MOLECULAR AND CELLULAR BIOLOGY, May 2004, p. 3849–3859 Vol. 24, No. 9
0270-7306/04/$08.00+0 DOI: 10.1128/MCB.24.9.3849–3859.2004

GIT1 Activates p21-Activated Kinase through a Mechanism Independent of p21 Binding

Tsui-Han Loo,[1] Yuen-Wai Ng,[1] Louis Lim,[1,2] and Ed Manser[1]*

GSK-IMCB Group, Institute of Molecular and Cell Biology, Singapore 117609, Singapore,[1] and Institute of Neurology, University College London, London WC1N 1PJ, United Kingdom[2]

Received 5 August 2003/Returned for modification 23 September 2003/Accepted 30 January 2004

p21-activated kinases (PAKs) associate with a guanine nucleotide exchange factor, Pak-interacting exchange factor (PIX), which in turn binds the paxillin-associated adaptor GIT1 that targets the complex to focal adhesions. Here, a detailed structure-function analysis of GIT1 reveals how this multidomain adaptor also participates in activation of PAK. Kinase activation does not occur via Cdc42 or Rac1 GTPase binding to PAK. The ability of GIT1 to stimulate αPAK autophosphorylation requires the participation of the GIT N-terminal Arf-GAP domain but not Arf-GAP activity and involves phosphorylation of PAK at residues common to Cdc42-mediated activation. Thus, the activation of PAK at adhesion complexes involves a complex interplay between the kinase, Rho GTPases and protein partners that provide localization cues.

The Rho family of small GTPases, of which the best studied are Cdc42, Rac, and Rho, plays a crucial role in the cell biology of the actin cytoskeleton as well as intermediate filament and microtubule organization (12). It is well established that cell attachment via integrins leads to rearrangement of the actin cytoskeleton and cell spreading, which is controlled by Cdc42 and Rac. Activation of Cdc42 and Rac promotes the formation of filopodia and lamellipodia, respectively (18). In the last 10 years, numerous effector molecules have been identified for the Rho family GTPases (3). The p21-activated kinase (PAK) was one of the first to be identified and is a serine/threonine kinase that is activated directly by Cdc42-GTP or Rac-GTP (24). PAK has been implicated in the organization of the actin cytoskeleton, microtubule network, and adhesion complexes (reviewed by Bokoch [4]). PAK activation results in a conformational change and autophosphorylation of the protein at several sites (19, 23). The effects of PAK are thought to be controlled in part by regulation of its subcellular localization: for example, when the cells are stimulated by a variety of agents, PAK is targeted and activated at focal adhesions complexes (FCs) and leading-edge membrane ruffles (10, 31). The PAK-interacting exchange factor (PIX) has been implicated in this process since PIX has exchange activity toward Cdc42 and Rac1 but not RhoA in vitro (25). The identification of PIX provides an example of a regulatory protein of the GTPases being operationally coupled to an effector to facilitate the interactions of components of signaling pathways. This complex has also been suggested to provide the link for cross talk between Cdc42 and Rac1 pathways (27).

The PIX proteins in turn associate with G protein-coupled receptor kinase-interacting target (GIT1), an Arf GTPase activating protein (GAP) which can bind paxillin through its C terminus. Paxillin binding is required to localize GIT1 and associated proteins to focal adhesion complexes (38). The mammalian GITs are derived from either of two related genes, GIT1 and GIT2 (30) which encode proteins with conserved Arf GTPase-activating protein (ArfGAP), ankyrin repeat, Spa2 homology and paxillin binding domains. Paxillin is an abundant protein that is tyrosine phosphorylated following integrin stimulation by focal adhesion kinase, a kinase that also binds to GIT1 (38). At least one function of GIT1 family proteins (also known as protein kinase linker, PKL, or APP) is to serve as a link between the PAK, PIX, and FCs; GIT probably functions in other contexts, for example as a synaptic component (35). It is suggested that Arf6 is a target of GIT1, with which it colocalizes on the plasma membrane and on recycling endosomes, where it participates in Rac-mediated membrane dynamics (33).

In this paper, we report that GIT1 in cooperation with PIX can robustly activate αPAK and that this process can occur in the absence of a direct interaction between GIT1 and PIX. This activity requires the ArfGAP domain of GIT1, but does not require the CRIB domain of αPAK that binds to small GTPases such as Rac1 and Cdc42. While overexpression of GIT1 alone can activate αPAK to a limited extent, PIX isoforms are not of themselves capable of αPAK activation. Because PAK activation leads to dissociation of the kinase from the complex (37), this provides a feedback mechanism to control PAK dynamics. Thus, it appears that these PAK partners are poised to provide kinase activation signals in the absence of small GTPase signaling, and points to the importance of studies addressing the temporal and spatial activation of these kinases.

MATERIALS AND METHODS

Materials. A reticulocyte lysate system was obtained from Promega (TNT T7 coupled), and assays were performed using 1 μg of DNA per 25 μl of lysate per the under the manufacturer's conditions. Anti-Flag M2 monoclonal antibody (MAb), anti-Flag MAb M2-Sepharose, and antivinculin hVIN-1 were from Sigma. Antipaxillin MAb and anti-RhoGDI MAb were from Transduction Laboratories. Rabbit anti-hemagglutinin rabbit anti-green fluorescence protein (GFP) were from Zymed. Alexa Fluor 388 goat anti-rabbit immunoglobulin G (IgG) and Alexa Fluor 546 goat anti-mouse IgG were from Molecular Probes (working concentration, 1:200). Rabbit anti-PAK C20 and anti-GIT1 (Santa

* Corresponding author. Mailing address: GSK-IMCB Group, Institute of Molecular and Cell Biology, 30 Medical Dr., Singapore 117609, Singapore. Phone: (65) 6874-6167. Fax: (65) 6774-0742. E-mail: mcbmansr@imcb.a-star.edu.sg.

Cruz sc-13961) and anti-RhoGDI (Transduction Laboratories) were used at 0.5 μg/ml for Western blotting (WB). Anti-phospho-PAK1 antibodies directed towards Ser144, Pak1 Ser199/204, and Pak1 Thre423 (Cell Signaling) were used at a 1:1,000 dilution for WB. The working concentrations for other primary antibodies were 0.5 to 1 μg/ml for Western blotting and 2.5 to 10 μg/ml for immunofluorescence assays. Secondary antibodies used were horseradish peroxidase-conjugated rabbit anti-mouse IgG and goat anti-rabbit IgG and were from Dako (used at 1:4,000).

Mammalian and bacterial expression constructs. Plasmid pXJ-GST-αPAK, pXJ-Flag GIT1, and pXJ-hemagglutinin β1PIX are as described previously (25, 38). Plasmids encoding GIT1 and β1PIX carrying various mutations were constructed using a QuikChange site-directed mutagenesis kit (Stratagene) under the manufacturer's conditions. Each new mutant plasmid was sequenced at the Institute of Molecular and Cell Biology central sequencing facility. GIT deletion mutants were generated using internal restriction sites in the cDNA. The N terminus deletion Δ1-118 (Δ1ArfGAP) corresponded to the HindIII-KpnI fragment, and Δ1-375 (GIT1 C) was cloned as a XhoI-KpnI fragment. The C terminus deletion mutant Δ647-770 was constructed using a BamHI-SmaI fragment from the original GIT1 expression plasmid. For internal deletions, oligonucleotides were designed to amplify corresponding N- and C-terminal fragments of the protein spliced using an introduced unique restriction site. GIT1 lacking the coiled-coil domain lacked residues 424 to 480. The mammalian expression vector allows for in vitro translation using T7 RNA polymerase in rabbit reticulolysate (Promega).

Kinase assays. Each kinase reaction contained 50 μl of Sepharose immobilized with glutathione *S*-transferase (GST)–αPAK or Flag-αPAK, containing 10 μCi of [γ-^{33}P]ATP, 10 μM ATP, and 10 μg of GST-substrate protein in kinase buffer (50 mM HEPES [pH 7.3], 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol, 0.05% Triton X-100). The mixture was incubated at 30°C for 30 min. Samples were resolved by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) and processed for autoradiography. The peptide substrate (fused to GST) consists of two tandem repeats of the sequence GDKRDSMVGAP in which the aspartic acid at position −1 confers specificity towards PAK. The reactions were stopped by adding SDS-PAGE loading buffer and heating for 3 min at 95°C. Proteins were resolved by SDS–9% PAGE and visualized by PhosphorImager analysis (Amersham Biosciences) prior to immunoblotting.

Cell transfection, fractionation, and immunoprecipitation. Relevant cDNAs were cloned in pXJ-Flag, pXJ-Ha or pXJ-GST mammalian expression vectors (23). COS-7 cells at 70 to 80% confluency (60-mm-diameter culture dish) were starved 1 h with serum-free Dulbecco's modified Eagle medium (SF-DMEM) prior to addition of DNA-Lipofectamine (Gibco BRL). For each dish 4 μg of total plasmid DNA (in 200 μl of SF-DMEM) was mixed with 25 μg of Lipofectamine reagent and incubated for 30 min at room temperature. This mix was diluted with 1.6 ml of SF-DMEM and added to the cells (60-mm-diameter dish). After 3 h fetal bovine serum was added to a 1% final concentration. NIH 3T3 cells were transfected using a standard calcium-phosphate precipitate protocol and harvested 40 h afterwards. Transfected cells were harvested by scraping in ice cold cell lysis buffer (500 μl of 50 mM HEPES [pH 7.3], 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 20 mM β-glycerophosphate, 5% glycerol, 1% Triton X-100, 1 mM dithiothreitol, and a protease inhibitor cocktail [Roche]). Cells were broken by 10 passages through a 29-gauge insulin syringe before clarification at 14,000 × *g* for 10 min. Pellets were washed with lysis buffer for 15 min on ice, repelleted, and suspended in appropriate volume of 1× SDS sample loading buffer. For SDS-PAGE samples were heated (3 min at 95°C), run immediately, and transferred to polyvinylidene difluoride membranes for Western blot analysis. For immunoprecipitation 200 μl of lysate was passed through 25 μl of anti-Flag M2 Sepharose, and the beads were washed with 1 ml lysis buffer. For GST-αPAK purification, 400 μl of lysate purified using 50 μl of glutathione agarose (Pharmacia) an the amount of protein recovered quantified by Coomassie staining or using anti-αPAK antibodies.

Immunocytochemistry, microscopy and image processing. HeLa cells were plated on coverslips in 30-mm-diameter culture dishes and grown to 60% confluency. Plasmid DNA (1 μg per 5 μl of Lipofectamine reagent) was mixed in 200 μl of SF-DMEM for 30 min and then diluted to 1 ml for addition to cells. After 2 h, fetal bovine serum (Gibco BRL) was added to 1% final concentration. After 16 h cells were fixed in 3% paraformaldehyde for 5 min and permeabilized (10 min) with phosphate-buffered saline (PBS) containing 0.5% Triton X-100. Primary antibodies were added (100 μl per coverslip in PBS plus Triton X-100). Primary antibodies were incubated 2 h (30°C) on the coverslips. Secondary fluorescent antibodies (Molecular Probes) were incubated for 1 h before washing in PBS containing 0.1% Triton X-100. Secondary antibodies were coupled with Alexa 488 or Alexa 546 dyes (Molecular Probes). For fluorescence observation these secondary antibodies were visualized on a Zeiss Axioplan II microscope using a Plan-Apochromat 63×/1.40 objective, and images were collected on a Coolsnap HQ charge-coupled device.

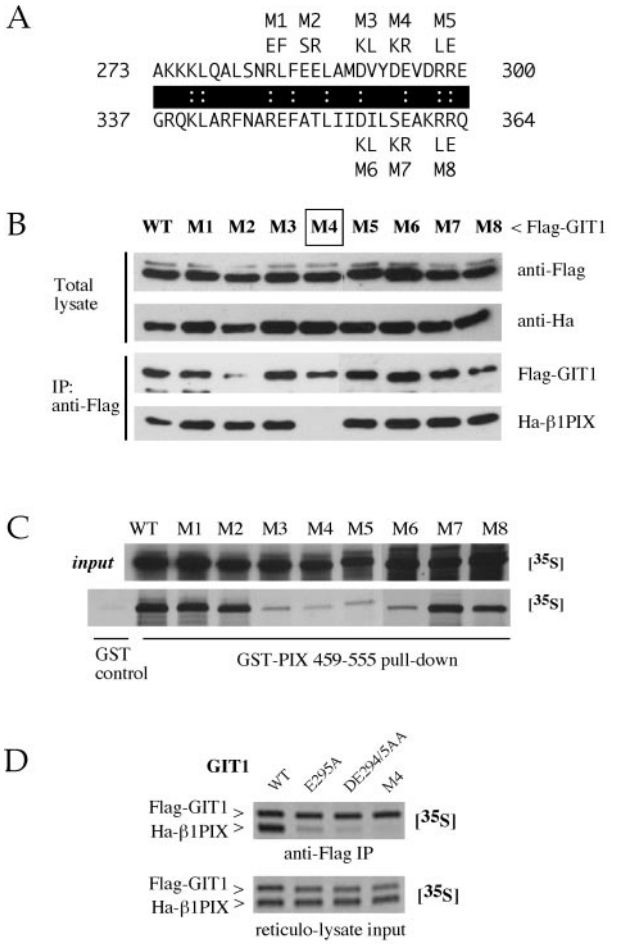

FIG. 1. Mutational analysis of the GIT1 Spa2 homology domain 1. (A) SHD-1 of GIT1 showing residues selected for mutagenesis on the basis of conservation in the yeast Spa2p. The two repeats in GIT1 are aligned: charged residues were selected for mutagenesis on the basis that these are likely to occur to be on the protein surface. Mutants M1 to M8 are as indicated. (B) Testing the effects of SHD-1 mutations on the interaction between Ha-β1PIX and Flag-GIT1 by coexpression in COS-7 cells and immunoprecipitation (IP) as indicated. Of those tested, GIT1(D294K/E295R, M4) severely reduced β1PIX binding (lower panel). (C) The GIT1 mutants expressed by translation in vitro: [^{35}S]-Met labeled proteins (input) were passed through recombinant GST-β1PIX(459-555). Four GIT1 mutants (M3-M6) exhibited decreased β1PIX binding relative to wild type. (D) Analysis of residues substituted in the GIT1 M4: E295 alone or D294/E295 were changed to alanine. In vitro-translated proteins were subjected to immunoprecipitation with tagged β1PIX.

RESULTS

Generating mutants that disrupt the GIT1-PIX interaction. β1PIX binds to GIT1 within residues 254 to 376 (38), which contain two direct repeats homologous to the Spa2 homology domain 1 (SHD-1) present in *Saccharomyces cerevisiae* Spa2-like proteins (Fig. 1A). Conserved residues in the GIT1 repeats were substituted with opposite-charged amino acids (as likely

to be surface residues) and tested for β1PIX binding by coimmunoprecipitation (Fig. 1B). Of the eight mutants generated, only GIT1(D294K/E295R) (M4) did not coprecipitate with β1PIX. M7 with a corresponding mutation in the second repeat retained βPIX binding, suggesting βPIX binding resides primarily in the first repeat. These SHD-1 mutants were also tested for binding to recombinant GST-βPIX(449-555). In this case (Fig. 1C) GIT1 proteins were generated by in vitro translation. This assay appeared to be more sensitive, since several mutants with substitutions in the first SHD-1 repeat (M3, M4, and M5) or second repeat (M6) did not bind well. This may reflect more efficient binding of GIT1 by full-length PIX than the GST-βPIX(449-555) polypeptide.

When the M4 mutant (D294/E295) was further analyzed by replacing the acidic residues with alanine, the D294A and D294A/E295A mutants still displayed some residual ability to coimmunoprecipitate βPIX (Fig. 1D). Thus, GIT1(D294K/E295R) represents a protein that is βPIX-binding defective by coimmunoprecipitation criterion. Whether these residues directly contact βPIX is unclear and requires further study.

We previously showed PIX to be important for the focal adhesion localization of PAK (25). Others subsequently showed that for correct focal adhesion localization of PIX, its interaction with GIT1 interaction is required (6). Unlike wild-type GIT1(Fig. 2A, upper panels), GIT1 M4 was poorly localized to focal adhesions (lower panels), although it had an intact paxillin binding domain (residues 646 to 770). Since the latter domain suffices to target GIT1 to focal adhesions (21), this poor localization of M4 supports our proposal that the PIX/GIT1 interaction drives the paxillin association (38). High-resolution images of GIT1 or PIX costained with paxillin (Fig. 2B) show the distribution of GIT1 and PIX to be asymmetric within focal adhesions, with PIX and GIT1 being enriched proximal to the cell edge side of adhesion complexes (Fig. 2B). By contrast GFP-paxillin and vinculin are perfectly colocalized in the same cell (data not shown). This asymmetry of distribution maybe a useful means of identifying other proteins associate with the GIT1/PIX complex.

GIT1 binds to a βPIX region containing residues 496 to 554 (38), which shares 89% identity in the two mammalian α and β PIX isoforms (16). The corresponding *Drosophila* dPIX region is more divergent (Fig. 3A), although likely to bind dGIT1 in an analogous manner. This N-terminal limit of the GIT1 binding region (13) is boxed; because this region is predicted to adopt an alpha-helical fold, residues I539/E540 were replaced with the "helix breakers" glycine and proline, respectively. This mutant, like Cool-1 V538A (13), failed to coprecipitate with GIT1 (Fig. 2B). The disposition of GFP-PIX(I539P/E540G) in HeLa cells was as expected: while wild-type PIX associated with punctate focal adhesions, the mutant did not (data not shown).

Interaction of GIT1 and βPIX alters their intracellular distribution. We noted that when GIT1 and βPIX were coexpressed in COS-7 cells, a substantial portion of both proteins could not be extracted with 1% Triton X-100. It was also notable that introducing PAK with GIT1 and PIX detergent-insoluble phase (Fig. 3C, fraction marked P). GIT1 partitioned primarily into the soluble (S) fraction, while coexpression of GIT1 and β1PIX shifted both to the pellet (P) fraction (Fig. 3C, lane 6); however, when we used a PIX(I539P/E540G) that cannot bind GIT1, both proteins were predominantly found in the soluble fraction. It was also noted that expression of GIT1 altered the mobility of PAK with the appearance of higher mobility species typical of the active kinase: this was particularly evident in the presence of β1PIX or the β1PIX(I539P/E540G). There was no obvious difference in the relative mobility of the triton-soluble versus triton-insoluble αPAK species. Because of this effect under these conditions we used the PIX(I539P/E540G) mutant for many experiments in which we wished to recover the majority of GST-PAK for further analysis.

GIT1 but not PIX activates αPAK. αPAK is largely inactive when expressed in mammalian cells although the kinase is activated in bacteria through an undefined mechanism (23). Coexpression of GIT1 with αPAK (Fig. 4A) clearly increased PAK activity (lanes 2). GIT1(1-646) lacking the paxillin α-binding domain and the D294K/E295R (M4) mutant were equally effective in increasing αPAK activity, suggesting neither paxillin nor β1PIX-binding was necessary for GIT1-induced activation of αPAK. Since GIT1 could activate PAK, we were interested in whether the PAK partner β1PIX was involved. PIX is characterized by two isoforms (αPIX and βPIX) with the latter exhibiting many alternate spliced versions (33); β1PIX is the predominant form in cultured cells such as HeLa and fibroblast lines (16). It has been reported that αPIX but not β1PIX (Cool-1) activates PAK (13). We therefore tested the studied effects of PIX alone (Fig. 4B): PAK autophosphorylation was assessed by phospho-specific antibodies targeted to known sites (23). In common with many kinases phosphorylation within the activation loop (T422 in αPAK) is critical for catalytic activity towards substrates (7, 34). Unlike GIT1 no full-length isoform of PIX that we tested was capable of driving PAK autophosphorylation.

In order to assess whether GIT1 levels affect the activity of endogenous Pak1 we transfected NIH 3T3 cells (where αPAK is the predominant isoform). Cell lysates were used for anti-αPAK immunoprecipitation and assessment of PAK activity as well as levels of associated PIX and GIT1 (Fig. 5A). Both of these proteins were enriched to a similar level to αPAK in the immunoprecipitate relative to input (lane 1) indicating a significant proportion of cellular αPAK is part of the trimeric complex. Both wild-type GIT1 and GIT1(1-504), which cannot associate with adhesion complexes, caused an increase in αPAK activity relative to the mock-transfected control (lane 2 and 3). Increasing the level of GIT1 increased the yield of PAK-associated GIT1 (lane 4), but the increased activity does not appear to result from adhesion complex targeting per se since GIT1(1-504) had a similar effect.

The effect of GIT1 was also tested in combination with either αPIX or βPIX (Fig. 5B lanes 5 and 6). Expression plasmids were transfected and total cell lysates then probed with antibodies against GIT1, PIX and PAK, as well as phospho-specific antibodies to the pS198/203 and T422 sites on αPAK. The exogenous levels were estimated as ~18-fold higher for GIT1 and ~12-fold higher for βPIX based on densitometry of the Western blot bands (top panels). Hyper-phosphorylated forms of PAK are evident from the anti-PAK Western blot: inclusion of the two PIX isoforms caused a more complete shift in αPAK and some increase in pS198 relative to that with GIT1 alone.

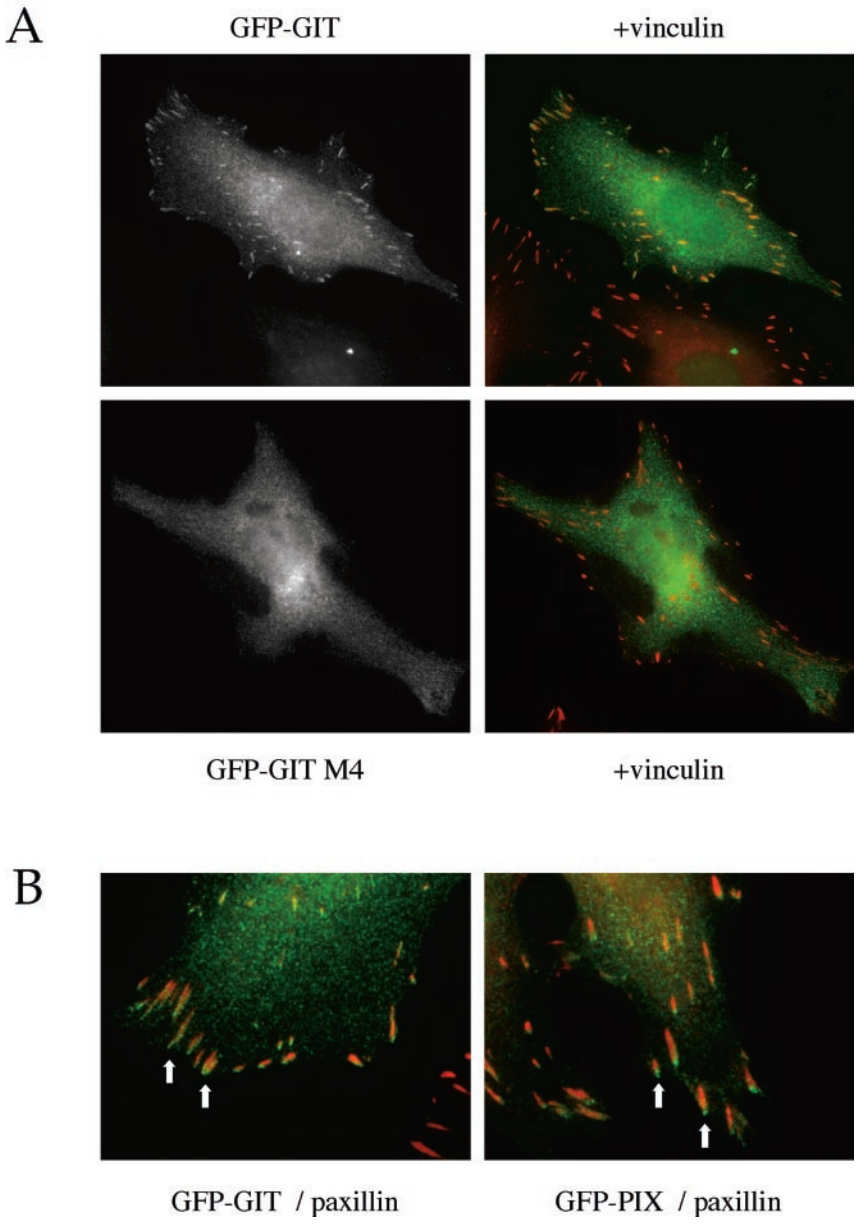

FIG. 2. GIT1 localization to focal adhesions requires interaction with PIX. (A) Constructs encoding GFP fused to GIT1 were transiently transfected to HeLa cells and processed for costaining with antivinculin. GIT M4 stained poorly at adhesion complexes. The wild-type GFP-GIT1 protein was apparent in those regions of focal adhesions distal to the stress fibers. Bar = 10 μM. (B) Plasmids encoding GFP-βPIX or GFP-GIT1 were transfected into HeLa cells; 16 h later cells were fixed and stained for rabbit anti-GFP and mouse antipaxillin (red). Arrows indicate the asymmetric localization of PIX and GIT1 in paxillin containing adhesion complexes.

The ArfGAP domain of GIT1 is required for PAK activation. To further analyze PAK activation by GIT1 while avoiding translocation of PAK to the Triton X-100 insoluble fraction (Fig. 3), the β1PIX I539P/E540G was used since this mutant complexes to PAK (via its SH3 domain) and remains as a homodimer (via the coiled-coil C-terminal domain) but behaves similarly to wild-type PIX terms of kinase activation when expressed with GIT1. Analysis was performed using transient transfection of plasmids encoding GST-αPAK, Flag-GIT1, and Ha-β1PIX. GIT1 expression induced αPAK autophosphorylation at residues S144 and S198/203 more robustly than for T422. However GIT1 constructs alone did result in significant phosphorylation at T422 with longer exposure times (Fig. 5B). Various GIT1 constructs (C1 to C5) were used to assess the GIT region required to cause PAK autophosphorylation. The GIT1 leucine zipper deletion mutant (C2) is expected to remain monomeric (15, 28) but was reasonably active in terms of its effect on αPAK. Notably, constructs encoding GIT1 N terminus deletion mutants (C3 and C4) that likely affect its interaction with Arfs failed to activate αPAK. The deletion of amino acids 1 to 118 removes the ArfGAP domain (schematically shown in Fig. 6). The amount of β1PIX copu-

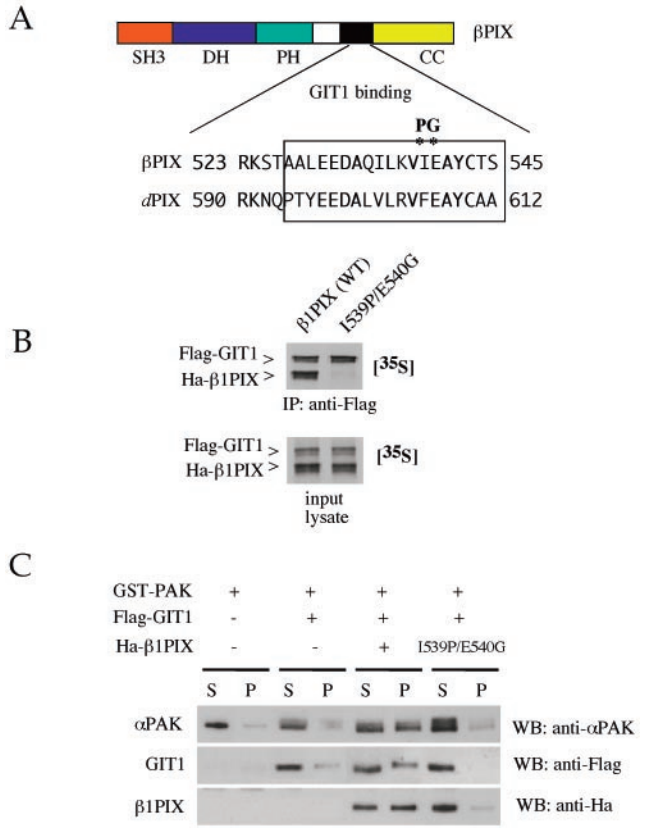

FIG. 3. Generation of β1PIX defective for GIT1 binding. (A) Schematic figure showing the location of the various protein domains in βPIX. Rat β1PIX sequences within the GIT1-binding domain are aligned to *Drosophila* dPIX sequences to identify conserved residues (in boldface type). The isoleucine 539 and glutamate 540 residues (asterisk) were selected for substitution with proline and glycine, respectively. (B) Plasmids encoding Flag-GIT1, Ha-β1PIX, and Ha-β1PIX(I539P/E540G) were expressed by in vitro translation with [^{35}S]methionine and tested for coimmunoprecipitation using anti-Flag M2 beads. Note that the wild-type protein coprecipitates very efficiently under these conditions, while the PIX mutant does not. (C) Plasmids encoding Flag-GIT1 and Ha-β1PIX or Ha-β1PIX (I539P/E540G) were coexpressed with GST-PAK in COS-7 cells and lysed with a buffer containing 1% Triton X-100. Lysates were processes as described the methods section to yield the detergent soluble (S) fraction. The pellet was washed and reextracted with 2% SDS loading buffer (P). Equal proportions of the S and P fractions were resolved by SDS–9% polyacrylamide gel electrophoresis for Western blot analysis. Both αPAK and GIT1 were predominantly located in the S fraction (lane 1 and 3); however, coexpression of GIT1 with β1PIX resulted in a shift to the pellet (lanes 5 and 6). The β1PIX(I539P/E540G) mutant when expressed with GIT1 is, by contrast, Triton X-100 soluble (lane 8).

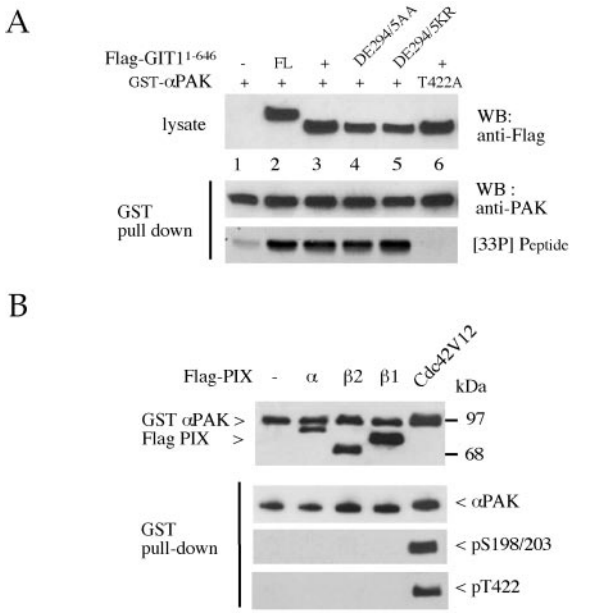

FIG. 4. GIT1 but not PIX promotes αPAK activation. (A) Full-length Flag-GIT1 (WT) or GIT1(1-646) was coexpressed with GST-αPAK or a kinase-inactive αPAK (T422A). Purified GST-αPAK activity recovered on glutathione-Sepharose (GST pull-down) was determined with [γ-^{33}P]ATP and 10 μg of substrate as described in the methods section. The substrate contains two tandem repeats of the peptide GDKRDSMVGAP. Coexpression of GIT1 increased the activity of αPAK independent PIX binding (i.e., D294/E295 mutants) or the C-terminal paxillin binding region (residues 647 to 770). (B) Full-length (FL) isoforms of αPIX, β1PIX, and β2PIX were cotransfected with GST-αPAK. As control Cdc42(G12V) was cotransfected (last lane). GST-PAK was pulled down and incubated with 10 μM ATP (15 min, 30°C) to allow additional in vitro phosphorylation. The PIX expression is not associated with increased phospho-PAK levels.

rified with αPAK (as shown) did not significantly vary with the activation status of αPAK (cf. lane 3 versus lane 9). In subsequent pull-down experiments, we often used GIT1(1-504) as an activator since it was well separated from the PIX (~78 kDa) and GST-PAK (~95 kDa).

The GIT1 ArfGAP domain is essential to activate αPAK. Since a critical region for αPAK activation lay within residues 1 to 117, two mutants were generated in a GIT1(1-504) background to address the role of the ArfGAP domain (Fig. 7A). The C11/14G mutant (changing the Zn^{2+} coordinating cysteines) which disrupts the zinc finger is expected to cause localized protein misfolding, while the R39K mutant is anticipated to abolish ArfGAP activity only (22). The R39K mutant appeared to be as effective as the wild type in promoting PAK autophosphorylation (Fig. 7B), while the GIT1(C11/14G) mutant was not. Thus, the ability of GIT1 to drive PAK autophosphorylation in the presence of PIX depends on the structural integrity of the Arf GAP domain but does not require GAP activity per se.

The physiological target of the GIT1 ArfGAP domain has been suggested to be Arf6 (33). The dominant inhibitory Arf6(T27N) was therefore tested (Fig. 7B right panel). This Arf6 mutant had a minor effect on GIT1 induced PAK autophosphorylation (compare lanes 2 and 3) but the PAK doublet remains clearly visible. A constitutively active Arf6 Q67L mutant similarly produced a minor inhibition. Both of these might arise from binding competition between Arf6 and another protein factor(s) interacting with the N terminus of GIT1. Direct binding between Arf6 and GIT1 has been detected by coimmunoprecipitation (data not shown). In any case it seems unlikely that Arf6 is the mediator in GIT1-induced PAK activation since neither alone, nor in combination with GIT1, could active or dominant inhibitory Arf6 enhance αPAK autophosphorylation.

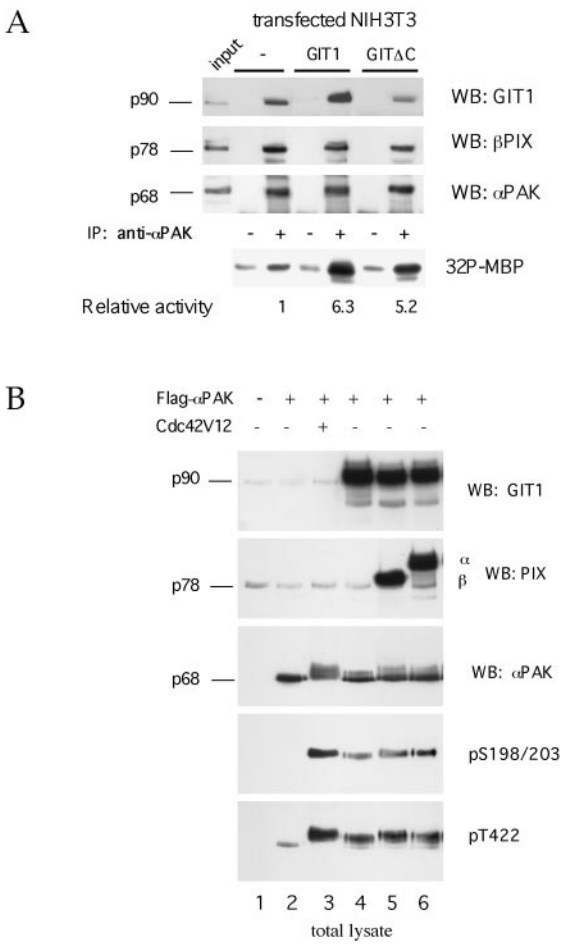

FIG. 5. GIT1 drives autophosphorylation and activates endogenous αPAK. (A) NIH 3T3 cells were transfected with Flag-GIT1 or Flag GIT1(1-504) [GIT1ΔC]. The cell lysate (lane 1) indicates endogenous proteins detected in 50 μg of total protein. For immunoprecipitation (IP), αPAK was recovered from 250 μg of total cell lysate and assayed for activity towards myelin basic protein (MBP) in the presence of 10 μM ATP containing [γ-^{32}P]ATP. In control lanes (left side of each pair) the equivalent volumes of lysates were incubated with protein A-Sepharose in the absence of antibody and nonspecific bound material subjected to identical kinase assays. Phosphorylation levels were determined on a PhosphorImager (Molecular Dynamics); in each case the nonspecific phosphorylation level was subtracted. GIT1 produced a sixfold enhancement of activity as indicated at the bottom of the figure. (B) COS-7 cells were transfected with GIT1 alone or in the presence of α- or β1PIX. The level of Flag-GIT1 or PIX was compared to those of the endogenous proteins by probing total cell lysates with anti-GIT1 (top panel) or anti-PIX (SH3) antibodies. The levels of PAK and (active) phosphorylated forms of the kinase were assessed by antibodies directed to the Ser198/203 or Thr422 as indicated. As a control Flag-Cdc42(G12V) was cotransfected with αPAK (lane 3). Both endogenous GIT1 (p90) and PIX (p78) are seen at the expected positions; however, αPAK is not detected in COS7 cells. The mobility shift of transfected αPAK was more complete (relative to Cdc42) in the presence of both GIT1 and PIX.

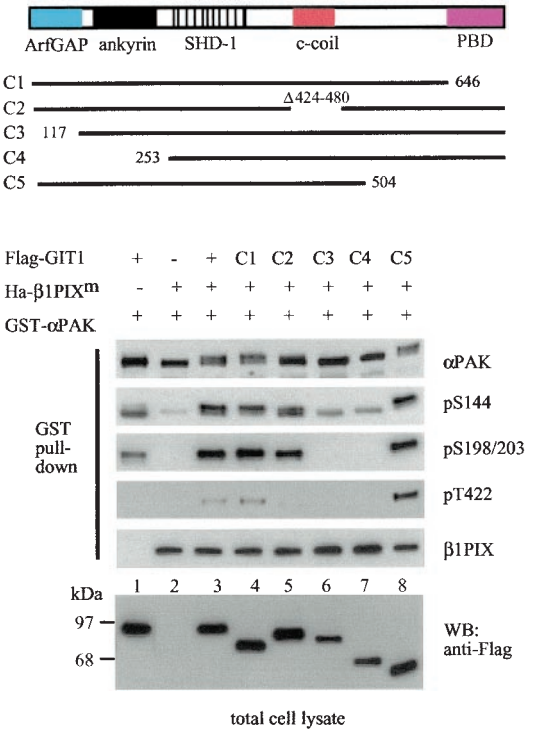

FIG. 6. Regions of GIT1 involved in PAK activation. Various constructs as shown in the schematic of the domain structure of GIT1 indicates the truncation of rat GIT1 used. The GST-αPAK was purified (GST pull-down) and probed with anti-PAK or phosphorylation-specific antibodies against sites present in rat αPAK (abbreviated as pS144, pS198/203, and pT422). Equal exposure times for each phospho-specific antibody are shown. GIT1 causes kinase autophosphorylation (lane 1). Wild-type PIX (Fig. 5) and non-GIT1 binding PIX(I539P/E540G) synergized with GIT1 to activate PAK (lane 3). Constructs of GIT1 encoding N-terminal deletions (lanes 7 and 8) were ineffective. Removal of the dimerization domain (C2) only modestly affected PAK activation (compared with full-length. The C-terminal truncation mutants (C1 and C5) were as effective as wild-type GIT1. Abbreviations: c-coil, coiled coil; PBD, protein-binding domain.

αPAK activation need not require Cdc42 and Rac1 binding. Because β1PIX is an activator of Rac1 (25), we next examined whether GIT1/PIX acted on αPAK via Cdc42 or Rac1 binding through the regulatory domain. The experiments involved cotransfection of dominant-inhibitory mutants (T17N) of Cdc42 and Rac1, or use of a PAK mutant that cannot interact with these GTPases (Fig. 8). We show that neither T17N GTPase mutant blocked αPAK activation by GIT1/PIX as assessed by T422 phosphorylation (Fig. 8A, left panel). Most surprisingly, αPAK(S76P), which was nonresponsive to Cdc42G12V (Fig. 8A, right panel, lane 4), underwent robust autophosphorylation when cotransfected with GIT and PIX (last lane), indicating the activation mechanism is unlikely to involve any GTPase of the Rho family.

The αPAK N terminus contains the binding site for the second SH3 domain of Nck (2, 5, 14, 37). As shown in Fig. 8B, a deletion construct (αPAK ΔN22) lacking the Nck-binding site was similarly activated (as assessed by mobility shift) as for Cdc42-mediated activation. The mechanism of GIT1 activation is conserved among PAK isoforms, since βPAK behaved in an identical manner to αPAK. It seems likely that the three conventional PAK1/2/3 isoforms can be activated via associated PIX and GIT.

Activation of PAK at adhesion complexes. Because transient expression experiments were performed 16 to 20 h after DNA addition there remained is issue of whether GIT1 could lead to

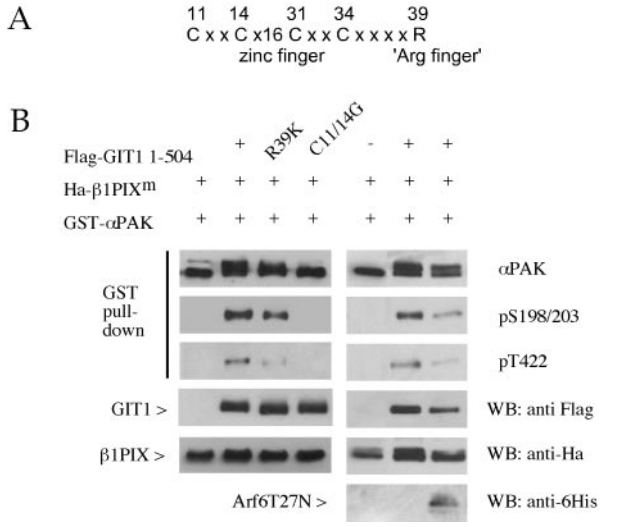

FIG. 7. The GIT1 Arf GAP domain is critical for αPAK activation. (A) Position of GIT1 residues involved in the zinc coordination or catalytic activity via the arginine finger. The mutant GIT1(C11/14G) fails to coordinate zinc with disruption to the ArfGAP domain, while GIT1(R39K) should abolish the ArfGAP activity of the protein without affecting the protein fold. (B) PAK autophosphorylation in response to coexpression of GIT1 mutants. GIT1 (R39K) was similar to the wild type in its effect on PAK (lane 3), while a C11/14G mutant failed to activate αPAK (lane 4). The right-hand panel shows the effect of a dominant inhibitory Arf6(T27N) expression plasmid on αPAK activation by GIT1. Inhibition of Arf6 had little effect on the relative amount of mobility shifted PAK.

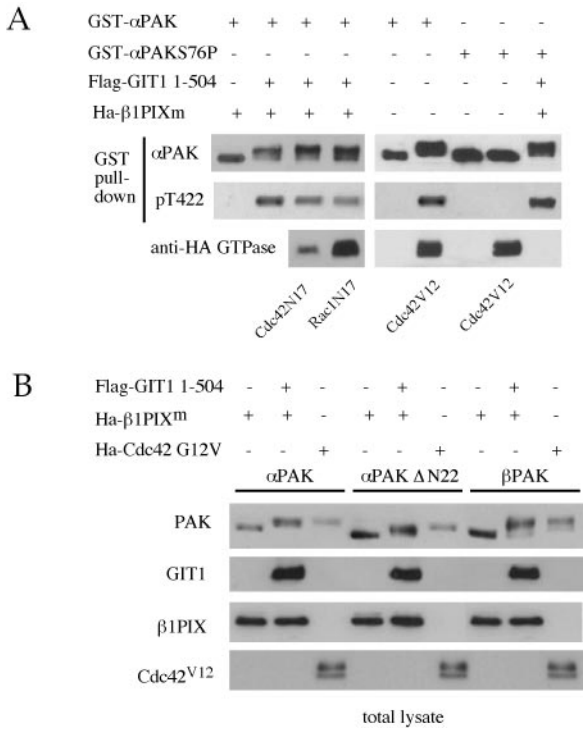

FIG. 8. PAK activation by GIT1 does not require Cdc42 or Rac1 binding. (A) αPAK activation was assessed in the presence of dominant inhibitory (T17N) versions of Cdc42 or Rac1 following cotransfection of expression plasmids in COS7 cells. Neither GTPase effected the level of phospho-PAK. In the right-hand panel, αPAK(S76P), which cannot bind Cdc42.GTP (36), is compared with wild-type kinase. This mutant is not activated by Cdc42(G12V) in vivo (lane 4). However, activation by GIT1/β1PIX was to the same extent as for wild-type αPAK (right lane). (B) αPAK or βPAK was tested for activation by GIT1 or Cdc42(G12V). Activation can be observed as assessed by the shift in mobility that accompanies autophosphorylation.

acute activation of αPAK. To assess this we microinjected NIH 3T3 cells with plasmids encoding Cdc42V12 or GIT1 (Fig. 9A, upper and lower panels). After 2 h autophosphorylated kinase was detected at adhesion complexes (Fig. 9A), which are predominantly peripheral with Cdc42. The neither anti-pT422 nor anti-pS144 antibody detected the active PAK after cell fixation.

Although GIT and PIX associate with adhesions complexes (Fig. 2), we have previously shown that PAK association is transient and but readily detected by inhibition of the kinase. We therefore reasoned that if the association of PAK with PIX/GIT at focal adhesions can promote kinase activation, then preventing the latter should maintain αPAK at these sites. We then looked at the disposition of αPAK in the presence of GIT1(C11/14G), which is defective for PAK activation (Fig. 6). Cells expressing low levels of GFP-PAK and this GIT1 mutant indeed showed clear focal adhesion localization (Fig. 9B). This was comparable with the localization of PAK when coexpressed with the kinase-inhibitory domain (KID) (right panel). These experiments explain why we fail to detect stable association of αPAK with PIX in RhoA-dependent adhesion complexes: although these structures may not contain active Cdc42 or Rac1 the GIT/PIX complex of itself drives activation which leads to the consequential loss of the αPAK.

Concentration-dependent autoactivation of PAK. Since the CRIB region is not implicated in PAK activation, we sought other conserved regions in surrounding sequences that might play a role in disinhibiting PAK in the presence of GIT1. A crystal structure of the autoinhibited catalytic domain of αPAK unfortunately does not include residues 1 to 69 and 150 to 248 (19), which may play additional roles in kinase activation. However, various strategies to identify the residues involved in the GIT1-mediated PAK activation failed. During these experiments, we uncovered a property of αPAK that could provide the means for its activation in vivo. When PAK is immobilized at higher concentration and incubated with Mg^{2+}-ATP it is able to undergo a GTPase-independent activation. As shown in Fig. 10A, over a relatively modest concentration range (from 0.5 to 2.5 μM) the protein behaves dramatically differently with respect to autoactivation. These samples were normalized with respect to protein loading per lane after elution from the matrix (lower panel). In Fig. 10B the CRIB mutant S76P PAK demonstrated the same behavior but is refractory to Cdc42 activation at the lower concentration. Thus, a local recruitment of PAK may predispose the kinase to activation in vivo. This effect can explain why membrane-targeted PAK is active (20) as well as the generation of predominantly active kinase in *Escherichia coli* (23).

DISCUSSION

The targeting of protein kinases to their site of action often requires accessory factors such as protein scaffolds, as exem-

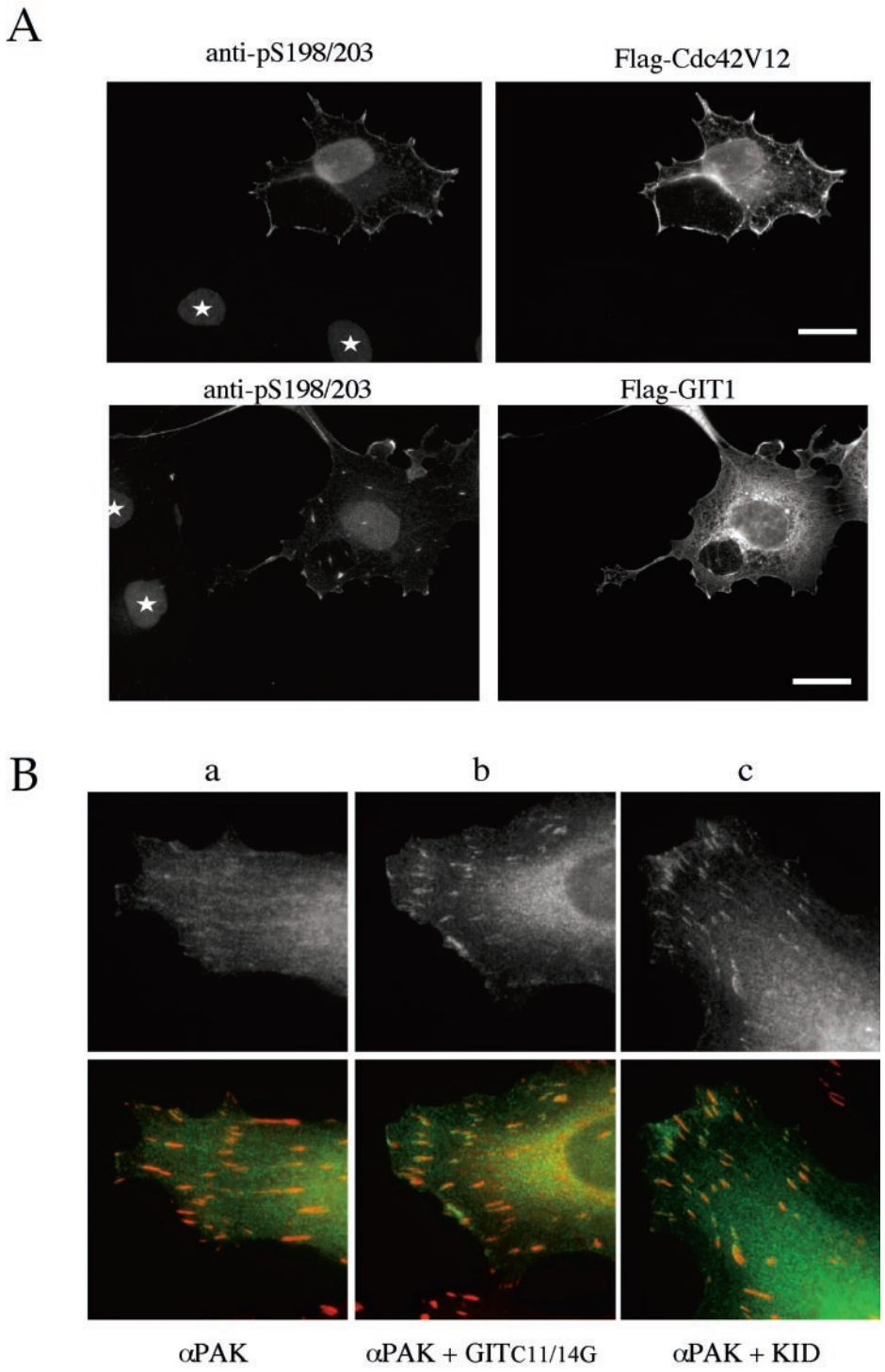

FIG. 9. GIT1 promotes activation of PAK at focal adhesions. (A) Plasmids encoding Flag-Cdc42V12 or Flag-GIT1 were microinjected into nuclei of NIH 3T3 cells and incubated for 2 h before fixation. Expression of the Flag epitope (right-hand panels) allowed location of injected cells. Anti-phosphorylated endogenous PAKs were detected by anti phospho-S198/203 staining: in both cases the signal was detected predominantly at punctate structures that were identified in other experiments as focal adhesion complexes. Noninjected cell nuclei are indicated by stars. Bar = 10 μM. (B) The top panels show the disposition of GFP-αPAK as detected with anti-GFP antibodies in the presence of GIT1(C11/14G) or αPAK(83-148) KID. Both lead to an accumulation of the αPAK at focal adhesion complexes marked by vinculin (red channel) relative to the control (left panel).

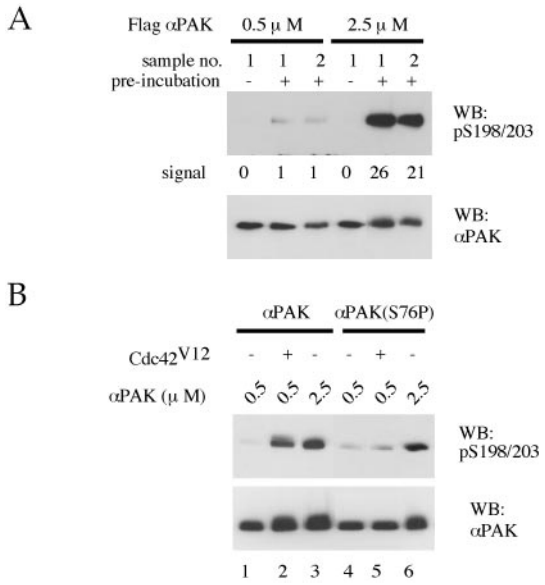

FIG. 10. Concentration-dependent autoactivation αPAK. (A) Flag-αPAK at 0.5 or 2.5 μM as indicated was immobilized on 20 μl of anti-Flag M2 beads (corresponding to 10 or 50 μl of COS7 total cell lysates from two different experiments as indicated). The beads were incubated with 200 μM ATP in a solution containing 50 mM HEPES (pH 7.3), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.05% Triton X-100 for 15 min at 37°C, and the reaction was quenched by adding SDS-PAGE sample buffer. Samples were diluted to the same final concentration of αPAK (40 or 200 μl, respectively) for Western blot analysis with anti-phospho-S198/203 or anti-αPAK as shown. The concentration of αPAK was determined by Coomassie blue staining relative to bovine serum albumin standard. The increased immunoreactivity at higher protein concentration was quantified (as indicated) by densitometry of the bands: anti-phospho S198/203 signal at 0.5 μM αPAK is defined as 1. (B) PAK autophosphorylation does not require intact CRIB sequence. Different concentrations of Flag-αPAK or Flag-αPAK(S76P) were immobilized and allowed to undergo autophosphorylation under conditions described for A. To assess Cdc42-mediated activation, recombinant GST-Cdc42V12 (1 μM) was added with the same ATP-containing buffer and incubated as described for panel A (15 min at 37°C). Reactions were quenched by adding SDS-PAGE sample buffer. Note the appearance of lower-mobility species in parallel with anti-phosphoS198/203 signal.

plified by the large family of AKAPs that target protein kinase A. For PAKs, it seems clear that PIX provides an important targeting cue, and indeed the proline-rich region of PAK that binds the PIX SH3 domain is nonconventional and probably cannot interact with other SH3 domains (37). PIX in turn is complexed to the ArfGAP scaffolding protein GIT1 (cf. Fig. 3), whose avian forms are referred to as protein kinase linker (PKL) (32) or p95-APP1/2 (11). A key question is what influence do these proteins have on the activation status of PAK? Intuitively, PIX as a Cdc42 and Rac1 guanine nucleotide exchange factor suggests itself as an activator of PAK. Nonetheless, this has not been convincingly shown, although in the context of neuronal cells PAK can drive Rac1 activation via PIX (27). The PIX dbl-homology domain may rather provide a means of recruiting PIX to Cdc42 type adhesions. To experimentally assess the role of these interacting protein partners, various point mutants and deletions have been generated. We show that substitution of D294K/E295R in GIT1 both severely affects β1PIX binding (Fig. 1) and leads to a loss of focal adhesion targeting (Fig. 2) but does not affect the ability of GIT1 to activate PAK (Fig. 4).

The targeting of GIT1-β1PIX to focal complexes is likely to be intimately linked to αPAK kinase activation since β1PIX recruits PAK to focal complexes (25). In a different context, *Drosophila* dPak was found to be mislocalized at synapses in dPIX mutant flies (29). By contrast, dPIX was localized normally in Pak mutant strains underlining that the kinase requires its partner dPIX to localize correctly. Interestingly Rac1-PAK signaling is regulated in a spatial manner; Rac1-GTP only couples to PAK on the plasma membrane in adherent cells (9). In this case, it appears the sequestration of active Rac1 by RhoGDI at internal sites prevents interaction with effectors such as PAK but the mechanism of integrin-mediated translocation of PAK has not been established.

Domains involved in GIT1/β1PIX targeting to focal complexes. We and others have observed that GIT1 and β1PIX proteins are enriched in focal complexes induced by Rac1 or Cdc42 (6). In RhoA-type focal adhesions, the PAK-associated complex of GIT and PIX appears to be destabilized by the activity of PAK (6) which is blocked by the KID of PAK. In transfection experiments, only inactive kinase was found to be enriched in these structures (23). In most cultured cells, integrin-dependent focal adhesions are predominantly of the RhoA-type and unaffected by introduction of dominant inhibitory Rac1 or Cdc42. The point mutants of GIT1 and β1PIX that prevent their mutual association enable us to probe the role of this interaction. Neither GIT1(D294K/E295R) nor β1PIX(I539P/E540G) localizes significantly to FCs. However, even if incapable of associating with FCs, both mutants would be expected to oligomerize with the endogenous wild-type protein, allowing (weaker) indirect FC localization. That GIT1(D294K/E295R) is so poorly FC-localized indicates β1PIX provides an input to allow association with focal adhesions: one possibility is the up-regulation of GIT1-paxillin α interaction by β1PIX (38).

Aside from the presence of GIT1 at adhesion complexes the protein may reside in a specific endo-membrane compartment (26) or form insoluble cytoplasmic protein complexes (21). It is not clear at this stage what protein or lipid interaction underlies this localization, and is the subject of ongoing studies.

PIX and GIT1 as mediators of αPAK activation. Previous studies of PAK activation in vivo have focused on PIX, as it was shown that β1PIX promoted GTP loading and activation of Rac1 (25). Since Cdc42(G12V/Y40C), which lacks an ability to bind and activate αPAK, was synergistic with β1PIX in αPAK activation, a model was proposed whereby β1PIX recruits αPAK for activation by Rho GTPases such as Rac1 (generated by guanine nucleotide exchange factor activity of β1PIX). A truncated form of βPIX (residues 155 to 545) apparently activated PAK1 whereas full-length βPIX had no effect (8), which is line with our data (Fig. 6). These observations are complicated by the fact that PIX can also recruit negative regulators such as the type 2C phosphatase POPX (17). We have looked for the interaction between GIT1 and a variety of other protein phosphatases (data not shown), since GIT1 might activate PAK by sequestering a different phosphatase. Although we demonstrate no requirement for direct interaction between GIT1 and PIX to drive this PAK activation, it is

clear in vivo that it is their mutual interaction that leads to accumulation of both proteins at adhesion complexes (cf. Fig. 2). Our data provide two important insights: firstly that microenvironments in the cell where PIX and GIT1 accumulate are sites of PAK activation regardless of Rho GTPase status: secondly that elevated levels of GIT1 or increased association of PAK with the GIT/PIX complex in cells is likely to be associated with increased kinase activity.

There has been no previous report that GIT1 increases αPAK activity; GIT1 (CAT1) and GIT2 (CAT2) being reported not to affect Pak3 (βPAK) activity (1). Full-length GIT1 has similar effects on PAK as the C-terminal truncated GIT1(1-504) (Fig. 6). Surprisingly, the ArfGAP domain of GIT1 plays a key role in activation of PAK, with structural integrity of this domain rather than GAP activity being important. GIT1 has potential as an Arf6 effector signaling to αPAK; however we have been unable to enhance the GIT activation of PAK with active Arf6 (data not shown).

Three lines of evidence indicate that GIT1's effect on αPAK is independent of the binding of GTPases such as Cdc42 or Rac. Firstly, αPAK activation was not driven by βPIX alone (i.e., Rac1 activator); secondly, dominant inhibitory Cdc42 or Rac1 mutants failed to block the GIT1 effect; thirdly and most telling, the αPAK(S76P) mutant which cannot bind the small GTPases was activated by the PIX-GIT1 combination. This activation mechanism was tested for two rat isoforms, α- and βPAK (i.e., equivalent to human PAK1 and PAK3), and did not require the N terminus that binds to Nck.

The translocation of cytoplasmic proteins to new sites of action is a common theme in signal transduction. Such mechanisms are likely to increase the local concentration of proteins and facilitate interaction with targets. What is interesting about the autoactivation of PAK is that the kinase has already been shown to exist as an autoinhibited dimer (19); if dimer "breathing" were a mechanism that allowed autoactivation, then increasing PAK concentration should counter this tendency. PAK has been shown to be activated by a variety of lipids in vitro, including sphingosine and phosphatidic acid (5, 7); thus, these are candidate intermediaries for the GIT1/PIX effect, but to date we have been unable to derive PAK mutants that are lipid insensitive.

In conclusion, our data suggest a model in which the GIT1/PIX complex serves as a docking site for PAK at adhesion complexes which leads to the activation of PAKs regardless of the local activation status of Cdc42 or Rac1. They also indicate that PAK activity is dependent on physiological levels of GIT1 and PIX.

ACKNOWLEDGMENT

This work was supported by the Glaxo Singapore Research Fund.

REFERENCES

1. **Bagrodia, S., D. Bailey, Z. Lenard, M. Hart, J. L. Guan, R. T. Premont, S. J. Taylor, and R. A. Cerione.** 1999. A tyrosine-phosphorylated protein that binds to an important regulatory region on the cool family of p21-activated kinase-binding proteins. J. Biol. Chem. **274:**22393–22400.
2. **Bagrodia, S., S. J. Taylor, C. L. Creasy, J. Chernoff, and R. A. Cerione.** 1995. Identification of a mouse p21Cdc42/Rac activated kinase. J. Biol. Chem. **270:**22731–22737.
3. **Bishop, A. L., and A. Hall.** 2000. Rho GTPases and their effector proteins. Biochem. J. **348:**241–455.
4. **Bokoch, G. M.** 2003. Biology of the p21-activated kinases. Annu. Rev. Biochem. **72:**743–781.
5. **Bokoch, G. M., Y. Wang, B. P. Bohl, M. A. Sells, L. A. Quilliam, and U. G. Knaus.** 1996. Interaction of the Nck adapter protein with p21-activated kinase (PAK1). J. Biol. Chem. **271:**25746–25749.
6. **Brown, M. C., K. A. West, and C. E. Turner.** 2002. Paxillin-dependent paxillin kinase linker and p21-activated kinase localization to focal adhesions involves a multistep activation pathway. Mol. Biol. Cell. **13:**1550–1565.
7. **Chong, C., L. Tan, L. Lim, and E. Manser.** 2001. The mechanism of pak activation. autophosphorylation events in both regulatory and kinase domains control activity. J. Biol. Chem. **276:**17347–17353.
8. **Daniels, R. H., F. T. Zenke, and G. M. Bokoch.** 1999. AlphaPix stimulates p21-activated kinase activity through exchange factor-dependent and -independent mechanisms. J. Biol. Chem. **274:**6047–6050.
9. **del Pozo, M. A., L. S. Price, N. B. Alderson, X. D. Ren, and M. A. Schwartz.** 2000. Adhesion to the extracellular matrix regulates the coupling of the small GTPase Rac to its effector PAK. EMBO J. **19:**2008–2014.
10. **Dharmawardhane, S., A. Schurmann, M. A. Sells, J. Chernoff, S. L. Schmid, and G. M. Bokoch.** 2000. Regulation of macropinocytosis by p21-activated kinase-1. Mol. Biol. Cell **11:**3341–3352.
11. **Di Cesare, A., S. Paris, C. Albertinazzi, S. Dariozzi, J. Andersen, M. Mann, R. Longhi, and I de Curtis.** 2000. p95-APP1 links membrane transport to Rac-mediated reorganization of actin. Nat. Cell Biol. **2:**521–530.
12. **Etienne-Manneville, S., and A. Hall.** 2002. Rho GTPases in cell biology. Nature **420:**629–635.
13. **Feng, Q., J. G. Albeck, R. A. Cerione, and W. Yang.** 2002. Regulation of the Cool/Pix proteins: key binding partners of the Cdc42/Rac targets, the p21-activated kinases. J. Biol. Chem. **277:**5644–5650.
14. **Galisteo, M. L., J. Chernoff, Y. C. Su, E. Y. Skolnik, and J. Schlessinger.** 1996. The adaptor protein Nck links receptor tyrosine kinases with the serine-threonine kinase Pak1. J. Biol. Chem. **271:**20997–21000.
15. **Kim, S., J. Ko, H. Shin, J. R. Lee, C. Lim, J. H. Han, W. D. Altrock, C. C. Garner, E. D. Gundelfinger, R. T. Premont, B. K. Kaang, and E. Kim.** 2003. The GIT family of proteins forms multimers and associates with the presynaptic cytomatrix protein Piccolo. J. Biol. Chem. **278:**6291–6300.
16. **Koh, C. G., E. Manser, Z. S. Zhao, C. P. Ng, and L. Lim.** 2001. Beta1PIX, the PAK-interacting exchange factor, requires localization via a coiled-coil region to promote microvillus-like structures and membrane ruffles. J. Cell Sci. **114:**4239–4251.
17. **Koh, C. G., E. J. Tan, E. Manser, and L. Lim.** 2002. The p21-activated kinase PAK is negatively regulated by POPX1 and POPX2, a pair of serine/threonine phosphatases of the PP2C family. Curr. Biol. **12:**317–321.
18. **Kozma, R., S. Ahmed, A. Best, and L. Lim.** 1995. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol. Cell. Biol. **15:**1942–1952.
19. **Lei, M., W. Lu, W. Meng, M. C. Parrini, M. J. Eck, B. J. Mayer, and S. C. Harrison.** 2000. Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch. Cell **102:**387–397.
20. **Lu, W., S. Katz, R. Gupta, and B. J. Mayer.** 1997. Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck. Curr. Biol. **7:**85–94.
21. **Manabe, R.-I., M. Kovalenko, D. J. Webb, and A. R. Horwitz.** 2002. GIT1 functions in a motile, multi-molecular signaling complex that regulates protrusive activity and cell migration. J. Cell Sci. **115:**1497–1510.
22. **Mandiyan, V., J. Andreev, J. Schlessinger, and S. R. Hubbard.** 1999. Crystal structure of the ARF-GAP domain and ankyrin repeats of PYK2-associated protein beta. EMBO J. **18:**6890–6898.
23. **Manser, E., H. Y. Huang, T. H. Loo, X. Q. Chen, J. M. Dong, T. Leung, and L. Lim.** 1997. Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes. Mol. Cell. Biol. **17:**129–1143.
24. **Manser, E., T. Leung, H. Salihuddin, Z. S. Zhao, and L. A. Lim.** 1994. Brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature **367:**40–46.
25. **Manser, E., T. H. Loo, C. G. Koh, Z. S. Zhao, X. Q. Chen, L. Tan, I. Tan, T. Leung, and L. Lim.** 1998. PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. Mol. Cell **1:**183–192.
26. **Matafora, V., S. Paris, S. Dariozzi, and I. de Curtis.** 2001. Molecular mechanisms regulating the subcellular localization of p95-APP1 between the endosomal recycling compartment and sites of actin organization at the cell surface. J. Cell Sci. **114:**4509–4520.
27. **Obermeier, A., S. Ahmed, E. Manser, S. C. Yen, C. Hall, and L. Lim.** 1998. PAK promotes morphological changes by acting upstream of Rac. EMBO J. **17:**4328–4339.
28. **Paris, S., R. Longhi, P. Santambrogio, and I. de Curtis.** 2003. Leucine-zipper-mediated homo- and hetero-dimerization of GIT family p95-ARF GTPase-activating protein, PIX-, paxillin-interacting proteins 1 and 2. Biochem J. **372:**391–398.
29. **Parnas, D., A. P. Haghighi, R. D. Fetter, S. W. Kim, and C. S. Goodman.** 2001. Regulation of postsynaptic structure and protein localization by the Rho-type guanine nucleotide exchange factor dPix. Neuron **32:**415–424.
30. **Premont, R. T., A. Claing, N. Vitale, S. J. Perry, and R. J. Lefkowitz.** 2000. The GIT family of ADP-ribosylation factor GTPase-activating proteins. Functional diversity of GIT2 through alternative splicing. J. Biol. Chem. **275:**22373–22380.

31. **Sells, M. A., A. Pfaff, and J. Chernoff.** 2000. Temporal and spatial distribution of activated Pak1 in fibroblasts. J. Cell Biol. **151:**1449–1458.
32. **Turner, C. E., M. C. Brown, J. A. Perrotta, M. C. Riedy, S. N. Nikolopoulos, A. R. McDonald, S. Bagrodia, S. Thomas, and P. S. Leventhal.** 1999. Paxillin LD4 motif binds PAK and PIX through a novel 95-kD ankyrin repeat, ARF-GAP protein: a role in cytoskeletal remodeling. J. Cell Biol. **145:**851–863.
33. **Vitale, N., W. A. Patton, J. Moss, M. Vaughan, R. J. Lefkowitz, and R. T. Premont.** 2000. GIT proteins, a novel family of phosphatidylinositol 3,4, 5-trisphosphate-stimulated GTPase-activating proteins for ARF6. J. Biol. Chem. **275:**13901–13906.
34. **Zenke, F. T., C. C. King, B. P. Bohl, and G. M. Bokoch.** 1999. Identification of a central phosphorylation site in p21-activated kinase regulating autoinhibition and kinase activity. J. Biol. Chem. **274:**32565–32573.
35. **Zhang, H., D. J. Webb, H. Asmussen, and A. F. Horwitz.** 2003. Synapse formation is regulated by the signaling adaptor GIT1. J. Cell Biol. **161:**131–142.
36. **Zhao, Z. S., E. Manser, X. Q. Chen, C. Chong, T. Leung, and L. Lim.** 1998. A conserved negative regulatory region in αPAK: inhibition of PAK kinases reveals their morphological roles downstream of Cdc42 and Rac1. Mol. Cell. Biol. **18:**2153–2163.
37. **Zhao, Z. S., E. Manser, and L. Lim.** 2000. Interaction between PAK and Nck: a template for Nck targets and role of PAK autophosphorylation. Mol. Cell. Biol. **20:**3906–3917.
38. **Zhao, Z. S., E. Manser, T. H. Loo, and L. Lim.** 2000. Coupling of PAK-interacting exchange factor PIX to GIT1 promotes focal complex disassembly. Mol. Cell. Biol. **20:**6354–6363.