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A GTPase-independent Mechanism of p21-activated Kinase Activation

REGULATION BY SPHINGOSINE AND OTHER BIOLOGICALLY ACTIVE LIPIDS*

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p21-activated kinases (PAKs) are serine/threonine kinases that have been identified as targets for the small GTPases Rac and Cdc42. PAKs have been implicated in cytoskeletal regulation, stimulation of mitogen-activated protein kinase cascades, and in control of the phagocyte NADPH oxidase. Membrane targeting of PAK1 induced increased kinase activity in a GTPase-independent manner, suggesting that other mechanisms for PAK regulation exist. We observed concentration- and time-dependent activation of PAK1 by sphingosine and several related long chain sphingoid bases but not by ceramides or a variety of other lipids. Although phospholipids were generally ineffective, phosphatidic acid and phosphatidylinositol also had stimulatory effects on PAK1. Lipid stimulation induced a similar level of PAK1 activity as did stimulation by GTPases, and the patterns of PAK1 autophosphorylation determined after partial tryptic digestion and two-dimensional peptide analysis were similar with each class of activator. Lipid stimulation of PAK1 activity was dependent upon intact PAK kinase activity, as indicated by studies with a kinase-dead PAK1 mutant. Treatment of COS-7 cells expressing wild type PAK1 with sphingosine, fumonisin B, or sphingomyelinase, all of which are able to elevate the levels of free sphingosine, induced increased activity of PAK1 as determined using a p47^{phox} peptide substrate. Studies using PAK1 mutants suggest that lipids act at a site overlapping or identical to the GTPase-binding domain on PAK. The inactive sphingosine derivative *N,N*-dimethylsphingosine was an effective inhibitor of PAK1 activation in response to either sphingosine or Cdc42. Our results demonstrate a novel GTPase-independent mechanism of PAK activation and, additionally, suggest that PAK(s) may be important mediators of the biological effects of sphingolipids.

The p21-activated kinases (PAKs)[1] are members of a growing family of Ser/Thr kinases whose catalytic domains contain significant homology to the STE20 kinase of *Saccharomyces cerevisiae* (1). PAKs have been implicated as regulators of MAP kinase cascades (2–6) and the phagocyte NADPH oxidase (7) through their ability to phosphorylate regulatory substrates in these systems. PAKs have also been shown to modulate the activity of the actinomyosin system in lower organisms and in mammalian cells. ATPase activity of the heavy chain of *Acanthamoeba* myosin is enhanced when phosphorylated by PAK (8), and PAKs have been reported to phosphorylate the light chain of mammalian myosin (9, 10). PAKs localize to cortical actin structures in growth factor-stimulated cells (11), and introduction of active PAK1 mutants into mammalian fibroblasts induces cytoskeletal reorganization, including a polarized phenotype characterized by membrane ruffling, extension of filopodia, formation of focal complexes, and loss of actin stress fibers (12, 13).

PAK activity can be stimulated by a variety of external stimuli that act via cell-surface receptors. These include chemoattractants acting on G protein-coupled receptors (7), growth factors interacting with receptor tyrosine kinases (11), cytokines (2), Fc receptor stimuli,[2] and extracellular matrix molecules binding to integrins.[3] In response to some of these stimuli, PAK translocates into a membrane fraction where it can interact with membrane-bound receptors and activators (11, 14). Whereas interactions of PAK with adapter proteins such as Nck have been implicated in translocation and stimulation of PAK activity by growth factors (14–16), the molecular mechanisms involved in PAK regulation by these and other receptors has not yet been determined.

Activity of PAK is dramatically stimulated *in vivo* and *in vitro* by the binding of GTP-Rac or GTP-Cdc42 (7, 17). These GTPases interact with a region at the PAK N terminus which contains the highly conserved CRIB motif described in a number of Cdc42/Rac-interacting proteins (17, 18). Binding of the active GTPase stimulates PAK autophosphorylation at several sites, presumably changing the protein's conformation and activity toward exogenous substrates (13, 19). An additional consequence of activation is increased binding of SH3-containing proteins to the PAK N terminus (12, 14, 15). The potential for other mechanisms of PAK activation in cells was established by the demonstration of PAK2 activation resulting from proteolytic removal of the regulatory N terminus by caspases during apoptosis (20). A recent study has also described enhanced activity of intact PAK as a result of membrane association initiated by introduction of a membrane-targeting sequence, although the mechanism through which membrane targeting causes activation has not been investigated (16).

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[1] The abbreviations used are: PAK, p21-activated kinase; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; DMS, *N,N*-dimethylsphingosine; MBP, myelin basic protein; HPLC, high pressure liquid chromatography.

[2] Jones, S. L., Knaus, U. G., Bokoch, G. M., Springer, T. A., and Brown, E. J. (1998) *J. Biol. Chem.* **273,** in press.

[3] L. S. Price, J. Leng, M. A. Schwartz, and G. M. Bokoch, submitted for publication.

In this paper, we describe the ability of specific lipids, particularly sphingolipids, to stimulate PAK activity both *in vitro* and *in vivo*. Lipids regulate PAK by a direct interaction that is independent of the action of Rac or Cdc42. Both PAK autophosphorylation and activity toward exogenous substrates are enhanced by active lipids. These results suggest that PAKs may be cellular targets of sphingolipids and may account for some of their biological activities.

EXPERIMENTAL PROCEDURES

Materials—The lipids utilized in these studies and their sources are listed in Table I. Lipids were routinely prepared as $CHCl_3$, ethanol, or Me_2SO stocks, as appropriate for each type of lipid. Prior to use the lipids were dried under N_2 and resuspended by sonication in a bath sonicator in 50 mM Hepes, pH 8.0, plus 1 mM EDTA or in Tris-HCl, pH 7.5, until a translucent suspension was obtained. In some instances (*e.g.* with Me_2SO stocks), the lipids were diluted into the same buffer or added as a complex with 4 mg/ml bovine serum albumin. Mixed lipid vesicles were generated by mixing the lipid stocks in molar ratios varying from 1:10 to 1:3 prior to sonication in buffer. Fumonisin B was obtained from Sigma (F-1147).

Plasmids and Protein Expression—PAK1 wild type and the indicated mutants were prepared essentially as described (12, 15) in the pCMV6M vector, which adds a 9E10 Myc epitope tag to the N terminus. The wild type PAK1 tagged with the C-terminal 17 amino acid farnesylation sequence of K-Ras 4B (KDGKKKKKKSKTKCVIM) in the pCMV6M vector was a gift of Jon Chernoff (Fox Chase Cancer Center). All other CAA*X*-tagged PAK1 constructs were generated by subcloning into the *Bam*HI and *Nhe*I sites of this vector. Mutation of the terminal cysteine in the CAA*X* motif to alanine was by polymerase chain reaction amplification using a 3′ oligonucleotide containing the mutated base pairs (5′ CCGGAATTCTCACATAATTACAGCCTTTGTCTT 3′). The PAK1 ED mutant was generated by mutating all aspartic acid residues to asparagine and all glutamic acid residues to glutamine between amino acids 175 and 183 of PAK1 wild type. This was achieved by using internally derived oligonucleotides containing the mutated sequence in both the 5′–3′ (5′ CAAAATCAGAATAACAATAACAACAATGCTAC-CCCACCACCAGTGATT 3′)and 3′–5′ (5′ ATTGTTGTTATTGTTAT-TCTGATTTTGTGAAACTGGTGGCACTGCA 3′) direction. Polymerase chain reaction was carried out with vector-derived oligonucleotides to generate two overlapping strands containing the mutated sequences. These were then used as polymerase chain reaction templates to allow synthesis of full-length PAK1 containing the ED domain mutation.

Expression in COS-7 cells was performed by seeding the cells at 1×10^6 per 100-mm dish and then transfecting with 5 μg of the plasmid using LipofectAMINE (Life Technologies, Inc.). After 30 h, the cells were either utilized for *in vivo* experiments, or lysates were prepared for use in *in vitro* solid phase kinase assays (7, 15). Transfected cells were serum-starved overnight in Dulbecco's modified Eagle's medium containing 10 mM Hepes, 2 mM glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin and then incubated with various stimuli for the indicated times. Cells (100-mm dish/stimulus) were then lysed in 250 μl of Laemmli sample buffer and used for in-gel analysis or were lysed in cell lysis buffer for immunoprecipitation, as described below.

PAK Kinase Assays—Solid phase PAK1 kinase assays and in-gel kinase assays were performed essentially as described previously (7, 15, 21).

Analysis of Membrane-targeted PAK1—COS-7 cells (1×10^6 cells/10-cm dish) were transiently transfected with pCMV6 plasmids containing various N-terminally Myc-tagged PAK1 constructs, including WT-CAA*X* and HL-CAA*X* PAK1. As an added control, constructs were used in which the terminal cysteine residue in the CAA*X* sequence was replaced with an alanine to ensure that there was no effect of adding the additional C-terminal residues in the absence of membrane targeting. We verified the predominant membrane localization of the CAA*X*-tagged constructs by homogenizing the transfected cells in lysis buffer without detergent (see below) and, following a low speed spin to remove the unbroken cells, the homogenate was fractionated into a high speed ($100{,}000 \times g$) membrane pellet and the cytosol-enriched supernatant. The relative localization of each construct was then determined by SDS-polyacrylamide gel electrophoresis and Western blotting for Myc-tagged protein in each fraction.

To evaluate kinase activity, cells were allowed to express protein for 48 h post-transfection and were then lysed into 500 μl/dish lysis buffer with 1% Nonidet P-40. Portions of each lysate were run on SDS-polyacrylamide electrophoresis gels and transferred to nitrocellulose prior to blotting for the expression of myc-tagged protein. The lysates (normalized for expression levels) were then immunoprecipitated with the anti-Myc antibody. *In vitro* kinase assays using 1 μg of MBP/assay as a substrate were carried out in 50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.2 mM dithiothreitol with 5 μCi of [γ-^{32}P]ATP and 20 μM ATP in each 100-μl reaction (7).

Immunoprecipitations—To immunoprecipitate PAK1 from COS-7 cell lysates, 9E10 Myc antibody was prebound to protein G-Sepharose beads (Amersham Pharmacia Biotech) by incubation overnight and then washed extensively in lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1% Nonidet P-40, 10% glycerol). In activation experiments, 250 μl of lysate from cells (100-mm dish) activated with the indicated stimulus was incubated with 30 μl of a 1:1 slurry of Myc antibody-coated beads for 1 h at 4 °C, and then the beads were pelleted by centrifugation. The bead pellets were washed twice in lysis buffer containing 1% Nonidet P-40 and twice without Nonidet P-40, suspended in 100 μl of Laemmli sample buffer, and then analyzed by in-gel kinase assay.

Measurement of Sphingosine—Sphingosine levels were determined by an enzymatic method as described previously (22) or by HPLC analysis (23) with slight modifications. Lipids were extracted with chloroform/methanol/concentrated HCl (100:200:1 v/v), and the phases were separated as described (22). For HPLC analysis, aliquots of the organic layer (approximately 50–100 nmol of total cellular phospholipid) were saponified by addition of 0.5 ml of 0.1 N KOH/methanol and incubation at 37 °C for 60 min. The reactions were terminated by addition of 10 μl of concentrated HCl, and samples were extracted with 1 ml of chloroform, 1 M KCl (1:1, v/v). The organic phases were dried under N_2, resuspended in 50 μl of methanol, and derivatized with *o*-phthalaldehyde for subsequent HPLC analysis (23). Sphingosine was separated isocratically on a Cosmosil 5C18-AR column (4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan) using a model 600E pump (Waters) with methanol, 5 mM Na_2HPO_4, pH 7.0 (60:40, v/v), at a flow rate of 1 ml/min. A model 420-AC fluorescence detector (Waters) was used to detect fluorescent derivatives with an excitation filter transmitting at a maximum of 340 nm and a cut-off emission filter at 400 nm. Sphingosine levels were quantified using the Rainin Dynamax HPLC software package (Woburn, MA).

Measurement of Cellular Phospholipids—Total phospholipids present in cellular lipid extracts used for sphingolipid analysis were quantified as described previously (24) with minor modifications. Briefly, to dried aliquots of cellular lipid extracts, 40 μl of a mixture of 10 N H_2SO_4/70% perchloric acid (3:1, v/v) was added, and samples were incubated for 30 min at 210 °C. After cooling, 75 μl of water and 400 μl of 4.2% ammonium molybdate in 4 N HCl, 0.045% (w/v) malachite green (1:3 v/v) was added. Samples were incubated at 37 °C for 15 min, and absorbance was measured at 660 nm.

Two-dimensional Peptide Mapping—Immunoprecipitated PAK1 was incubated with 1 μg of GTPγS-Cdc42 or 200 μM sphingosine and labeled with [γ-^{32}P]ATP, as described above. Autophosphorylated PAK1 bands were separated on 6.5% polyacrylamide gels, dried on HiDry hydrophobic drying sheets (Diversified Biotech, Newton Center, MA), and detected by autoradiography. Each band was separately excised from the gel and resuspended in ammonium bicarbonate, pH 8.3 (50 mM), containing trypsin (1.0 μg) for 14 h at 37 °C. The resulting tryptic peptides were extracted from the gel pieces with Kontes disposable pestles (Fisher) and centrifuged at 14,000 rpm in a table top microcentrifuge, and the supernatant was lyophilized. Two-dimensional phosphopeptide mapping of the tryptic peptides was performed according to the procedure of Boyle *et al.* (25). The lyophilized samples were resuspended in 2 μl of pH 1.9 electrophoresis buffer (2.2% formic acid, 8% acetic acid), spotted onto 100-μm coated cellulose plates (EM Science, Gibbstown, NJ) in 0.5-μl aliquots, and electrophoresed for 40 min at 1300 V in a Multiphor II horizontal electrophoresis unit (Pharmacia Biotech, Uppsala, Sweden) in pH 1.9 electrophoresis buffer. Plates were air-dried and chromatographed in buffer containing 62.5% isobutyric acid, 1.9% *n*-butanol, 4.8% pyridine, and 2.9% glacial acetic acid. ^{32}P-Labeled tryptic peptides were detected by autoradiography on Kodak X-AR film for up to 12 h.

RESULTS

Activation of PAK1 by Membrane Targeting Is Independent of GTPases—The association of wild type PAK1 with the plasma membrane induced by the addition of a membrane targeting sequence caused increased activity of PAK1 toward an exogenous substrate (Fig. 1), as also reported (16). Activation did not occur in the absence of membrane association, as determined with a PAK1 construct in which the cysteine residue in the

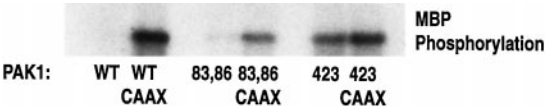

FIG. 1. **Activation of PAK1 by membrane targeting is GTPase-independent.** Various forms of PAK1 were expressed in COS-7 cells either with or without an added C-terminal membrane targeting CAA*X* sequence (CVIM), as described under "Experimental Procedures." Cell lysates were prepared and assayed for PAK-induced MBP phosphorylation as described, with detection by autoradiography on Kodak X-AR film for less than 12 h. PAK1 protein expression was determined by immunoblotting and adjusted within each pair to have equal levels of the tagged and untagged constructs. *WT*, wild type PAK1; *83,86*, PAK1(H83L, H86L) which does not bind Rac or Cdc42; *423*, PAK1(T423E) which is a constitutively active PAK. The relative increase in MBP phosphorylation quantified by PhosphorImager for each PAK1 construct in this experiment was as follows: *WT*, 1.0-fold; *WT-CAAX*, 23-fold; *83,86*, 2.0-fold; *83,86-CAAX*, 7.0-fold; *423*, 11-fold; *423-CAAX*, 16-fold.

CAA*X* motif was mutated to alanine. Of particular interest, we observed that membrane association also stimulated the activity of PAK1(H83L, H86L), a form of PAK1 which is unable to bind Rac or Cdc42 (12). In contrast, a fully active PAK1 (T423E) construct was not further activated significantly by membrane targeting (Fig. 1). These data strongly indicate that PAK activation resulting from membrane association is at least partially GTPase-independent and may occur through a distinct mechanism. We therefore investigated the possibility that certain membrane lipids may have the ability to directly stimulate PAK.

Stimulation of PAK Activity by Sphingolipids—We observed that sphingolipids were able to dramatically stimulate the Ser/Thr kinase activity of PAK1. As shown in Fig. 2, sphingosine and several related long chain sphingoid bases were able to increase autophosphorylation of PAK1 in an *in vitro* kinase assay to levels similar to that achieved in the presence of GTPγS-loaded Cdc42 or Rac1. A concentration-response curve for PAK1 activation by sphingosine is shown in Fig. 3*A*. Stimulation of PAK1 was observed at sphingosine concentrations as low as 10 μM and peaked at ~200 μM lipid. PAK activation was observed with sphingosine obtained from several commercial sources and with the lipid prepared in several different buffers, in Me_2SO, or when suspended with bovine serum albumin. Sphingosine was also effective in stimulating PAK1 when presented in the form of a mixed phospholipid micelle, either with phosphatidylcholine or phosphatidylethanolamine (Fig. 2*C*). Stimulation by sphingosine was not stereospecific, as each of the DL-*erythro* or DL-*threo* forms produced similar levels of activation. Even dihydrosphingosine, which lacks the 4,5-*trans* double bond, or phytosphingosine, which contains an additional hydroxyl group, stimulates PAK1 *in vitro*. In contrast to sphingosine, the sphingosine precursor *N*-acetylsphingosine or ceramide was inactive, as were a variety of other structurally related and unrelated lipids (Fig. 2 and Table I). In particular, the inability of sphingosylphosphorylcholine and octylamine (not shown) to activate PAK1 suggests this was not a general effect resulting from the presence of a highly positively charged group associated with the lipid backbone.

Activation of PAK1 with either Cdc42- or Rac1-GTPγS resulted in the formation of three distinct species of PAK1 with slightly varying migration on the SDS-polyacrylamide gels. These represent variably phosphorylated forms of PAK1[4] whose formation correlates with increased activity of PAK toward exogenous substrates. Interestingly, activation of PAK1 with sphingosine only resulted in the formation of the two faster migrating phosphorylated PAK species (Fig. 2*A*). This

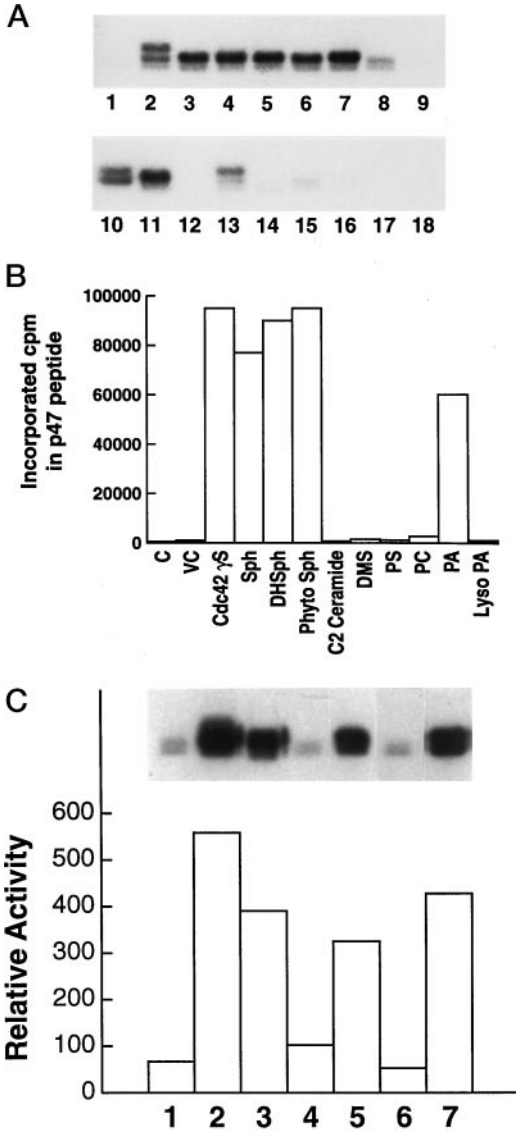

FIG. 2. **Stimulation of PAK1 kinase activity by lipids.** The effect of various lipids evaluated in *A* and *B*, all at a concentration of 400 μM, on PAK1 kinase activity was determined as described under "Experimental Procedures" and detected either by autophosphorylation of PAK1 (*A*) or phosphorylation of a $p47^{phox}$ peptide (2 μg, amino acid residues 324–331) substrate (*B*). *C*, mixed lipid micelles were prepared and used as described under "Experimental Procedures." *A*, *lane 1*, buffer control; *lane 2*, 1 μg of Cdc42-GTPγS; *lane 3*, sphingosine; *lane 4*, DL-dihydrosphingosine; *lane 5*, DL-*erythro*-dihydrosphingosine; *lane 6*, DL-*threo*-dihydrosphingosine; *lane 7*, phytosphingosine; *lane 8*, *N*,*N*-dimethylsphingosine; *lane 9*, sphingosylphosphorylcholine; *lane 10*, monosialoganglioside (GM3); *lane 11*, phosphatidic acid; *lane 12*, lysophosphatidic acid; *lane 13*, lysophosphatidylinositol; *lane 14*, phosphatidylserine; *lane 15*, lysophosphatidylcholine; *lane 16*, C_2-ceramide; *lane 17*, phosphatidylethanolamine; *lane 18*, phosphatidylcholine. *B*: *C*, no addition; *VC*, lipid vehicle control; *Cdc42γS*, 1 μg of Cdc42-GTPγS; *Sph*, sphingosine; *DHSph*, DL-dihydrosphingosine; *PhytoSph*, phytosphingosine; C_2-ceramide; *DMS*, *N*,*N*-dimethylsphingosine; *PS*, phosphatidylserine; *PC*, phosphatidylcholine; *PA*, phosphatidic acid; *LysoPA*, lysophosphatidic acid. *C*, *lane 1*, buffer control; *lane 2*, 1 μg of Cdc42-GTPγS; *lane 3*, 400 μM sphingosine; *lane 4*, 400 μM phosphatidylcholine (*PC*); *lane 5*, 400 μM sphingosine in a mixed PC/sphingosine micelle at 1:3 lipid molar ratio; *lane 6*, 400 μM phosphatidylethanolamine (*PE*); *lane 7*, 400 μM sphingosine in a mixed PE/sphingosine micelle at 1:3 lipid molar ratio. Similar results were obtained using mixed lipid vesicles with molar ratios varying from 1:10 to 1:3.

modification is sufficient for sphingosine to effectively stimulate phosphorylation of a $p47^{phox}$-derived peptide substrate by PAK (Fig. 2*B*), as well as intact $p47^{phox}$ protein and Nck (15). However, when PAK1 was activated by sphingosine we were

[4] C. C. King and G. M. Bokoch, unpublished observations.

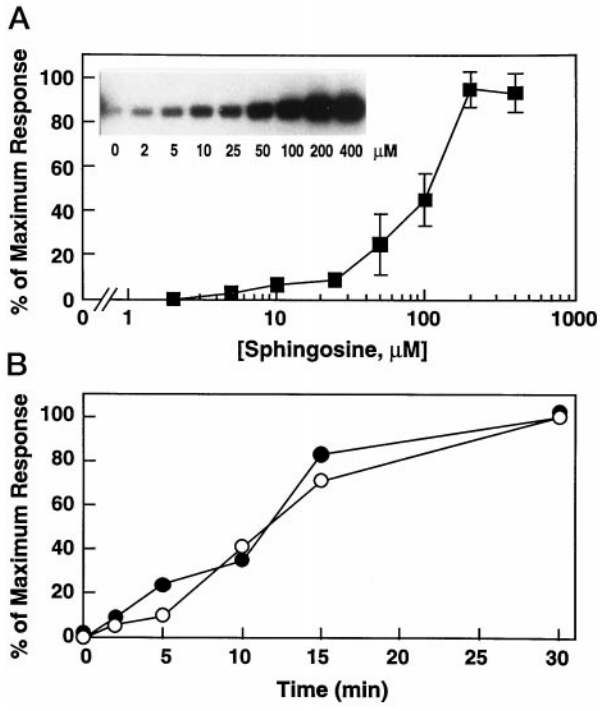

FIG. 3. **Characterization of sphingosine effects on PAK1 activity.** *A*, PAK1 activity was determined as described under "Experimental Procedures" in the presence of the indicated concentration of sphingosine. The data plotted represent the average ± S.D. for at least three separate determinations using PAK1 autophosphorylation as the end point; similar results were obtained using p47^{phox} peptide as exogenous substrate. *B*, the time course for PAK1 activation as assessed by autophosphorylation was determined in the presence of 1 μg of Cdc42-GTPγS (*closed circles*) or 200 μM sphingosine (*open circles*), as described under "Experimental Procedures." The *inset* shows the autoradiogram from a representative time course.

unable to detect effective phosphorylation of myelin basic protein, which serves as an excellent substrate for PAK1 when it is activated by Cdc42 or Rac (7, 17). We are uncertain at this time whether this represents a real difference in the ability of lipid-activated PAK1 to utilize certain specific substrates or whether this is a competitive effect of the lipid with the MBP substrate. This could also be due to differences in substrate utilization by the differentially phosphorylated PAK1 species since, in contrast to sphingosine and related compounds, stimulation of PAK1 by phosphatidic acid, phosphatidylinositol, and/or monosialoganglioside GM3 induced the formation of all three autophosphorylated PAK1 species, and these lipids also stimulated the phosphorylation of both p47^{phox} peptide and MBP by PAK1. For reasons which are not clear, the action of phosphatidic acid and phosphatidylinositol upon PAK1, while highly reproducible, was only observed with very fresh lipid preparations, and their ability to activate PAK1 was lost upon storage even under N_2 at −20 °C.

The use of immunoprecipitated PAK precludes us from conclusively stating that activation of PAK by lipids is due to a direct effect on PAK1 itself. However, to address the possibility of a contaminating lipid-regulated kinase that could phosphorylate PAK1 directly co-precipitating with Myc-tagged PAK1 from COS-7 cells, we performed similar experiments using several distinct PAK antibodies and using different sources of PAK1. We used PAK1 antibody R626 (7), two other PAK1 antibodies directed against distinct portions of PAK1,[5] or 9E10 Myc antibody to immunoprecipitate PAK from bovine brain cytosol, human neutrophil cytosol, or PAK1-transfected COS-7 cells. No sphingosine or Cdc42-sensitive kinase activity was immunoprecipitated by the 9E10 Myc antibody from brain or neutrophil cytosol, indicating that this antibody did not nonspecifically precipitate such a kinase. The R626 PAK1 antibody and the other PAK antibodies, however, precipitated sphingosine-activated PAK from all three sources, strongly supporting the likelihood that sphingosine was acting directly on PAK itself. We have also observed stimulation by sphingosine of PAK2 expressed in COS-7 cells and immunoprecipitated via a distinct antibody directed against its N-terminal hemagglutinin epitope tag. Finally, sphingosine increases activity of a pure recombinant PAK1 (~99% homogeneous by silver staining) that is already partially active in the absence of activating agents (we have been unable to purify a completely inactive recombinant PAK1) to an extent (2–3-fold) similar to that attained by addition of Cdc42-GTPγS, again suggesting a direct effect of the lipid on PAK itself.

Sphingosine-activated PAK1 Is Phosphorylated at Sites Similar to Those Resulting from Activation by GTPase—Fig. 3*B* shows the time course for PAK1 activation *in vitro* by sphingosine *versus* Cdc42-GTPγS. There was essentially no difference in the time course for autophosphorylation with either activator. Both sphingosine and Cdc42-GTPγS stimulated PAK1 to a similar extent at optimal concentrations of each. In addition, stimulation of PAK1 with sphingosine in the presence of Cdc42-GTPγS did not induce additive PAK1 autophosphorylation nor additive activity toward exogenous p47^{phox} peptide substrate, indicating each stimulus alone was able to fully activate PAK1. When we prephosphorylated PAK1 in the presence of sphingosine and unlabeled ATP, then rephosphorylated in the presence of Cdc42-GTPγS plus [γ-^{32}P]ATP, there was no incorporation of radiolabel into PAK, suggesting that sphingosine had caused all the available autophosphorylation sites on PAK1 to be occupied. We examined the pattern of PAK1 phosphorylation when activated by GTPase or lipid by two-dimensional mapping of tryptic peptides. As shown in Fig. 4, the phosphorylation patterns were qualitatively quite similar. There was a single minor phosphopeptide spot evident with Cdc42-GTPγS that was not detected with sphingosine activation, and two additional spots seen with sphingosine activation were not evident with Cdc42-GTPγS, but the pattern of the major phosphorylated peptide species was identical. Differences in relative spot intensity seen between PAK activated by Cdc42-GTPγS *versus* sphingosine in Fig. 4 were not consistently observed from experiment to experiment. These data suggest that the mechanism of PAK activation induced by the sphingolipid is similar to that induced by the binding of the GTPase.

Regulation of PAK by Stimuli That Increase Cellular Sphingosine Metabolism—To determine if PAK1 activity could be stimulated by endogenous production of sphingosine, we treated COS-7 cells that had been transiently transfected to express PAK1 with either sphingosine itself or agents that would be expected to elevate intracellular sphingosine levels. As shown in Table II, treatment of the cells with sphingosine, fumonisin B, or bacterial sphingomyelinase all increased intracellular sphingosine content. Addition of 15 μM sphingosine to the cells stimulated the activity of several kinases when lysate was directly assayed using the p47^{phox} peptide as substrate in an in-gel kinase assay (Fig. 5*A*). A prominent activity that comigrated with a constitutively active PAK1(T423E) standard was observed. Stimulation of this activity was rapid, occurring within 5 min of sphingosine addition. This kinase was not stimulated by the addition of 15 μM C2- or C8-ceramides nor by the addition of *N,N*-dimethylsphingosine to the cells. Similarly, treatment of the COS-7 cells with 5–10 μM fumonisin B, a

[5] U. G. Knaus and G. M. Bokoch, unpublished observations.

TABLE I
Stimulatory effects of lipids on PAK1 kinase activity

Lipid (400 μM)	Autophosphorylation	p47 peptide phosphorylation	Source/product
Sphingoid base-related lipids			
Sphingosine	+++	+++	Sigma S-6879 and S-6136, Matreya 1304
D-*erythro*-Sphingosine	+++	+++	Matreya 1802
L-*erythro*-Sphingosine	+++	+++	Matreya 1826
D-*threo*-Sphingosine	+++	+++	Matreya 1827
L-*threo*-Sphingosine	+++	+++	Matreya 1806
Phytosphingosine	++	++	Sigma P-2795
N-Acetyl-D-sphingosine	−	ND[a]	Sigma A-7191
DL-dihydro-sphingosine (sphinganine)	++	++	Sigma D-6783
DL-*erythro*-Dihydrosphingosine	++	++	Sigma D-6908
DL-*threo*-Dihydrosphingosine	++	++	Sigma D-7033
C_2-Ceramide	−	−	Sigma D-7033
C_2-Dihydroceramide	−	ND	Biomol SL-101
C_8-Ceramide	−	−	Biomol SL-112
Sphingomyelin	−	−	Sigma S-7006, LS-10
N,N-Dimethylsphingosine	+	−	Sigma D-1047
Monosialoganglioside (GM3)	+++	+++	Matreya 1515
Psychosine	−	ND	Matreya 1305
Sphingosylphosphorylcholine	−	ND	Sigma S-4257
Sphingosine 1-phosphate	−	ND	Sigma S-9666
Non-sphingosine-related lipids			
Arachidonic acid	−	−	LC Labs A-4879
Phosphatidylcholine	−	−	Sigma P-3841
Dimyristoylphosphatidylcholine	−	−	Sigma P-0888
Phosphatidylethanolamine	−	−	Sigma P-0888
Phosphatidylserine	−	−	Avanti 840021, Sigma P-6641 and P-8518
Phosphatidylinositol	+++	+++	Avanti 830042
PIP	−	ND	Calbiochem 524647
PIP_2	−	ND	Calbiochem 524644
Lysophosphatidylinositol	++	++	Avanti 850091
Phosphatidic acid	+++	+++	Sigma P-9511 and P-1293
Dimyristoylphosphatidic acid	++	++	Avanti 830845
Dilaurylphosphatidic acid	−	−	Avanti 840653
Lysophosphatidic acid	−	−	Avanti 857120
Lysophosphatidylcholine	−	−	Sigma L-5254

[a] ND, not determined.

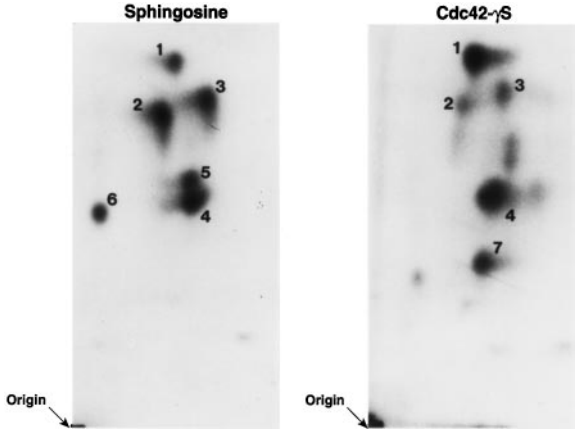

FIG. 4. **Two-dimensional tryptic digest map of autophosphorylated PAK1 stimulated by Cdc42-GTPγS or sphingosine.** Mapping was performed as described under "Experimental Procedures" and visualized by autoradiography for ~12 h on Kodak X-AR film. The point at which the sample was applied is indicated as *Origin* and the major phosphopeptide spots are indicated as *1–7*.

mycotoxin that inhibits ceramide synthase and thereby blocks the acylation of sphingosine and sphinganine to form ceramide, for 10 min or with 1 unit/ml sphingomyelinase from *Bacillus cereus* for 30 min also caused a stimulation of this ~68-kDa kinase which was of even greater magnitude than was observed with exogenously added sphingosine. Cell viability was maintained at greater than 90% for all treatments.

To verify that the ~68-kDa activity observed was indeed PAK1, we immunoprecipitated the introduced PAK1 from the cell lysates with the 9E10 Myc epitope antibody. The activity of

TABLE II
Changes in levels of sphingosine

After various treatments of COS-7 cells for the indicated times, cellular lipids were extracted, and sphingosine levels were measured as described under "Experimental Procedures." Sphingosine levels are expressed as picomoles normalized to total phospholipids (PL) (nanomoles) and are the means ± S.D. of triplicate cultures.

Treatment	pmol/nmol PL
None	0.32 ± 0.04
Sphingosine (15 μM, 5 min)	0.54 ± 0.04
Fumonisin B (5 μM, 10 min)	0.51 ± 0.006
Sphingomyelinase (1 unit/ml, 30 min)	0.83 ± 0.006
DMS (20 μM, 5 min)	0.33 ± 0.02
C_2-ceramide (20 μM, 5 min)	0.32 ± 0.02

the precipitated PAK1 was stimulated in cells that had been treated with 15 μM sphingosine, 10 μM fumonisin B, or 1 unit/ml sphingomyelinase but was not stimulated by 20 μM C_2-ceramide, C_8-ceramide (not shown), or *N,N*-dimethylsphingosine (Fig. 5*B*). We determined that the stimulated activity at 68 kDa was immunodepleted from the lysate after precipitation with Myc antibody (not shown).

The Site of Sphingolipid Action on PAK1—To gain insight into the requirements for PAK1 activation by sphingosine, we evaluated the effect of this lipid on a number of PAK1 structural mutants we had generated (Fig. 6). Introduction of a K299R mutation into the PAK1 catalytic domain results in loss of PAK1 Ser/Thr kinase activity. Sphingosine had no stimulatory effect on the catalytically inactivate PAK, again consistent with a direct stimulatory effect of the lipid on PAK itself rather than via a co-precipitating kinase. When PAK1 was constitutively activated by introduction of the T423E mutation, there was no further stimulation of autophosphorylation by sphingo-

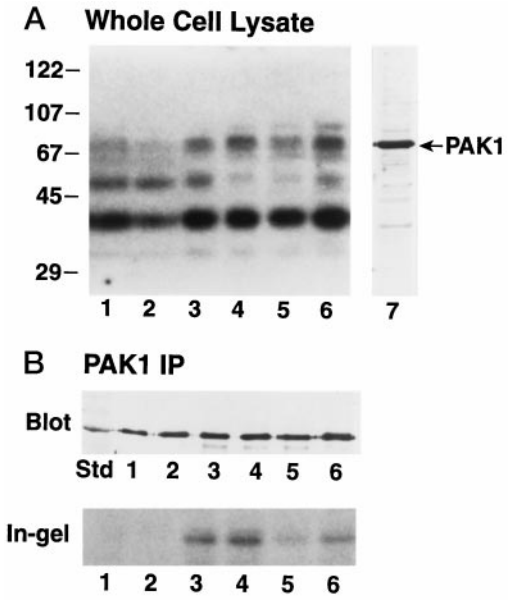

FIG. 5. **Activation of PAK1 expressed in COS-7 cells by treatments that elevate intracellular free sphingosine.** *A*, whole cell lysates were obtained from COS-7 cells transiently expressing PAK1 in the presence or absence of the indicated stimuli. Lysates were analyzed by in-gel kinase assay, as described. *Lane 1*, Me_2SO control 10 min; *lane 2*, 20 μM C_2-ceramide 10 min; *lane 3*, 15 μM sphingosine 10 min; *lane 4*, 5 μM fumonisin B 10 min; *lane 5*, 1 unit/ml sphingomyelinase 10 min; *lane 6*, 1 unit/ml sphingomyelinase 30 min; *lane 7*, immunoblot of control lysate for PAK1 using antibody R626 (7). *B*, PAK1 was immunoprecipitated from lysates of treated COS-7 cells, and the precipitates were assayed for PAK1 kinase activity as described under "Experimental Procedures." The *upper panel* shows the levels of PAK1 in each sample as determined by immunoblotting with the 9E10 Myc epitope antibody. *Std* represents a recombinant PAK1 standard. The *lower panel* shows an in-gel kinase assay using $p47^{phox}$ peptide substrate. *Lane 1*, Me_2SO control 10 min (relative activity 1.00); *lane 2*, 20 μM C2 ceramide 10 min (relative activity 1.50); *lane 3*, 1 unit/ml sphingomyelinase 30 min (relative activity 16.4); *lane 4*, 5 μM fumonisin B 10 min (relative activity 23.6); *lane 5*, 15 μM sphingosine 10 min (relative activity 6.0); *lane 6*, 20 μM monosialoganglioside (GM3) 10 min (relative activity 13.2). Relative kinase activities indicated above were determined by PhosphorImager analysis of the in-gel kinase assays shown.

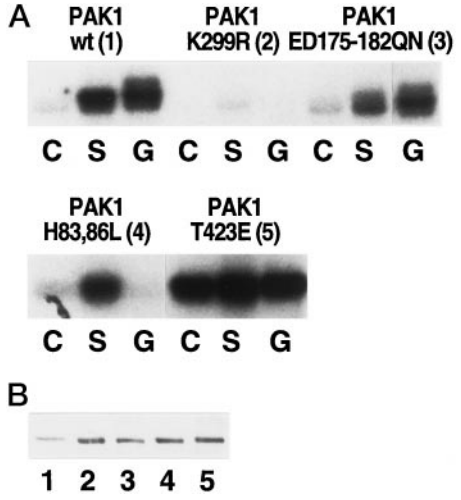

FIG. 6. **Activation of PAK1 mutants by sphingosine and Cdc42.** The indicated PAK1 constructs were expressed in COS-7 cells, and lysates were prepared and matched for equivalent levels of expressed protein by comparison of Myc immunoblot intensity, shown in the immunoblot of *B*. *A*, equalized samples were then assayed for autophosphorylation in the absence (*C*, control) or presence of 400 μM sphingosine (*S*) or 1 μg Cdc42-GTPγS (*G*), as described. *PAK1 wt*, wild type; *PAK1 K299R*, kinase-dead mutant; *PAK1 ED175–183QN*, mutant in which the glutamic acid and aspartic acid residues at positions 175–183 were replaced with glutamine and asparagine; *PAK1 H83/86L*, double mutation in the p21-binding domain which prevents GTPase binding; *PAK1 T423E*, mutation in the catalytic domain resulting in a highly constitutively active form of PAK1. The *numbers* in *parentheses* in *A* refer to the respective immunoblots in *B*.

sine, nor by GTPase. This supports the hypothesis that both stimuli induce activation by a similar mechanism.

The H83L/H86L mutation in the p21-binding domain of PAK1 inhibits GTPase binding and induces partial constitutive activation of the kinase (12). Sphingosine effectively stimulated autokinase activity of PAK1 (H83L/H86L), and the level of stimulation was even greater than that observed with wild type PAK1. We have previously shown that the PAK1 (H83L/H86L) mutant has a conformationally modified N terminus that is capable of enhanced interaction with SH3-containing proteins and of inducing polarized actin assembly (12). We interpret the current data as suggestive evidence that this partially activated conformation of the PAK1 (H83L/H86L) protein is also more sensitive to the stimulatory action of sphingosine. PAKs also contain a glutamic acid- and aspartic acid-rich region (ED domain) suggestive of a potential interaction domain for positively charged lipids. However, mutation of these residues to neutral amino acids had no effect on autokinase activity of PAK1 induced by sphingosine or GTPase (Fig. 6). Results with each mutant were also confirmed by phosphorylation of an exogenous substrate ($p47^{phox}$ peptide, data not shown).

Since a number of sphingoid base analogs exhibited little or no stimulation of PAK1 activity, we examined the possibility that they might compete with activating stimuli. As shown in Fig. 7, PAK1 activation induced by Cdc42-GTPγS was dramatically reduced in the presence of as little as 10 μM *N,N*-dimethylsphingosine (DMS). This inhibitory effect extended to both autophosphorylation and phosphorylation of exogenous substrates (including p47 peptide). DMS also antagonized PAK1 activation by sphingosine itself (not shown). A number of other lipids, including C_2- or C_8-ceramide, DL-dihydrosphingosine, phosphatidylserine, and dimyristoyl-phosphatidylcholine, had little or no inhibitory effect at concentrations up to 400 μM (Fig. 7). DMS thus appears to be a specific lipid inhibitor of PAK1 activation.

DISCUSSION

PAKs Are Lipid-regulated Kinases—The data presented here describe a third physiological mechanism through which mammalian PAKs can be regulated, *i.e.* through the stimulatory action of long chain sphingoid bases and several other biologically active lipids. It has previously been established that PAKs are also regulated by two other mechanisms as follows: the binding of GTP-bound Rac or Cdc42 to an N-terminal p21 interaction domain (7, 17) and, in the case of PAK2, by the caspase-mediated proteolytic removal of the regulatory N terminus (20). Only three classes of lipid out of a large variety of lipids tested were capable of effectively stimulating PAK activity, indicating this is a specific process that is not due solely to cationic/anionic groups associated with the lipid moiety nor to nonspecific hydrophobic- or detergent-like properties of lipids in general. In fact, we find that PAK activity tends to be inhibited in the presence of most ionic detergents.

We have utilized immunoprecipitates of PAK1 for most of the studies described here. We cannot definitively rule out that there is not a second kinase which tightly associates with and co-precipitates cellular PAK1 that is in fact the target for sphingosine and the other active lipids, a finding which would certainly be important in itself if true. However, the following several lines of evidence suggest this is unlikely: 1) PAKs isolated from at least three different cellular sources with five distinct antibodies are all responsive to sphingosine; 2) auto-

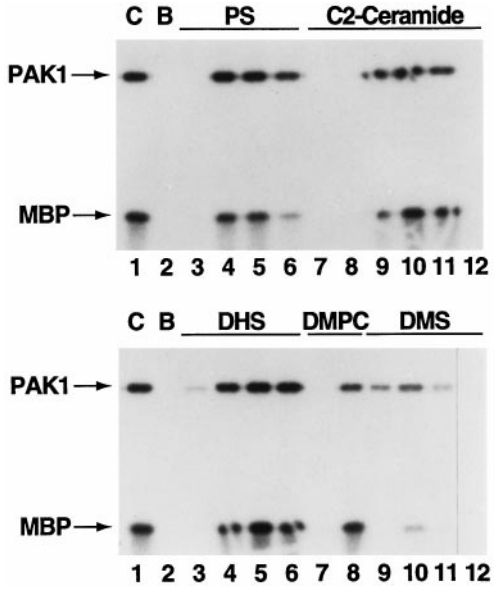

FIG. 7. **Inhibition of Cdc42-stimulated PAK1 by *N,N*-dimethylsphingosine.** PAK1 was expressed and immunoprecipitated from COS-7 cells and assayed for kinase activity in the presence of the indicated concentrations of lipid. All lanes contain immune precipitated PAK1. *Upper panel*: *lane 1*, *C*, 1 μg of Cdc42-GTPγS added; *lane 2*, *B*, buffer control; *lane 3*, 400 μM phosphatidylserine (*PS*) phosphatidylserine added; *lanes 4–6*, phosphatidylserine at 10, 40, and 400 μM with 1 μg of Cdc42-GTPγS; *lane 7*, buffer control; *lane 8*, 400 μM C_2-ceramide added; *lanes 9–11*, C_2-ceramide at 10, 40, and 400 μM with 1 μg of Cdc42-GTPγS; *lane 12*, buffer control. *Lower panel*: *lane 1*, 1 μg of Cdc42-GTPγS added; *lane 2*, buffer control; *lane 3*, 400 μM DHS (DL-dihydrosphingosine) added; *lanes 4–6*, DHS at 10, 40, and 400 μM with Cdc42-GTPγS; *lane 7*, 400 μM dimyristoylphosphatidylcholine (*DMPC*) added; *lane 8*, 400 μM dimyristoylphosphatidylcholine with Cdc42-GTPγS; *lane 9*, 400 μM DMS added; *lanes 10–12*, DMS at 10, 40, and 400 μM with 1 μg of Cdc42-GTPγS. *MBP*, 1 μg of myelin basic protein included as exogenous substrate in the assay.

phosphorylation and activation of PAK1 by sphingosine is dependent on the ability of PAK1 itself to bind ATP and exhibit catalytic activity; and 3) a recombinant, but partially activated, PAK1 of 99% purity is stimulatable by sphingosine to an extent similar to that induced by addition of Cdc42-GTPγS.

There were similarities and differences in PAK activation by lipids *versus* GTPases. Stimulation of PAK by lipid was not additive with activation by Cdc42 or Rac1, and the level of activity attained was similar with each class of activating agent. Tryptic mapping of PAK phosphorylated by either type of stimulus indicated that the major sites that became autophosphorylated on PAK1 were similar. However, there was an obvious difference between the number of phosphorylated species formed after stimulation by sphingosine (two) *versus* Cdc42 (three). Interestingly, phosphatidic acid, phosphatidylinositol, and monosialoganglioside (GM3) all induced formation of three phosphorylated species that appeared to comigrate with the three forms produced after activation by Rac1 or Cdc42, suggesting these lipids may act somewhat differently than do the sphingoid bases. The ability to induce formation of three autophosphorylated PAK species also correlated with the ability of each stimulus to enhance phosphorylation of myelin basic protein substrate by PAK1. Sphingosine did not stimulate PAK1 activity toward MBP but did enhance the phosphorylation of a $p47^{phox}$ peptide substrate, as well as of $p47^{phox}$ protein and Nck. Whether this indicates differential substrate specificity for the different phosphorylated PAK species remains to be determined.

Although we cannot determine the exact site(s) on PAK1 that activating lipids interact with, several pieces of evidence suggest this may be within or overlapping the p21-binding domain of the N terminus. We observed that mutations in the p21-binding domain either enhanced (H83L/H86L) or inhibited (Y107F, not shown) stimulation by sphingosine. Additionally, the inactive derivative DMS effectively blocked the ability of both sphingosine and Cdc42-GTPγS to activate PAK1. This inhibitory effect was specific for DMS *versus* several structurally related or distinct lipids (Fig. 7). DMS represents the first small molecular weight inhibitor of GTPase-mediated PAK activity that has been identified. Previous studies have reported potent inhibition of sphingosine kinase and protein kinase C by DMS. Inhibition of these three kinases by DMS suggests that they might possess similar sphingolipid binding domains. However, other data indicate differences in the lipid interaction sites of these enzymes as follows: D-*erythro*-sphingosine is a stereospecific substrate for sphingosine kinase (26), whereas all other stereoisomers of sphingosine, as well as sphinganine which lacks the 4,5-*trans* double bond, stimulate PAK kinase activity. Similarly, all stereoisomers of sphingosine, as well as sphinganine, are equipotent inhibitors of protein kinase C (27). It is also interesting to note that both PAK1 and sphingosine kinase, although the former is a protein kinase and the latter is a lipid kinase, are stimulated by acidic phospholipids such as phosphatidic acid and phosphoinositols (28). Moreover, in diverse cell types, sphingosine has been shown to uniformly increase the level of phosphatidic acid either by activation of phospholipase D and diacylglycerol kinase or by inhibition of phosphatidic acid phosphohydrolase (29). Thus, sphingosine can also potentially act as a positive feedback regulator of PAK activity due to its effect on phosphatidic acid levels.

Regulation of PAK by Lipids: a Mechanism for Activation by Membrane Association?—It is interesting to note that the *Acanthamoeba* myosin heavy chain kinase, which shares significant sequence homology with PAKs, has been reported to bind to and be activated by membranes and acidic phospholipids, particularly phosphatidylserine (30, 31). The activation of mammalian PAK1 by membrane association induced by the addition of membrane targeting sequences directly to PAK1 or to the PAK-interacting adapter protein Nck has been described (Ref. 16 and the current study). We show here that this stimulation of PAK induced by membrane association is not dependent upon the ability of PAK to interact with an activated GTPase, since stimulation of PAK activity also took place when mutation of the p21-binding domain of PAK prevented binding of Rac or Cdc42 (Fig. 1). Based upon our current findings, we propose that it is the direct association of PAK with lipids in the plasma membrane that stimulates its activity. This effect would potentially be regulated by agonist binding to receptors that stimulate the formation or metabolism of the stimulatory lipid species we have described here, most of which are believed to be largely present within the membrane rather than being cytosolic. We have shown in a previous study that stimulation of Swiss 3T3 cells with platelet-derived growth factor induces the association of PAK1 with a plasma membrane-enriched fraction, and this is associated with PAK1 activation and effects on the actin cytoskeleton (11). Platelet-derived growth factor is known to be an effective stimulus for the formation of sphingosine (28). It will be important to examine the role of sphingosine in growth factor-induced PAK regulation in future studies.

A Role for PAKs in Sphingolipid Signaling?—Sphingosine and related long chain sphingoid bases have been implicated in intracellular signaling pathways regulating a variety of cell functions. Among these are regulation of cell proliferation and cell death (27, 32), modulation of phagocyte oxidant production (33), and effects on cell morphology, adhesion, and the cytoskeleton (34, 35). The latter is perhaps most interesting in terms of

the possible involvement of PAK in sphingolipid signaling, since PAK is known to have regulatory effects on cell morphology and the cytoskeleton. Indeed, treatment of cells with sphingosine has been shown to stimulate the activity of several unidentified Ser/Thr kinases, including one with a molecular weight similar to that of PAK (36). Our data establish PAK(s) as potential downstream mediators of sphingolipid signaling and indicate that further investigation into the role of PAKs in sphingolipid signaling, as well as into the role of sphingolipids in PAK activation by physiological stimuli should be of substantial interest.

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