A variety of intracellular signaling pathways are linked to cell surface receptor signaling through their recruitment by Src homology 2 (SH2)/SH3-containing adapter molecules. p21-activated kinase 1 (PAK1) is an effector of Rac/Cdc42 GT-Pases that has been implicated in the regulation of cytoskeletal dynamics, proliferation, and cell survival signaling. In this study, we describe the specific interaction of PAK1 with the Grb2 adapter protein both in vitro and in vivo. We identify the site of this interaction as the second proline-rich SH3 binding domain of PAK1. Stimulation of the epidermal growth factor receptor (EGFR) in HaCaT cells enhances the level of EGFR-associated PAK1 and Grb2, although the PAK1-Grb2 association is itself independent of this stimulation. A cell-permeant TAT-tagged peptide encompassing the second proline-rich SH3 binding domain of PAK1 simultaneously blocked Grb2 and activated EGFR association with PAK1, in vitro and in vivo, indicating that Grb2 mediates the interaction of PAK1 with the activated EGFR. Blockade of this interaction decreased the epidermal growth factor-induced extension of membrane lamellae. Thus Grb2 may serve as an important mechanism for linking downstream PAK signaling to various upstream pathways.

Growth factors, cytokines, and many other hormones signal through specific cell surface receptors that contain intrinsic tyrosine kinase activity (1, 2). Activation of these receptors stimulates autophosphorylation at tyrosine residues within the cytoplasmic receptor tail. Many of these residues are contained in specific motifs that, when phosphorylated, recruit various intracellular effectors (3–5). These adapter molecules, including Nck and Grb2, serve as scaffolds to recruit and/or stimulate additional downstream signaling pathways, leading to cellular responses in vivo (6). The binding of Nck to PAK acts to recruit and couple PAK to signaling by the T cell receptor (26), the platelet-derived growth factor receptor (22, 23), the FcγRII (27), and to integrins (24, 28). Proteins interacting with the other PAK N-terminal SH3 binding sites remain to be identified.

In this study, we describe the specific interaction of PAK1 with the Grb2 adapter protein both in vitro and in vivo. We identify the site of this interaction as the second proline-rich SH3 binding domain of PAK1. Stimulation of the EGF receptor (EGFR) in HaCaT cells greatly enhances the level of EGFR-associated PAK1 and Grb2, although the PAK1-Grb2 association is itself independent of this stimulation. Evidence is presented that Grb2 mediates the coupling of PAK1 to the activated EGFR. Grb2 may thus serve as an important mechanism for linking downstream PAK signaling to various upstream pathways that assemble signaling modules containing the Grb2 adapter protein.

EXPERIMENTAL PROCEDURES

Plasmids and Constructs—PAK1 N-terminal fragment (aa 1–235), PAK1 N-terminal fragment (aa 1–74), and point mutants of these N-terminal constructs were generated by polymerase chain reaction and cloned into pET-28a (Novagen) for expression as N-terminal His-tagged proteins. The point mutants included PAK1 P13A and PAK1 P42A; these mutations disrupt the PXXP SH3 binding motifs at these sites (23). Proteins were isolated using nickel beads according to the manufacturer’s instructions (Qiagen) and gave predominantly single bands on both silver- and Coomassie Blue-stained SDS-polyacrylamide gels, with the exception of His full-length PAK1 N-terminus (aa 1–235), which degraded to yield several smaller fragments.

Preparation and Use of TAT-PAK1 Peptides—PAK1 contains several N-terminal proline-rich motifs that have the characteristic PXXP

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Lorena A. Puto, Kersi Pestonjamasp, Charles C. King‡, and Gary M. Bokoch§
From the Departments of Immunology and Cell Biology, The Scripps Research Institute, La Jolla, California 92037

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PAK1 Binds Grb2 Adapter Protein

Fig. 1. Identification of Grb2 as a PAK1 binding partner in vitro. A, purified His-tagged PAK1 N-terminal fragments 1–235 and 1–74 bound to nickel beads were incubated with Jurkat T cell lysates, and pull-down assays were performed as described under “Experimental Procedures.” As detected by silver staining, an ~25-kDa band specifically bound to both the PAK1 1–235 and 1–74 fragments. This band was not detected using either nickel beads in the absence of PAK1 N-terminal fragments (Control) or with a PAK1 C-terminal fragment bound to the nickel beads (data not shown). B, immunoblotting of PAK1 pull-downs from Jurkat T cell lysates using Grb2 monoclonal antibody at 1:5000 dilution. The blot shown is representative of three separate experiments. C, pull-down assays were performed as described for A. Equal amounts (5 μg of protein) of the mutated versions of the N-terminal PAK1 1–235 and 1–74 fragments, P13A and P42A, corresponding to the first and second proline-rich SH3 binding motifs, respectively, were used to pull down the 25-kDa Grb2 protein from Jurkat T cell lysate. The blots were immunostained with anti-Grb2 antibody as described earlier. The graph shows the densitometric quantitation (in arbitrary units) of three similar experiments, with the mean ± S.D. shown. The asterisks indicate statistically significant differences as compared with corresponding wild-type constructs. The decrease observed with the 1–74 p13 mutation was not statistically different from the wild-type (p > 0.5), whereas that with the 1–235 p13 mutation was statistically different at the p < 0.05 level. The decrease observed with both the p42 mutants, on the other hand, were highly significant (p < 0.003). D, TAT-tagged peptides encompassing the first (P1) or the second (P2) proline-rich domains of PAK1 were added to pull-down assay incubations with HaCaT lysates at a concentration of 200 μg/ml. Control and TAT control indicate controls in which lysates with either no peptide added or 200 μg/ml of a control TAT peptide were added, respectively. Immunoblotting of pull-down assays was performed at 1:5000 dilution with Grb2 monoclonal antibody. E, His-tagged purified PAK1 1–74 fragment was incubated with GST beads alone (lane 1), GST-Grb2 (lane 2), GST beads plus a protein A/protein G bead mixture, included as a specificity control (lane 3), or GST-Grb2 beads plus a protein A/protein G bead mixture (lane 4). Immunoblottting with the Grb2 antibody reveals the direct binding of PAK1 to Grb2 (lanes 2 and 4 versus control lanes 1 and 3). The results shown are representative of at least three separate experiments. (where X indicates a variable amino acid) structure of SH3 binding domains (29). Two of these consist of the sequences PPAPP (aa 12–16) and PLPPNP (aa 40–45). We prepared peptides encompassing these domains that were coupled to a membrane-permeant TAT-derived sequence (YARAAARQAR) (30). These are as follows: P1, YARAAARQARADKPPAPPF and P2, YARAAARQARASKPLPPNPPEA. A TAT peptide-only control was obtained from Dr. Steven Dowdy (University of California, San Diego).

Antibodies—A rabbit polyclonal antibody (R2124) raised against amino acids 174–306 of PAK1 (as in Ref. 31) was used for PAK1 immunoprecipitation (1:50) and immunoblotting (1:1,000). Grb2 immunoprecipitation (1:50) and immunoblotting (1:5,000) was performed with Grb2 mouse monoclonal antibody (G16720) from BD Biosciences. Phosphotyrosine-containing proteins (i.e. the stimulated EGFR) were detected using mouse monoclonal anti-phosphotyrosine 4G10 and Nck antibody. EGFR was detected using mouse monoclonal antibody E12020 from BD Biosciences.

Cell Culture and Immunoprecipitation Analyses—HaCaT cell lines were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal calf serum, 10 mm HEPES, 2 mm l-glutamine at 37 °C in an atmosphere of 10% CO_{2}. Jurkat T cells were maintained in RPMI 1640 medium (Invitrogen) with 10% fetal calf serum, 10 mm HEPES, 2 mm l-glutamine at 37 °C in an atmosphere of 10% CO_{2}.

For immunoblotting and immunoprecipitation studies, HaCaT cells were plated in 10-cm tissue culture dishes and serum-starved for 18 h prior to the start of experiment. As indicated, TAT-tagged peptides were added at the time of plating (200 μg/ml) and left for the entire time (18 h). The cells were then stimulated with EGF (200 ng/ml) for 2 or 30 min

Fig. 2. PAK1 interacts with Grb2 in cells. A, immunoprecipitation of endogenous Grb2 with PAK1 antibody. The PAK1 R2124 rabbit polyclonal antibody prepared against aa 174–306 of PAK1 was used to immunoprecipitate (1:50) either Jurkat T cell or HaCaT lysates. The Grb2 antibody was used for immunoblotting at 1:5000 dilution. Controls shown are a direct load of [1/50] volume of the original Jurkat T cell lysate used for precipitations (Jurkat IgG control) and a precipitation using a nonspecific rabbit polyclonal IgG antibody (IgM control). B, immunoprecipitation of endogenous PAK1 with Grb2 antibody. The monoclonal Grb2 antibody was used to immunoprecipitate (1:50) either Jurkat T cell or HaCaT lysates. The PAK1 antibody used for immunoblotting was R2124 (1:1,000), which detects multiple PAK isoforms. Also shown are a direct load of [1/50] volume of the original Jurkat T cell lysate used for precipitations (Jurkat lysate) and a control precipitation using a nonspecific mouse monoclonal antibody (IgM control). Note that the PAK doublet detected represents PAK1 (upper band) and PAK2 (lower band).
and then rapidly scraped from the dish into ice-cold lysis buffer (as in Ref. 23). After 1 h on ice, the lysates were pelleted for 10 min at 14,000 rpm at 4 °C, and the clarified supernatants removed and used for immunoprecipitation and/or immunoblotting.

Aliquots (~1 mg of total protein) of cell lysates were incubated with the indicated antibody overnight at 4 °C and then with 60 μl of a 1:1 slurry of protein A or protein G-Sepharose beads for 45–60 min. Beads were pelleted and washed four times with 1 ml (each time) of radioimmunoprecipitation assay buffer and then used for immunoblots. Radiography immunoprecipitation assay buffer consisted of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium cholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 50 IU/ml aprotinin.

Immunoblotting was done as follows: SDS-PAGE gels were transferred to polyvinylidene difluoride membranes (Immobilion-P transfer membrane, pore size 0.45 μm; Millipore) and incubated with blocking buffer (10 mM Heps, pH 7.4, 0.5 mM NaCl, 3% bovine serum albumin, 10% goat serum) overnight at 4 °C, followed by incubation with primary antibodies for 1 h at room temperature. After extensive washing with Tris-buffered saline, 0.1% Triton X-100 membranes were incubated for ~1 h at room temperature with secondary antibodies (anti-goat horseradish peroxidase) and visualized by enhanced chemiluminescence (Pierce).

Imaging—HaCaT cells suspended in either medium alone or medium containing indicated concentrations of the TAT peptide were seeded on 25-mm glass coverslips coated with 10 μg/ml fibronectin and allowed to spread for 18 h. The coverslips were placed into AutoCorr live cell chambers (Molecular Probes), and time-lapse imaging was carried out at 30-s intervals using an Olympus IX70 microscope fitted with a Princeton MicroMax 5-MHza 12-bit cooled CCD camera. The cells were stimulated on the microscope stage with EGF (200 ng/ml). Images were analyzed for total cell area using ISEE software from Inovision.

RESULTS

Identification of Grb2 as a PAK1-interacting Protein—We utilized various purified His-tagged PAK1 N-terminal constructs in an effort to affinity purify potential PAK1-interacting proteins from Jurkat T cell lysates. Along with a number of other protein bands detected by silver staining, both full-length PAK1 N terminus (aa 1–235) and a shorter version of the PAK1 N terminus (aa 1–74) specifically bound an ~25-kDa protein (Fig. 1A, lanes 2 and 3). This 25-kDa protein band was not observed in control incubations with nickel beads (Fig. 1A, lane 1) or when beads to which the PAK1 C terminus (aa 236–545) was coupled were used (data not shown).

Analysis of PAK1 structure using the ScanSite program (32) predicted that the 42PPNP sequence was only likely to bind to the SH3 domain of the 25-kDa adapter protein Grb2. We therefore tested whether the PAK1-associated protein was indeed Grb2 by immunoblotting the band pulled down out of Jurkat lysates with a specific Grb2 antibody (Fig. 1B). The bound protein strongly reacted with the Grb2 antibody, confirming its identity as Grb2. To further characterize the binding of Grb2 to the PAK N terminus, we prepared mutations of the first (aa P13A) and second (aa P42A) proline-rich SH3 binding motifs. As shown in Fig. 1C, Grb2 bound effectively to wild-type N-terminal aa 1–74 and aa 1–235 constructs and to a slightly lesser extent to the P13A mutant of these fragments. The latter may indicate some weak association of Grb2 with the first PAK1 proline-rich motif. In contrast, mutation of P42A resulted in a dramatic reduction in binding of Grb2 to PAK1 (p < 0.003). These data indicate that Grb2 interacts most effectively with the PAK1 N terminus via the second PXXP-SH3 motif interaction.

To confirm a specific interaction of Grb2 with the second PAK1 SH3 binding domain beginning at position 42, we examined the ability of TAT-tagged peptides that encompassed both the first (aa 10–17 = DKPPAPPMP) and second (aa 38–45 = SKPLPPNP) PAK1 proline-rich domains to block binding of Grb2 to PAK1. As shown in Fig. 1D, the peptide containing the second PXXP motif substantially inhibited Grb2 binding to...
PAK1 binds Grb2 adapter protein

PAK1. In contrast, there was a weak inhibitory effect observed with the first PXXP site peptide. We verified that PAK1 interacts directly with Grb2 using purified protein components. Fig. 1E shows that the His-tagged PAK1 1–74 fragment bound specifically to GST-Grb2 beads (lanes 2 and 4) but not to control beads (lanes 1 and 3).

PAK1 Interacts with Grb2 and EGFR in Intact Cells—We examined the interactions of endogenous Grb2 and PAK1 in several cell types. We observed that Grb2 could be specifically co-immunoprecipitated from cell lysates with a PAK1 antibody (Fig. 2A). Conversely, with a Grb2 antibody we co-immunoprecipitated PAK1 (Fig. 2B). The association of Grb2 with PAK1 was not significantly increased upon treatment of HaCaT cells with EGF (see below). These data indicate that endogenous Grb2 interacts constitutively with a pool of PAK1 in various cell types, including Jurkat and HaCaT cells.

Adapter proteins such as Grb2 are known to be important in the coupling of various growth factor receptors to downstream signaling pathways. Grb2 has been shown to associate with tyrosine-phosphorylated EGFRs via its SH2 domain (6). Activity of PAK1 is stimulated by EGF (22). To assess the physiological relevance of the Grb2-PAK1 interaction, we decided to examine this interaction in a cell line that responds well to EGF stimulation. The human keratinocyte HaCaT cell line exhibits a dramatic stimulus-dependent 13–15-fold increase in the amount of tyrosine-phosphorylated EGFR over a 30-min time course (Fig. 3A). Endogenous PAK1, Grb2, and Nck protein levels do not change during EGF stimulation over this period (Fig. 3A). We examined the EGF activation-dependent interactions among EGFR, Grb2, and PAK1. As shown in Fig. 3B, we observed that there was a pool of Grb2 that appeared to be constitutively associated with endogenous PAK1, and this did not change significantly with EGF stimulation. The association of PAK1 with the adapter Nck has similarly been shown to be largely independent of growth factor receptor stimulation (23). The recruitment of Grb2 to the activated EGFR was assessed in phosphotyrosine antibody immunoprecipitations of the receptor (Fig. 3C). We observed that Grb2 was co-precipitated with the stimulated, but not the unstimulated, EGFR. Similarly, the recruitment of PAK to the EGFR was dependent upon receptor stimulation (Fig. 3D).

The Interaction of PAK1 with the Phosphorylated EGFR Is Dependent upon Grb2 Adapter Function—The results of Fig. 3 suggest that Grb2, which has been shown to interact directly with stimulated EGFR, serves as an adapter for the PAK1-EGFR association. To test this hypothesis, we made use of the TAT-tagged PAK1 domain peptides that specifically blocked the PAK1-Grb2 interaction in vitro (see Fig. 1D). The polybasic sequence derived from the human immunodeficiency virus TAT protein has been shown to mediate the transport of peptides and proteins through cell membranes into the cytoplasm (30), enabling us to use these as a means to investigate the PAK1-Grb2 interaction in intact cells. As shown in Fig. 4A, the TAT-P2 domain peptide effectively blocked the association of endogenous Grb2 with PAK1, whereas the TAT-PACK1 domain peptide or a control TAT peptide both exhibited only slight (non-statistically significant) inhibitory effects. A similar pattern of inhibition was observed for the co-precipitation of the EGFR in PAK1 immunoprecipitates (Fig. 4B). In this case, however, for reasons that are not evident, both the control TAT peptide and the P1 peptide significantly decreased to a similar extent EGFR co-precipitating with PAK1. This is thus unlikely to reflect a specific inhibitory effect of the P1 peptide. Once again, however, the P2 peptide was significantly more effective, completely abolishing the EGFR-PAK1 interaction. The addition of TAT peptides did not affect cellular expression levels of EGFR, Grb2, or PAK1, or the level or time course of EGFR tyrosine phosphorylation in response to EGF (data not shown). These results demonstrate that blocking of the second proline-rich region of PAK1 causes a simultaneous decrease in the association of PAK1 with Grb2 and the EGFR. We interpret these data as evidence that the interaction of PAK1 with Grb2 mediates the interaction of PAK1 with the activated EGFR.

To establish that inhibition of the recruitment of PAK1 to the
EGFR via Grb2 had biological consequences, we examined cytoskeletal remodeling induced by EGFR stimulation. As shown in Fig. 5A, stimulation of control cells with EGF for 15 min induced the extension of broad lamellae-like ruffles. This could be quantified as an increase in cell area (Fig. 5B). The TAT-P2 peptide completely prevented lamellar extension, and the cells actually retracted slightly to give a decrease in the initial surface area. As opposed to effects observed in the EGFR-PAK1 co-precipitation experiments (Fig. 4B), no significant inhibition was observed with Tat-P1 peptide or Tat control peptide (Fig. 5B). PAK1 thus appears to be an important downstream mediator of EGF-induced cytoskeletal remodeling.

**DISCUSSION**

In the work presented here, we utilized an affinity-based approach in which the PAK1 N terminus was used as a probe to isolate specific binding partners from Jurkat T cell lysates. Grb2 was identified as a PAK1 N-terminal interacting protein. This was shown using both purified recombinant proteins and with the endogenous proteins from several cell lines. Grb2 specifically interacted with the second PAK1 SH3-binding P42PNP motif. Using cell-permeant TAT peptides encompassing the first and second SH3 binding regions of PAK1, we showed that we could block more than 95% of the interaction between PAK1 and the tyrosine-phosphorylated EGFR receptor in HaCaT cells with the peptide derived from the second SH3 binding domain. Although some nonspecific inhibition of this interaction by other TAT peptides was observed in the co-precipitation experiment for reasons that are not clear, these peptides were clearly less effective. We further demonstrated that the interaction of PAK1 with the activated EGFR mediated through the Grb2-binding second proline-rich motif was required for EGFR-induced cytoskeletal remodeling (Fig. 5). Extension of broad membrane lamellae induced by EGF was specifically blocked by the P2 peptide, with no significant inhibition by P1 or Tat control peptide. The EGFR thus appears to primarily utilize Grb2 to recruit PAK, consistent with the observation that dominant negative versions of Nck did not block PAK1 activation induced by EGFR stimulation in 293 cells (33). Although it remains possible that the P2 peptide might be blocking the interaction of an yet unidentified binding partner for the proline-rich SH3 binding motif of PAK1, the ScanSite analysis (32) of this region predicts that Grb2 is the only known SH3-containing protein likely to bind to this specific sequence.

Growth factor and cytokine tyrosine kinase receptors couple to downstream signaling pathways leading to cell proliferation, cell survival, and cytoskeletal rearrangements. Critical links in the coupling of such receptors to signaling elements are SH2/SH3-containing adapter proteins, including Nck and Grb2 (reviewed in Refs. 1–5). PAK1 activity is stimulated by various tyrosine kinase receptors, including the platelet-derived growth factor and EGFR receptors, the insulin receptor, interleukin-6 and interleukin-3, the T cell receptor, etc. (34–36). Activation of PAK may thus be important in the proliferative, cytoskeletal, and survival responses initiated by such receptors.

PAK1s 1, 2, and 3 contain several conventional and one non-conventional proline-rich SH3 binding domains in their regulatory N terminus (10, 11). It has been shown previously (22, 23) that PAK1 binds to the adapter protein Nck via its first proline-rich motif (aa 13–16) and that this mediates coupling to certain tyrosine kinase receptors. Indeed, using a strategy similar to that of the current study, Kiosses et al. (37) demonstrated with TAT peptides based upon the PAK1 first SH3 binding domain that the Nck-PAK1 interaction is important in endothelial cell motility and angiogenesis. In conjunction with the current study, these results demonstrate that TAT peptides containing PAK1 proline-rich protein interaction motifs can be effectively used to specifically block the interaction of PAK1 with SH3-containing adapter molecules in intact cells.

Taken together, these data indicate that PAK1 can interact with both the Nck and Grb2 adapter proteins for recruitment and activation by various receptors and/or signaling molecules that utilize these adapters. Including the molecular interactions mediated by PAK-interacting exchange factor via the unconventional SH3 binding motif (PRP) at aa 191–193, these results suggest that PAK1 may thus be a widely utilized signaling component. It will be of interest to determine whether the interaction of PAK with Grb2 can also be regulated by the phosphorylation of PAK by exogenous kinases (38), or by cell adhesion (24), as has been demonstrated for the PAK1-Nck interaction.

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