capacity to precipitate ERK from SMC lysates compared with the full N-terminal construct. Future mutagenesis experiments will aid in determining the relative contribution of the defined sites to direct high affinity binding to ERK. Notably, the 12-amino acid core of each of the defined binding sites in PAK1 are highly conserved across the Group I PAK family members (100, 100, and 75% identical for A, B, and C, respectively); thus, it is likely that ERK may interact with PAK2 and PAK3 as well as PAK1. In fact, a recent report by Shin *et al.* (49) highlights the possibility that ERK1 and PAK2 may indeed associate in cells in a fibro-

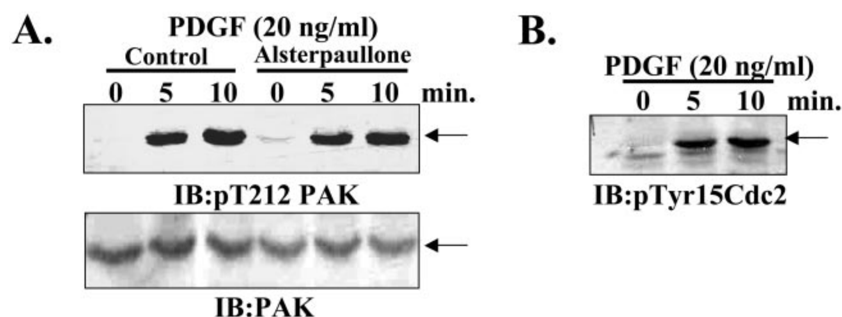

FIG. 9. **PDGF-BB-stimulated Thr^{212} PAK1 phosphorylation in SMC is not dependent on Cdc2/Cdk5 activity.** *A*, serum-starved SMC were pretreated for 3 h with alsterpaullone (10μM) prior to PDGF-BB (20 ng/ml) treatment for the indicated times. Samples were electrophoresed, and Western blotting was performed with anti-pThr^{212} PAK1 (*top panel*) or anti-PAK1 antibodies (*bottom panel*). *B*, serum-starved SMC were treated with PDGF-BB (20 ng/ml) for times indicated. Cell extracts were prepared, and samples were examined by Western blotting using an anti-pTyrCdc2 antibody. Data are representative of at least three separate experiments.

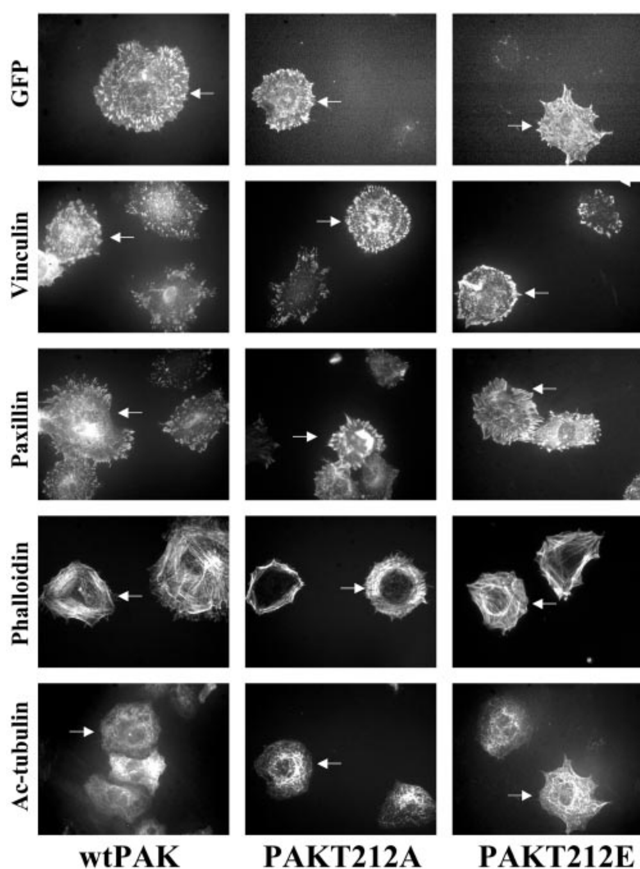

FIG. 10. **PAK1 Thr^{212} phosphorylation does not alter focal adhesion localization, formation, or cell spreading.** A7R5 cells transfected with GFP-PAK, GFP-PAKT212A, or GFP-PAKT212E were trypsinized and plated on fibronectin-coated chamber slides (10 μg/ml) for 1 h as described under "Experimental Procedures." Cells were fixed, permeabilized, and either observed by direct fluorescence (GFP, *top panel*) or stained with anti-vinculin or anti-paxillin antibodies to examine mature and nascent focal adhesions respectively, or phalloidin or anti-acetylated tubulin antibody to examine actin or tubulin polymerization. *Arrows* indicate GFP-PAK1-, GFP-PAK1 T212A-, or GFP-PAK1 T212E-expressing cells identified by direct fluorescence. Data are representative of at least three separate experiments scoring 100–150 transfected cells for each treatment.

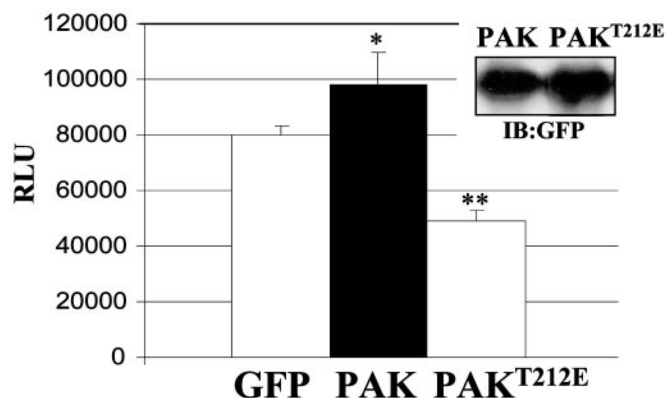

FIG. 11. **Thr^{212} phosphorylation suppresses SRE activation.** A7R5 cells were transfected with an SRE-luciferase reporter plasmid along with either GFP-C1 vector control, GFP-PAK1, or GFP-PAK^{T212E}. 48 h following transfection, cells were lysed and analyzed for luciferase activity as described under "Experimental Procedures." The *inset* shows equivalent expression levels of the GFP-PAK1 and GFP-PAK^{T212E} constructs. Data were normalized to total cellular protein. The graph represents mean ± S.E. of four separate experiments. The *single* and *double asterisks* indicate significant increases or decreases from control GFP-transfected cells, respectively ($p < 0.05$).

blast growth factor-dependent fashion as determined by co-immunoprecipitation. In addition, the authors reported that a GST-ERK1 fusion protein could precipitate exogenously expressed PAK2 from cell lysates, although a direct association between the two proteins has yet to be demonstrated (49).

The location of the ERK2 binding site indicates the likelihood that ERK association with full-length PAK *in vivo* may require preactivation of PAK by Rac and/or Cdc42. Recent structural studies have indicated that the inactive autoinhibited conformation of PAK1 is an asymmetric dimer, whereby the autoinhibitory domain of one molecule associates tightly with the kinase domain of another (34). Binding of GTP-loaded Cdc42 or Rac with the CRIB domain disrupts the dimer, unfolds the protein, and exposes the autoinhibitory domain allowing for subsequent protein-protein interactions (34). Indeed, the association of PAK1 and ERK following cell adhesion mimicked the time course we observed for Rac activation following plating cells on fibronectin (23). Thus, it is possible that recruitment of ERK to PAK may be dependent on integrin-dependent Rac activation.

We also show that adhesion-dependent association of PAK and ERK can provide an additional means of regulation by driving phosphorylation of PAK by ERK. The time course of PAK1 phosphorylation, inhibition of phosphorylation by UO126, and the fact that ERK2 directly phosphorylated PAK1 *in vitro* on the same site that is also phosphorylated after PDGF-BB treatment of SMC all support the possibility that PAK1 is an *in vivo* substrate for ERK. Interestingly, although the data presented in this report are consistent with PAK-Thr^{212} being an *in vivo* target for ERK in SMC, other reports have clearly shown that Thr^{212} is also a target for Cdc2/Cdk5 in cells undergoing mitosis (35–38). Two lines of evidence rule out the possibility that PAK1 is a target for Cdc2/Cdk5 following

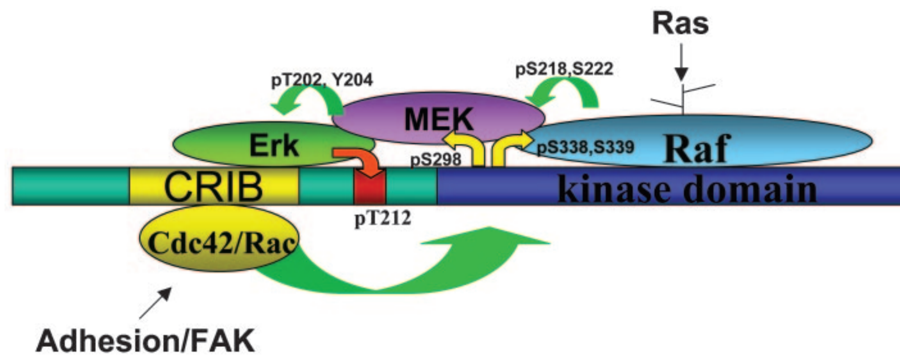

FIG. 12. **Adhesion-dependent PAK-ERK association facilitates ERK signaling.** Numerous extrinsic factors stimulate Ras activity and thereby initiate activation of the canonical Raf/MEK/ERK cascade wherein Raf phosphorylates MEK on serines 218 and 222, then MEK phosphorylates ERK on Thr[202] and Tyr[204] (*green arrows*). Adhesion signaling through FAK activates the smGTPase Rac1, which binds to CRIB domain of PAK1, enabling PAK1 activation. Our studies reveal that activated PAK associates with ERK through N-terminal sequences and past studies reveal that C-terminal sequences of PAK direct an interaction with Raf and mediate phosphorylation of Raf on serines 338 and 339 (*yellow arrow*). Although MEK has not been demonstrated to interact in a direct fashion with PAK, PAK can phosphorylate MEK on Ser[282] (*yellow arrow*) and enhance association between MEK and ERK. Each of these events facilitates ERK-dependent signal transduction. Following growth factor stimulation, ERK phosphorylates PAK on Thr[212] resulting in a negative feedback loop to depress downstream signaling (*red arrow*).

PDGF-BB-treatment of SMC. First, pretreatment of SMC with alsterpaullone (a potent pharmacological inhibitor of Cdc2/Cdk5) had no effect on PDGF-BB-stimulated Thr[212] phosphorylation, whereas treatment with the MEK inhibitor U0126 almost completely inhibited the response. Second, PDGF-BB-treatment of SMC caused a phosphorylation-dependent inactivation of Cdc2, similar to what has been reported following ERK activation in *Xenopus* extracts (50). Thus, at least in the context of adhesion-dependent growth factor signaling, phosphorylation of PAK1 in SMC appears to be directed by ERK and not Cdc2/Cdk5.

Unlike the ERK2 binding sites that are conserved in PAK1–3, Thr[212] is unique to PAK1. This consensus site is however conserved in all mammalian forms of PAK1, indicating that phosphorylation of the site may regulate an important function. Notably, a previous detailed temporal analysis of pThr[212] *in vivo* revealed that high levels were observed in the embryonic forebrain, lung, kidney, intestine, and skin, but that pThr[212] was virtually undetectable in adult tissues (51). In terms of function, previous studies indicate that the kinase activity of the PAK1 variants T212A and T212E were indistinguishable from wild-type PAK1 (36). Previous reports also indicate that Cdc2-mediated pThr[212] altered the rate and extent of postmitotic spreading of murine fibroblasts (37) and regulated microtubule dynamics and overall morphology in neurons (35, 36). We show herein that ectopic expression of PAK-T212A or PAK-T212E in SMC did not alter focal adhesion formation, microtubule formation, or the rate of cell spreading, although the consequence of PAK1 phosphorylation on focal adhesion turnover or directed cell migration has yet to be determined. PAK-T212E did, however, attenuate serum-stimulated ERK-dependent transcription, indicating that phosphorylation of this site might provide a negative feedback inhibition to limit adhesion and growth factor-stimulated ERK signaling. The precise mechanism of this inhibitory response is not yet clear. We have determined that pThr[212] within the context of GST-PAK1-(1–290) does not alter the association of ERK and PAK *in vitro*, indicating that ERK can still bind PAK-pThr[212]. However, Thr[212] is located within the core of a canonical SH3-binding motif, thus it is feasible that phosphorylation of this site could modulate association with a binding partner that could in turn regulate ERK phosphorylation or nuclear translocation. Experiments to address this question are presently underway.

In summary, we have shown that ERK2 binds within the N terminus of PAK1 and when activated by adhesion-dependent growth factor signaling, phosphorylates PAK1 on Thr[212]. Our present data, together with previous reports that illustrate a role for PAK in the regulation of the ERK cascade, indicate that PAK1 may function as a scaffold for this canonical pathway. Why would the ERK pathway require so many scaffolds? One possible explanation may be that scaffolding controls local activation of this seemingly promiscuous pathway. For example, KSR has been shown to shuttle from the cytoplasmic membrane to the plasma membrane following growth factor treatment, and p14, a partner of the putative scaffold, MP1, targets MP1-MEK1 and ERK1 to late endosomes/lysosomes (52–54). In the case of PAK, association of the complex could lead to the targeting of active ERK to focal adhesions. Since association of ERK with PAK may be dependent on adhesion-induced activation of Rac, one might speculate that adhesion-stimulated PAK-dependent targeting of ERK to these dynamic sites might regulate directional lamellipodial extension during cell migration. Notably, studies have shown that ERK activation enhances focal adhesion turnover by enhancing the rate of focal adhesion disassembly (55). This effect could be caused by the ability of ERK to phosphorylate and regulate several focal adhesion proteins such as paxillin, MLCK, and calpain II (56–58). We have also shown that coordinate signaling through adhesion and growth factors regulates phosphorylation of PAK on Thr[212], and provide evidence that this event may decrease subsequent ERK-dependent signaling. It is likely that ERK signaling would have to be tightly regulated in a temporal fashion in order for directed cell migration to occur, thus negative feedback phosphorylations such as Thr[212] may prove important in controlling this dynamic cellular process.

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