Reciprocal Signaling between Heterotrimeric G Proteins and the p21-stimulated Protein Kinase*

(Received for publication, June 18, 1999, and in revised form, July 21, 1999)

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p21-activated protein kinase (PAK)-1 phosphorylated Gaα, a member of the Gaα family that is found in the brain, platelets, and adrenal medulla. Phosphorylation approached 1 mol of phosphate/mol of Gaα in vitro. In transfected cells, Gaα was phosphorylated both by wild-type PAK1 when stimulated by the GTP-binding protein Rac1 and by constitutively active PAK1 mutants. In vitro, phosphorylation occurred only at Ser16, one of two Ser residues that are the major substrate sites for protein kinase C (PKC). PAK1 did not phosphorylate other Ga subunits (i1, i2, i3, o, s, or q). PAK1-phosphorylated Gaα was resistant both to RGSZ1, a Gz-selective GTPase-activating protein (GAP), and to RGS4, a relatively non-selective GAP for the Gi and Gq families of G proteins. Phosphorylation of Ser27 by PKC did not alter sensitivity to either GAP. The previously described inhibition of Gz GAPs by PKC is therefore mediated by phosphorylation of Ser16. Phosphorylation of either Ser16 or Ser27 by PAK1 or Ser27 by PKC decreased the affinity of Gz for Gβγ; phosphorylation of both residues by PKC caused no further effect. PAK1 thus regulates Gaα function by attenuating the inhibitory effects of both GAPs and Gβγ. In this context, the kinase activity of PAK1 toward several protein substrates was directly inhibited by Gβγ, suggesting that PAK1 acts as a Gβγ-regulated effector protein. This inhibition of mammalian PAK1 by Gβγ contrasts with the stimulation of the PAK homolog Ste20p in Saccharomyces cerevisiae by the Gβγ homolog Ste4p/Ste18p.

Protein kinases are the eventual downstream mediators of most signals initiated by G protein-coupled receptors. Mechanisms of kinase activation are diverse, however. They include both direct stimulation of cyclic AMP-dependent protein kinase and PKC by second messenger products of G protein-regulated effectors and less direct activation of tyrosine kinase and mitogen-activated protein kinase cascades. In yeast, heterotrimeric G proteins regulate members of the p21-activated protein kinase (PAK) family. In Saccharomyces cerevisiae, Ste20p is stimulated by Gβγ subunits (Ste4p/Ste18p) in response to mating pheromones (1, 2), and in Schizosaccharomyces pombe, the Ga subunit (Gpa1p) is the signal transducer to the Ste20p homolog Shk1p (3). The PAKs are mammalian homologs of Ste20p and Shk1p that were initially recognized as kinases that are activated by Rac and Cdc42, members of the Rho family of monomeric GTP-binding proteins (4). The PAKs also respond to heterotrimeric G proteins through pathways that include regulation of both GDP/GTP exchange factors and GAPs for Rac and Cdc42 (5–7).

Conversely, protein kinases modulate upstream signaling by heterotrimeric G proteins. Receptors are subject to feedback regulation by second messenger-activated kinases and G protein-coupled receptor kinases (8); G protein-regulated effectors are modulated by phosphorylation (9–12); and in a few cases, G proteins are themselves phosphorylated (13–16). Gaα, a sparsely expressed member of the Gi family, is phosphorylated by PKC both in platelets and in cells where it has been expressed artificially (13, 17–19). PKC-catalyzed phosphorylation decreases the affinity of Gaα for Gβγ subunits, potentially sensitizing Gα to activation because Gβγ inhibits GDP/GTP exchange. Phosphorylation by PKC also desensitizes Gz to the GAP activity of RGS proteins, which are widely thought to inhibit G protein signaling (20). PKC may potentiate Gz signaling through either of these mechanisms.

Gα is found primarily in neurons, platelets, and adrenal chromaffin cells, and its intracellular localization suggests that it may regulate formation, transport, or release of secretory granules (21–25). The ability of PAK1 to cause remodeling of cytoskeletal structures points to a role in regulating processes such as cell motility and secretion, and PAK1 has been implicated directly in the Fcy receptor-mediated respiratory burst and cytokine secretion (26). Few natural PAK substrates are known, however; but PAK1 probably phosphorylates proteins that regulate either cytoskeletal disassembly or the cytoskeletal elements themselves.

We report here that Gaα is phosphorylated specifically at Ser16 by PAK1, thus inhibiting its interaction with both Gβγ and RGS proteins. We have used this specificity to distinguish and delineate the functional consequences of phosphorylation at Ser27 and Ser16, which we show to be the two principal substrate sites for PAK. An unexpected outcome is the finding that Gβγ, which stimulates the PAK homolog Ste20p in Saccharomyces, inhibits the activity of mammalian PAK1 toward both Gaα and other substrates.

EXPERIMENTAL PROCEDURES

Plasmids and cDNA—Mammalian expression vectors for full-length PAK1, the constitutively active mutant PAK1-(165–544) (N-terminal truncation leaving residues 165–544) (27), and G12V Rac1 were prepared in pCMV5M (pCMV5 modified to include a Myc epitope tag (27)) as described previously (27). The G12V Rac1 mutation was prepared using a QuickChange mutagenesis kit (Stratagene), and the cDNA was inserted into pCMV5. Mammalian expression vectors for wild-type Gaα and its S16A, S23A, S27A, and S16A,S25A mutants were constructed in...
pDF5 and were gifts from D. Manning (University of Pennsylvania) (17). The S16A,S25A,S27A triple mutant was prepared using the QuickChange kit with the S16A,S27A construct as template. Recombinant baculoviruses expressing the Gα mutants were prepared as described previously for wild-type Gαs (28).

Protein Expression and Purification—Wild-type and mutant Gαs and Gβγ subunits, other than Gαo, were expressed in Sf9 cells and purified as described (28, 29). Gαq was expressed in Escherichia coli with or without yeast protein N-myristoyltransferase (30) and purified as described (31). Gαs and Gαq were gifts from S. Mukhopadhyay and T. Kozasa (this department). Wild-type PAK1 and PAK1-(232–544) were expressed in E. coli as glutathione S-transferase fusion proteins and purified by glutathione-agarose affinity chromatography (27). To prevent proteolysis, wild-type GST-PAK1 was purified in the presence of 20 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Purified PAK1 and PAK1-(232–544) were dialyzed against 20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1 mM diithiothreitol, 1 mM EDTA, and stored at −80 °C. The protein kinase TAO1 (32) was a gift from K. Berman (this department), and PKCα was a gift from T. Kozasa (this department). Purified phosducin (33) was a gift from R. Gaudet and P. Sigler (Yale University).

Protein Kinase Assays and Protein Phosphorylation—Phosphorylation of Gαs by PKCα was performed exactly as described (28, 29). Gα subunits were phosphorylated by PAK1 at 30 °C for 60 min or the times indicated in 50 mM Hapes (pH 8.0), 10 mM MgCl2, 1 mM diithiothreitol, and 0.5 mM ATP. Phosphoamino acid analysis and tryptic phosphopeptide mapping of Gαs were performed as described (34). Partial tryptic proteolysis after protection of phosphorylated Gαs by GTPγS was performed exactly as described (35). Under these conditions, trypsin cleaves Gαs, after Arg(23)(35). Protein kinase assays using MBP as substrate were performed as described (36).

In Vivo Phosphorylation—Human embryonic kidney fibroblasts (HEK-293 cells) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. For transfection, cells were grown in 60-mm culture dishes to ~70% confluence and then transfected by calcium phosphate precipitation (27). Twenty hours after transfection, the medium was replaced by Dulbecco’s modified Eagle’s medium without serum, and the cells were incubated for another 24 h. For determination of in vivo phosphorylation of Gαs, transfected cells were washed once with phosphate-free Dulbecco’s modified Eagle’s medium and incubated for 2–3 h in phosphate-free Dulbecco’s modified Eagle’s medium plus [32P]Pi (0.5 mM/ml). For harvesting, cells were washed once with phosphate-buffered saline and scraped into 0.5 ml of radioimmune precipitation assay buffer (50 mM sodium P, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1 mM diithiothreitol, 10 μg/ml aprotinin, 1% sodium deoxycholate, and 1% Nonidet P-40) that contained 0.2% SDS, 80 mM β-glycerophosphate, 0.5 mM Na3VO4, 50 mM NaF, 20 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A. Lysates were

![FIG. 1. Phosphorylation of Gα subunits by PAK1. Purified recombinant G protein subunits Gαs, Gαq, Gαq (non-myristoylated), Gαo (myristoylated; i1), Gαs, Gαo, Gαo, and Gαo (1.25 pmol each) and Gγ (0.75 pmol) were incubated with partially purified wild-type GST-PAK1 (~0.1 pmol) and [γ-32P]ATP (3 cpm/pmol) for 60 min at 30 °C as described under “Experimental Procedures.” All Gα subunits were expressed in SF9 cells except for Gαo, which was expressed in E. coli either with (i1) or without (i2) coexpression of N-myristoyltransferase. Samples were resolved by SDS-polyacrylamide gel electrophoresis, silver-stained (Ag; lower panel), and exposed to x-ray film (22P, upper panel). Molecular mass markers (in kilodaltons) are shown on the left. This complete experiment and a similar experiment using ~0.5 pmol of PAK1-(232–544) yielded identical results.]
Reciprocal Regulation of Ga, Gγ, and PAK

RESULTS

PAK1 Phosphorylates Ga Selectively at Ser16—The protein kinase PAK1 phosphorylated Ga in vitro, but did not phospho-
rylate several other Ga subunits tested (Fig. 1). PAK1-catalyzed phosphorylation of Ga was efficient relative to that catalyzed by PKCa, and the truncated protein PAK1-(232–544) displayed activity similar to that of the wild-type kinase. We were unable to detect phosphorylation of Ga by either TAO1 or protein kinase A (data not shown).

PAK1 phosphorylated Ga within the first 29 amino acid residues because all 32P was removed by limited tryptic proteolysis (Fig. 2A), which cleaves after Arg29 (35). Phosphoamino acid analysis of phosphorylated Ga detected only phosphoserine (data not shown), consistent with the absence of Thr residues in this region. In contrast to the site specificity displayed by PAK1, PKC phosphorylated at least one additional site C-terminal to Gln30, usually accounting for ~10% of the total incorporation of phosphate (Fig. 2A, lane 12). We then used serine mutants of Ga to determine the site of PAK1-catalyzed phosphorylation near the N terminus. PAK1 catalyzed the phosphorylation of Ga to ~1 mol of phosphate/mol of Ga and phosphorylation was blocked completely by mutation of Ser16 to Ala. Phosphorylation was not diminished by mutation of either Ser29 or Ser27 (Fig. 2, B and C). Longer incubation with PAK1 or the use of more PAK1 did not cause further phosphorylation of Ga (data not shown). PAK1 thus selectively phosphorylates Ser16 of Ga.

In contrast to PAK1, PKC catalyzed the addition of 2 mol of phosphate/mol of Ga, and phosphorylation was decreased by about half when either Ser16 or Ser27 was mutated (Fig. 2, B and C). Both residues are thus PKC substrate sites. The time courses of phosphorylation of S16A and S27A Ga suggest that Ser27 is the kinetically preferred PKC substrate (Fig. 2C). Such preference agrees with the conclusion of Lounsbury et al. (17) that Ser27 is the major phosphorylation site in transfected 293 cells, although cellular phosphorylation might also be influenced by selectivity of whatever protein phosphatases naturally dephosphorylate Ga. We have not attempted to map the minor, more C-terminal PKC phosphorylation site on Ga.

To determine whether PAK1 also phosphorylates Ga in vivo, we expressed Ga in HEK-293 cells and measured its differential steady-state phosphorylation upon coexpression with PAK1, with or without the PAK activator G12V Rac1 (Fig. 3). Ga was phosphorylated by endogenous HEK-293 cell kinases during the 3-h incubation with [32P]Pi, primarily at one

![Fig. 3. Racl-stimulated phosphorylation of Ga at Ser16 by PAK1 in HEK-293 cells. HEK-293 cells were transfected with plasmids that encode wild-type or mutant Ga, wild-type (WT) PAK1 or PAK1-(165–544), and G12V Rac1 as shown. [32P]Pi, was added to the medium; after 3 h, cells were harvested, and Ga was immunoprecipitated and analyzed by gel electrophoresis as described under "Experimental Procedures." In some cases, 12-O-tetradecanoylphorbol-13-acetate (TPA; 0.1 μM) was added 5 min before harvest to stimulate endogenous PKC. The amount of Ga in each immunoprecipitate was determined by comparing the density of bands in immunoblots of the immunoprecipitates (A, WB) with samples of purified Ga on the same blot (data not shown). Incorporation of [32P]Pi was determined by scintillation counting of radioactivity in each sliced](image)

![Fig. 4. Phosphorylation of Ga or MBP by PAK1 or PKC is independent of the presence of active or inactive Ga. PKCa (0.5 pmol) or PAK1-(232–544) (2 pmol) was incubated for 20 min at 30 °C under standard phosphorylation conditions with Ga (5 pmol) and/or MBP (10 pmol), with or without 10 mM NaF and 30 μM AlCl3 (AMF) as indicated. Samples were analyzed by electrophoresis, Coomassie Blue staining, and autoradiography. Similar results were obtained with Ga activated with GTPγS or with full-length PAK (data not shown).](image)
Reciprocal Regulation of Ga, Gbg, and PAK

Effects of phosphorylation of Ga, at Ser16 and/or Ser27 on its interaction with Gbg. Ga was phosphorylated by either PAK1 or PKCα by incubation at 30 °C for 90 min as described in the legends to Figs. 1 and 2. After adjusting free [Mg2+] to 1 mM with EDTA, the samples (2.2 pmol/assay point) were preincubated for 10 min at 0 °C in binding buffer with or without Gbg. Binding of [35S]GTPγS (20 μM, 1500 cpm/pmol) was then determined as described (28). A, GTPγS binding to either PAK1-phosphorylated (●, ○) or control (●) wild-type Ga was measured in the presence (●, ○) or absence (△, ○) of 100 pmol of Gbg. Each curve was fit to a first-order reaction scheme, and all four curves share the same value of maximal GTPγS binding. B–D, the rate constants for GTPγS binding to wild-type (WT) Ga, S16A Ga, S27A Ga, or S16A,S27P Ga, respectively, were determined at increasing concentrations of Gbg (closed symbols) or not (open symbols; no kinase added) as shown. Data are means of duplicate determination in two or three experiments (n = 4 or 6).

Phosphorylation of Ga was slightly increased by coexpression of wild-type PAK1 and was further increased when both PAK1 and the constitutively activated G12V mutant of Rac1 were present. It was difficult to quantitate the in vivo phosphorylation of Ga by PAK1 because an unknown fraction of Ser16 may already be phosphorylated prior to addition of 32P and because considerable phosphorylation by other kinases occurred at Ser27 (Fig. 3A, lanes 6–8). However, phosphorylation of the S25A,S27P Ga mutant showed that wild-type PAK1 plus G12V Rac1 can at least double the incorporation of 32P into Ser16 of Ga and that the activated mutant PAK1-(165–544) can increase labeling by 50%. For reference, phosphorylation of Ga by endogenous PKC was monitored by stimulating the cells with phorbol ester, which increased phosphorylation of both wild-type and S16A Ga by 2–2.6-fold (Fig. 3) (17). Phosphorylation of Ga at sites C-terminal to Arg28 was relatively minor (Fig. 3B, lanes 8–14).

Activation of Ga, and PAK1-catalyzed Phosphorylation—Activation of Ga by AIFc had no effect on its phosphorylation by either PAK1 or PKC (Fig. 4). Activation by GTPγS was also without effect (data not shown). Conversely, neither PAK1-catalyzed phosphorylation of MBP or PAK1 auto phosphorylation was altered by Ga when bound to GDP/AIFc (Fig. 4), GDP, or GTPγS (data not shown).

Phosphorylation of Ga at Either Ser16 or Ser27 Inhibits Binding to Gbg—To determine whether PAK1-catalyzed phosphorylation of Ga decreases its affinity for Gbg, as is true for PKC (18), we monitored the effect of phosphorylation on the concentration dependence with which Gbg inhibits GDP/GTPγS exchange. As shown in Fig. 5A, phosphorylation of Ga by PAK1 markedly attenuated the ability of Gbg to inhibit nucleotide exchange on Ga, but had no effect on the intrinsic nucleotide exchange rate. This attenuation reflected a 10–20-fold decrease in the affinity of phospho-Ga for Gbg (Fig. 5B and D). PAK1 and PKC inhibited Gbg binding equally. Furthermore, phosphorylation of Ser16 by PAK1 alone (Fig. 5, B and D) or of Ser27 by PKC in the S16A mutant (Fig. 5C) decreased Ga-Gbg affinity equally. Phosphorylation of both residues by PKC had no greater effect than phosphorylation of only one or the other. Although mutation of either residue to Ala itself decreased affinity for Gbg somewhat (IC50 shifted...
Reciprocal Regulation of $G_{\alpha_z}$, $G_{\beta\gamma}$, and PAK

Phosphorylation of $G_{\alpha_z}$ at Ser$^{16}$ Blocks the GAP Activity of RGS Proteins—Phosphorylation of $G_{\alpha_z}$ by PKC blocks the GAP activity of RGS proteins (29, 38). Selective phosphorylation of Ser$^{16}$ by PAK1 also substantially inhibited the GAP activities of both RGSZ1 and RGS4 (Fig. 6 and Table I). RGS4 was inhibited by $>$96%. RGSZ1 was inhibited by $\sim$15 and 25% respectively (Table I). Proteolysis of the N-terminal 29 amino acid residues of $G_{\alpha_z}$ provided a control to show that the effects of mutation or phosphorylation of Ser$^{16}$ and Ser$^{27}$ are local. Limited tryptic proteolysis increased the $K_{d_{\text{hydrol}}}$ for each of these proteins to $\sim$0.022 min$^{-1}$ (data not shown), the value characteristic of the proteolyzed wild-type, non-phosphorylated protein (35).

Addition of $G_{\beta\gamma}$ to phosphorylated $G_{\alpha_z}$ had little further inhibitory effect on GAP activity (Fig. 5). The slight inhibition that was observed occurred primarily below 200 nM $G_{\beta\gamma}$, well below the $K_i$ of phospho-$G_{\alpha_z}$ for $G_{\beta\gamma}$. Residual inhibition by $G_{\beta\gamma}$ probably reflects effects on the small fraction of $G_{\alpha_z}$ that was not phosphorylated during incubation with kinase.

In addition to blocking phosphorylation, mutation of either Ser$^{16}$ or Ser$^{27}$ to Ala also inhibited the intrinsic sensitivity of $G_{\alpha_z}$ to GAP activity (Fig. 6 and Table I). Sensitivity of non-phosphorylated S16A $G_{\alpha_z}$ to either RGSZ1 or RGS4 was equivalent to that of phospho-Ser$^{16}$ $G_{\alpha_z}$ and was not further altered by phosphorylation of Ser$^{27}$. S27A $G_{\alpha_z}$ was more sensitive to GAP activity than was S16A $G_{\alpha_z}$ but was still a far worse GAP substrate than wild-type $G_{\alpha_z}$. It was striking that PKC-catalyzed phosphorylation at Ser$^{27}$ in the S16A mutant reproducibly increased its sensitivity to GAPs. Although this effect was relatively small, it was a consistent finding in multiple experiments. The small inhibitory effect of $G_{\beta\gamma}$ on the sensitivity of mutated or phosphorylated $G_{\alpha_z}$ to GAPs was similarly reproducible.

The intrinsic rates at which S16A and S27A $G_{\alpha_z}$ hydrolyzed bound GTP were diminished in comparison to the wild type, by $\sim$15 and 25% respectively (Table I). Proteinase K digestion of the N-terminal 29 amino acid residues of $G_{\alpha_z}$ provided a control to show that the effects of mutation or phosphorylation of Ser$^{16}$ and Ser$^{27}$ are local. Limited tryptic proteolysis increased the $k_{d_{\text{hydrol}}}$ for each of these proteins to $\sim$0.022 min$^{-1}$ (data not shown), the value characteristic of the proteolyzed wild-type, non-phosphorylated protein (35).

$G_{\beta\gamma}$ Inhibits the Protein Kinase Activity of PAK1—During the course of the experiments described above, we noticed that $G_{\beta\gamma}$ reproducibly inhibited the protein kinase activity of PAK1 (232–544) (Fig. 7A). Inhibition was detectable by 80 nM and was half-maximal at $\sim$200 nM, well within the range of concentrations over which other GAPs have been described (39). Similar results were obtained with full-length, wild-type PAK both before and after removal of the fused GST domain (data not shown). The buffer used to store $G_{\beta\gamma}$ had no effect on PAK1 activity (Fig. 7A). $G_{\beta\gamma}$ also inhibited the ability of PAK1 to phosphorylate either MBP (Fig. 7) or MEK1 (data not shown). $G_{\beta\gamma}$ thus appears to inhibit PAK directly rather than simply binding the $G_{\alpha_z}$ substrate and blocking access to the kinase. The inhibitory activity of $G_{\beta\gamma}$ was relatively specific for PAK. It had no effect on the protein kinase activities of either protein kinase A or TAO1 in two separate experiments and inhibited PKC insignificantly (10–15% at the highest concentrations tested).

$G_{\beta\gamma}$ slightly but reproducibly stimulated PAK1 autophosphorylation in the presence or absence of added substrate (example in Fig. 7A), indicating that $G_{\beta\gamma}$ binds directly to PAK1 to alter its function. In yeast, $G_{\beta\gamma}$ also binds (and stimulates) the PAK homolog Ste20p directly (1, 2), and its stimulatory activity is blocked by binding to $G_{\alpha}$. Surprisingly, $G_{\beta\gamma}$ was a potent inhibitor of PAK1 protein kinase activity both when free...
or when complexed as a heterotrimer with GDP-bound Go or Go (Fig. 7, A and B) or Go (data not shown). Addition of the Gβγ-binding protein phosducin (up to 6.7 μM) also failed to reverse inhibition by Gβγ (Fig. 7B).

**DISCUSSION**

Mammalian PAKs were discovered as effectors of the small G proteins Rac and Cdc42, although the yeast homolog Ste20p was first identified as a protein kinase activated by Gβγ. Our current findings now indicate that PAKs function both upstream and downstream of heterotrimeric G proteins in animal cell signaling pathways. First, PAK1-catalyzed phosphorylation potentiates Gz activation by inhibiting the GAP activity of RGS proteins, including the RGSZ subfamily of Gz-selective GAPs. Second, phosphorylation of Go decreases its affinity for Gβγ subunits and thus attenuates the inhibitory effects of Gβγ on Gz activation. The net result is a two-pronged potentiation of Gz signaling by PAK. The reduced affinity of phospho-Go for Gβγ will promote Gβγ release and might thereby potentiate Gβγ signaling, but Go is expressed at such low levels that it may release too little Gβγ to have significant impact on intracellular signaling.

Several lines of evidence are consistent with the hypothesis that PAK phosphorylates Go under physiological conditions in cells. *In vitro*, PAK1-phosphorylated purified Goα to a stoichiometry of 1 mol of phosphate/mol of Goα. The rate of phosphorylation and therefore Gz activation was also reasonably fast in comparison with PKC, which phosphorylates Goα in platelets stimulated by either thrombin or phorbol ester (13, 19). Phosphorylation of Goα in HEK-293 cells was increased by expression of constitutively active PAK1 or of wild-type PAK1 and its activator Rac. PAK1-driven incorporation of [32P] into Go was of the same order as that catalyzed by PKC in response to 12-O-tetradecanoylphorbol-13-acetate despite the fact that PKC phosphorylates Goα on two sites rather than one. Finally, phosphorylation of Goα by PAK is associated with altered function of the protein as discussed more fully below. We conclude that stimulation of PAKs, via the activation of either Rac or Cdc42, provides a novel means of potentiating the cellular function of Goα.

PAK1 displays marked selectivity for Goα relative to other Gα subunits and for Ser16 relative to other potential phosphorylation sites in Gz. Selectivity for Ser16 allowed us to delineate the individual contributions of phosphorylation of Ser16 and Ser27 to regulation of Goα in a manner not possible using mutagenesis. Phosphorylation of Ser16 was both sufficient and necessary to decrease sensitivity to the GAP activity of RGS proteins (Fig. 6 and Table I). It was also sufficient to decrease affinity for Gβγ. PKC-catalyzed phosphorylation of Ser27 in the S16A mutant also decreased affinity for Gβγ. On the other hand, phosphorylation of both residues in wild-type Goα had no more effect than phosphorylation of Ser16 alone. These data confirm the idea that the extreme N-terminal helix of Gα subunits is crucial for interaction with RGS proteins (29, 35), despite the fact that this interaction was not observed in the crystal structure of the Gα1-RGS4 complex (40).

The ability of Gβγ subunits to inhibit the protein kinase activity of PAK1 is provocative for two reasons. First, it suggests that the PAKs may be a new family of heterotrimeric G protein-regulated effectors. Inhibition of PAK1 by Gβγ was nearly complete, was effective with multiple protein substrates, and occurred over a physiological range of Gβγ concentrations. Second, PAK inhibition by Gβγ extends the pattern of G protein regulation of the PAK family that was established in yeast. However, whereas Shk1p is activated, perhaps indirectly, by Go in *S. pombe* and Ste20p is directly activated by Gβγ in *S. cerevisiae*, PAK1 is directly inhibited by mammalian Gβγ. The ability of Gβγ to regulate PAK1 (232–544) also indicates that the Gβγ-binding site on PAK is unrelated to the binding site for Rac and Cdc42, which lies near the PAK N terminus (41). A tantalizing possibility is that mammalian G proteins may regulate PAKs through multiple mechanisms.

Inhibition of PAK by Gβγ in vitro fulfilled most criteria for physiological validity, so it was initially puzzling that inhibition was not blocked either by GDP-bound Goα or by phosducin. However, yeast Gβγ binds Ste20p through the N-terminal helix of Gβ (2, 42), which is not occluded in the phosducin-Gβγ complex (33). Whereas the N-terminal helix of Gβ makes extensive contact with Go (43, 44), the face of the helix that binds PAK may remain accessible in some conformations of the Goα-Gβγ heterotrimer. Contact sites for other Gβγ-regulated effectors cluster on the face of the Gβ torus rather than near its N terminus (45) and are thus fully blocked by Goα. It should be possible to use such structural information to evaluate the biological relevance of PAK inhibition by Gβγ in cells where PAK activity can be monitored in response to receptor-regulated Gβγ release.

**Acknowledgments**—We thank Jimmy Woodson and Steven Stipeck for excellent technical assistance, David Manning for the plasmids that encode mutant forms of Goα, Kevin Berman for TAO1, Tohru Kozasa for PKCα, and Rachel Gaudet and Paul Sigler for phosducin.

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