Gβγ subunits stimulate p21-activated kinase 1 (PAK1) through activation of PI3-kinase and Akt but act independently of Rac1/Cdc42

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Abstract The p21-activated kinase (PAK) family is homologous to the yeast sterile 20 (Ste20) and regulates a wide variety of cellular responses, including cell morphology, proliferation, and survival. In this study we examined the activation of PAK1 by Gβγ subunits. Co-transfection of COS7 cells with Gβ1γ2 or Gβ1γ5 was sufficient to induce agonist-independent activation of PAK1. Expression of dominant/negative Rac, Cdc42, or Ras did not inhibit this Gβγ-dependent activation. Wortmannin, which inhibits phosphoinositide 3-kinase (PI3-kinase) activity, and expression of a dominant/negative form of Akt were sufficient to abrogate the activation of PAK1 that was induced by Gβγ. These results reveal that stimulation of PAK1 by Gβγ can occur via a PI3-kinase and Akt pathway that does not require Rac1 or Cdc42.

Key words: p21-Activated kinase; G protein-coupled receptor; Serine–threonine kinase

1. Introduction

The protein kinases of the mitogen-activated protein kinase (MAPK) family are a highly conserved group from yeast to man. MAPKs phosphorylate specific serines and threonines of various target proteins and regulate cellular activities such as proliferation, cytoskeletal rearrangement, and cell survival [1–3]. There is evidence that activation of MAPK cascades can follow phosphoinositide 3-kinase (PI3-kinase) stimulation depending on the cell type and stimulus used [4,5]. The phosphorylated lipids produced by activated PI3-kinase, predominantly phosphatidyltinositol 3, 4, 5 trisphosphate (PIP3) and PI(3,4)P2, activate downstream protein kinases Akt and PDK. PIP3 and PI(3,4)P2 bind with high affinity and specificity to the PH domain of Akt [6]. This binding induces translocation of Akt to the plasma membrane and subsequent phosphorylation of Thr-308 and Ser-473 [7–9].

In addition to Akt, the Rho GTPases Rac1 and Cdc42 have been identified as downstream effectors of PI3-kinase [10,11]. The Rho family GTPases link the plasma membrane receptors to the assembly and organization of the actin cytoskeleton. The Rho GTPase cascade has been shown to be activated at different points by various extracellular stimuli [12]. The formation of actin stress fibers can be achieved by treatment of quiescent cells with lysophosphatidic acid (LPA), while growth factors such as PDGF and insulin stimulate polymerization of actin at the plasma membrane of many cell types to induce lamellipodia formation and surface ruffling in a PI3-kinase-dependent manner [13]. The activation of Cdc42 and Rac can stimulate the activity of downstream effectors, such as a family of serine–threonine kinases: p21-activated kinases (PAKs) [14–17].

Screens for binding partners of Rac and Cdc42 GTPases in rat brain slices revealed that a member of the Ste20-like serine–threonine protein kinase family, PAK, could bind and be activated by GTP bound forms of these GTPases [16]. In *Saccharomyces cerevisiae*, Ste20 is stimulated by Gβγ subunits (Ste4/Ste18) in response to mating pheromones [18,19]. PAKs 1, 2, and 3 comprise the Group I PAKs and PAKs 4, 5, and 6 comprise the Group II PAKs. In mammalian tissues PAK1 is highly expressed in brain, muscle, and spleen; PAK2 is expressed ubiquitously, PAK3 is expressed in the brain [20], PAK4 is expressed in most tissues, with high levels of expression in the testis and colon [21,22], PAK5 is expressed in the brain [23], and PAK6 is expressed at the highest level in the testis and prostate [24]. All PAK proteins identified to date share a similar N-terminal motif stretching over 18 amino acids that binds to Cdc42 and Rac and is termed the Cdc42/Rac-interactive binding domain (CRIB) [25,26]. Group I PAKs have an autoinhibitory domain (AID) that overlaps the CRIB domain, this AID is lacking in the Group II PAKs. The activation of Group I PAKs is enhanced when GTP-bound Rac or Cdc42 bind the CRIB domain. The activation of the Group II PAKs is distinct, however, as they are independent of Rac/Cdc42 binding [21,23,24].

In this study we investigated the role of Gβγ subunits in PAK1 activation. Cells that overexpress G protein β1γ5 and Gβ1γ2 subunits have agonist-independent activation of PAK1 that is insensitive to dominant-negative Rac1 or Cdc42. This Gβγ-dependent activation was also insensitive to a selective epidermal growth factor receptor (EGFR) inhibitor, AG1478, and a protein kinase C (PKC) inhibitor, bisindolylmaleimide (BIS), but was sensitive to an inhibitor of PI3-ki-

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Abbreviations: PAK, p21-activated kinase; MBP, myelin basic protein; LPA, lysophosphatidic acid; EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; MAPK, mitogen-activated protein kinase; HA, haemagglutinin; PI3-kinase, phosphoinositide 3-kinase; CRIB domain, Cdc42/Rac-interactive binding domain; AID, autoinhibitory domain

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nase, wortmannin, and to expression of dominant-negative Akt. Our results support a model where PAK1 can be stimulated by Gβγ subunits via PI3-kinase and Akt, but independently of Rac and Cdc42.

2. Materials and methods

2.1. Plasmids and antibodies

Antibodies to the N- and C-terminus (N20 and C19) of PAK1 were purchased from Santa Cruz Biotechnology, with the former used for immunoprecipitation and the latter for Western blotting. The antibodies to PhosphoPAK1 (Thr-423)/PAK2 (Thr-402), to PhosphoAkt (Ser-473), and to total Akt were purchased from Cell Signalling Technology. Plasmids used in this study include pCDhM2 [27], pRK7myc-PAK1 prepared from pCMV6mycPAK1 plasmid (that was a kind gift from Dr. Gary Bokoch) by subcloning a *Bam*HI/*Eco*RI fragment into pRK7, pRK5Gβ1, pRK7Gγ2, and pRK7Gγ5 and pKH3 with inserts for RacN17, Cdc42N17, and RasN17 [27]. The pKH3 plasmid expresses inserts with a triple haemagglutinin (HA) epitope tag at the N-terminus as described previously [12,28]. Plasmids expressing dominant-negative Akt, pCMV6.HA.Akt.K/M and active Akt, pCMV6.myr.Akt.HA, were kind gifts from Dr. Alex Toker.

2.2. Cell culture and transfection assays

COS7 cells were cultured as described previously [28–30]. COS7 cells were transfected by calcium phosphate precipitation with 2 μg pRK7myc-PAK1 plasmid and 3 μg vector control or test plasmids [30]. Cells were grown for 48 h after transfection. Cells that were treated with LPA were serum starved for 24 h prior to treatment with the agonist.

2.3. Agonist treatment and immunoprecipitation for COS7 transfectants

The COS7 cells were treated with agonists and inhibitors and then the PAK1 immunoprecipitated as described [31]. PAK1 activity was assayed [32] with modifications as described [31]. Representative results are shown with data reported as mean ± S.E.M. from three independent experiments. Whole cell lysates for Western blotting of endogenous PAK and Akt (Fig. 6) were prepared as described [29].

3. Results

3.1. Activation of PAK1 by Gβγ subunits can occur in the presence or absence of agonist stimulation

In yeast models it has been noted that Ste20, a PAK homologue, binds to and is activated by Gβγ subunits [17,18]. To investigate the potential role of Gβγ in cell signaling through PAK1 in mammalian cells, we co-transfected COS7 cells with mycPAK1 and combinations of Gβ1γ5 or Gβ1γ2. Overexpression of Gβ1γ5 or Gβ1γ2 yielded a 4.2-fold increase in PAK1 activity when compared to control mycPAK1 transfectants. Treatment of the Gβ1γ2 or Gβ1γ5 co-transfectants with LPA did not significantly further elevate the level of PAK1 activity. Similarly, carbachol, EGF, and phorbol 12-myristate 13-acetate (PMA) were also unable to increase PAK1 activity beyond that induced by Gβ1γ5 (Fig. 1). These results reveal that overexpression of Gβγ subunits is sufficient to induce PAK1 activation.

3.2. Inhibition of PI3-kinase decreases Gβγ activation of PAK1

Overexpression of Gβγ results in activation of PAK1. To investigate whether this stimulation was dependent on the activity of intermediate kinases, cells co-transfected with Gβ1γ5 and mycPAK1 were incubated with selective inhibitors of PI3-kinase or EGFR or PKC for 30 min prior to cell lysis. No inhibition of Gβγ-dependent PAK 1 activation was detected following AG1478 or BIS administration (Fig. 2), although these inhibitors will block EGF or phobol ester-dependent PAK1 activation [31]. Inhibition of PI3-kinase by wortmannin reduced the Gβγ-dependent activation of PAK1 from 4.2- to 1.4-fold over control (Fig. 2). We have previously shown that wortmannin does not by itself inhibit PAK1 activity, as it did not reduce phorbol ester- or LPA-dependent PAK1 activation [31]. The inhibition by wortmannin therefore indicates that Gβγ requires PI3-kinase to activate PAK1 in these cells. This result also suggests that PAK1 activation by Gβγ is mediated through a reversible mechanism, since the wortmannin is only present for the last 30 min of the incubation of the co-transfected cultures.

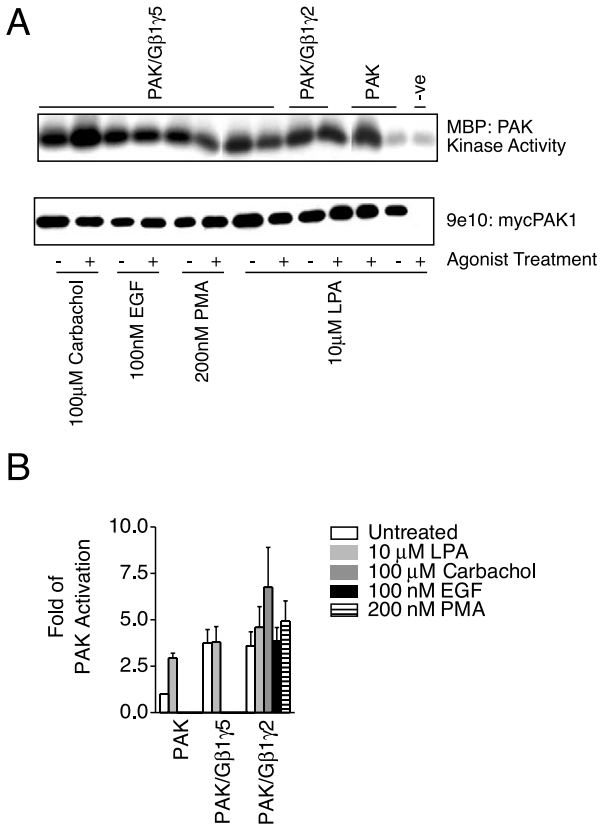


Fig. 1. Activation of PAK1 by Gβγ subunits can occur in the presence or absence of agonist stimulation. COS7 cells were transfected with mycPAK1 and Gβ1γ5 or Gβ1γ2 (plus hM2 for those to be stimulated with carbachol) and then treated as shown. A: Overexpression of Gβ1γ5 or Gβ1γ2 increased PAK1 activity when compared to untreated mycPAK1 transfectants. Western blots of the immunoprecipitates were probed with 9e10 antibody to detect myc-tagged PAK1. pRK7 plasmid was used as a negative vector control (−ve). MBP was used as a substrate to detect PAK1 phosphorylation in an in vitro kinase assay. B: Graph depicting activation of PAK1 in cells co-transfected with Gβγ subunits.

3.3. Gβ1γ5 activation of PAK1 is independent of Rac, Cdc42, and Ras

To investigate the role of Rac/Cdc42 binding to the N-terminal CRIB domain of PAK1 in the Gβγ-dependent activation, dominant-negative HARacN17 or HACdc42N17 were overexpressed in Gβ1γ5/PAK1 co-transfectants. Surprisingly, the overexpression of dominant-negative Rac or Cdc42 did not inhibit the activation of PAK1 (Fig. 3), indicating that the activation of PAK1 by Gβ1γ5 is independent of the interaction of these GTPases with the CRIB domain of PAK1.

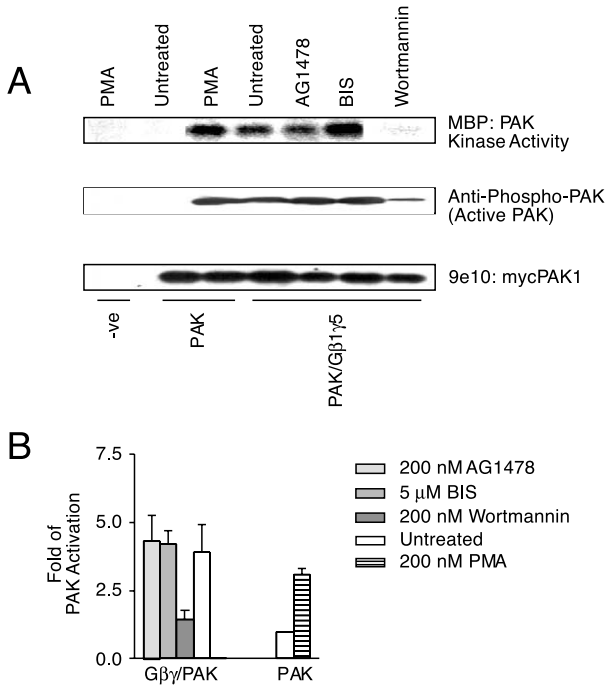


Fig. 2. Inhibition of PAK1 activation by wortmannin. COS7 mycPAK1/Gβ1γ5 transfectants were untreated or treated with the selective EGFR inhibitor AG1478 (200 nM), the PI3-kinase inhibitor wortmannin (200 nM), or the PKC inhibitor BIS (5 μM) for 30 min. A: MBP was used as a substrate to detect PAK1 phosphorylation in an in vitro kinase assay. Western blots of the immunoprecipitates were probed first with 9e10 to detect mycPAK1, then stripped and reprobed with a PhosphoPAK1 (Thr-423)/PAK2 (Thr-402) antibody (Cell Signalling Technology). pRK7 plasmid was used as a negative vector control (−ve). B: Graph depicting activation of PAK1 as observed by assay of MBP phosphorylation.

Under these same conditions, expression of dominant-negative Rac1 or Cdc42 is sufficient to completely block LPA (shown) or EGF or phorbol ester stimulation of PAK1 [31]. Co-transfections with dominant-negative Ras, HARasN17, also failed to inhibit Gβγ-dependent PAK1 activation, suggesting that Ras activation is also not required.

3.4. Gβ1γ5 activation of PAK1 occurs through Akt

It has been established that Akt is a downstream effector of PI3-kinase [33–35]. We therefore investigated the role of Akt in PAK1 stimulation by Gβγ. Co-transfection of mycPAK1 and a constitutively active Akt (myrAkt) revealed an activation of PAK1 that was similar to that induced by Gβ1γ5 (Fig. 4). The expression of dominant-negative Akt (AktK/M) in the mycPAK1/Gβ1γ5 co-transfectants blocked PAK1 activation. Stimulation of PAK1 activity was also assayed following treatment with 200 nM PMA, which activates PAK1 via PKC and is independent of PI3-kinase [31]. This activation of PAK1 following PMA treatment was not abrogated by transfection with dominant-negative AktK/M. These results indicate a selective role for Akt in PAK1 regulation in response to G protein βγ subunits.

3.5. Activation of endogenous PAK1 and Akt by EGF and LPA

We have previously shown that multiple agonists are able to activate PAK1 in COS7 cells [31]. To determine whether endogenous Akt and PAK1 are activated by the same agonists, we treated COS7 cells with LPA, EGF, or with EGF after pretreatment with wortmannin. Activation of Akt and PAK1 was assayed by Western blotting for the phosphorylated kinases in whole cell lysates (Fig. 5). Stimulation with LPA or EGF activated both Akt and PAK1. Inhibition of PI3-kinase activity with wortmannin blocked the ability of EGF to stimulate both Akt and PAK1. Wortmannin also blocked the ability of LPA to stimulate Akt (data not shown), although it does not prevent the stimulation of PAK1 by LPA [31].

3.6. Activation of endogenous PAK1 by Gβ1γ2 and Gβ1γ5 subunits

We have shown that Gβγ subunits can activate a co-transfected mycPAK1 construct. To determine whether endogenous PAK1 could also be activated by increased Gβγ, we transfected COS7 cells and assayed for the activity of native PAK1. There was a 3.5- and 4.8-fold increase in PAK1 activity following Gβ1γ2 and Gβ1γ5 overexpression, respectively (Fig. 6). This activity could be inhibited by pretreatment of the cells for 30 min with 200 nM wortmannin. We can conclude that endogenous PAK1 can also be stimulated by Gβγ through PI3-kinase activation.

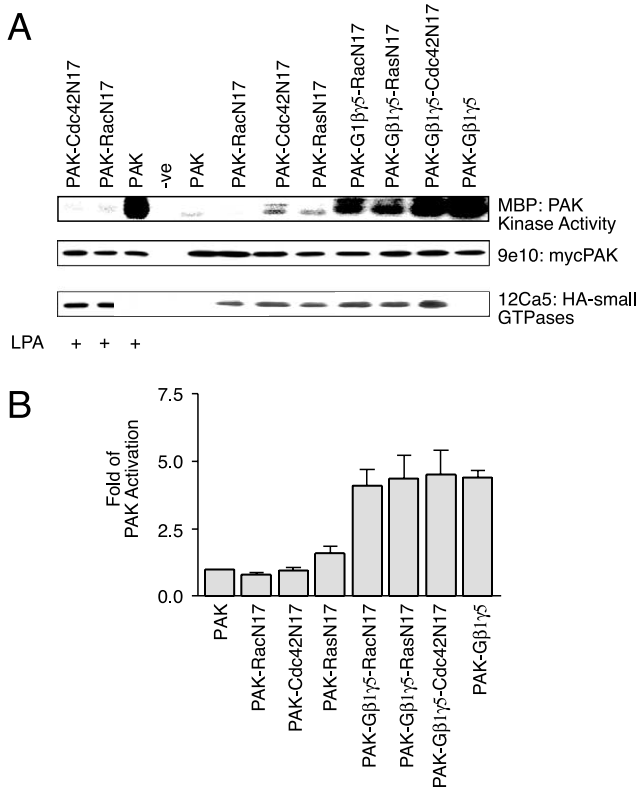
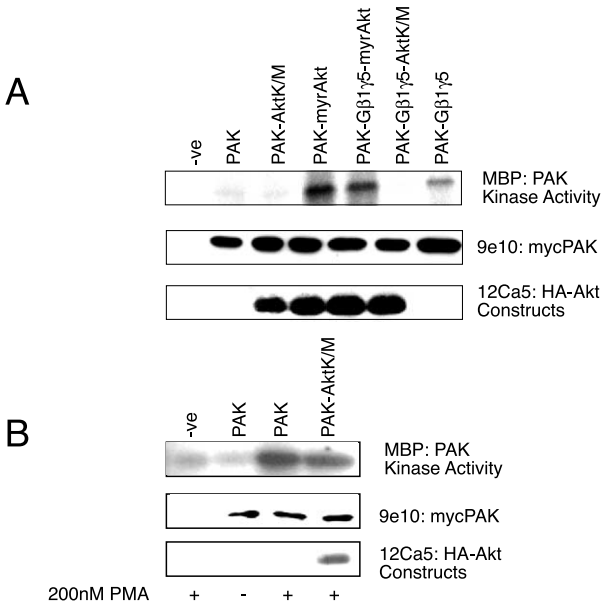


Fig. 3. Gβ1γ5 activation of PAK1 is independent of Rac, Ras, or Cdc42. COS7 cells were transfected with myc-tagged PAK1 alone or co-transfected as shown. A: MBP was used as a substrate to detect PAK1 phosphorylation in an in vitro kinase assay. Western blots of the immunoprecipitates were probed with 9e10 to detect myc-tagged PAK1. Western blots of cell lysates were probed with 12Ca5 to detect HA-tagged proteins. pRK7 plasmid was used as a negative vector control (−ve). B: Graph depicting activation of mycPAK1 by Gβγ in COS7 cells. The inhibition of LPA-induced PAK1 activation by dominant-negative Rac and Cdc42 is consistent with that previously described [31].



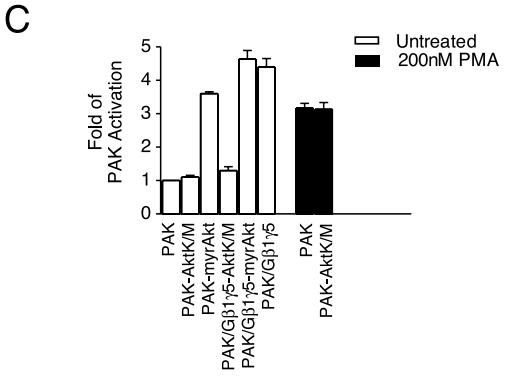


Fig. 4. Gβ1γ5 activation of PAK1 is dependent on Akt. Co-transfection of mycPAK1 and a constitutively active Akt, myrAkt, and co-transfection of mycPAK1, Gβ1γ5 and myrAkt in COS7 revealed activation of PAK1. The expression of dominant-negative Akt, AktK/M, in the mycPAK1/Gβ1γ5 co-transfectants inhibited PAK1 activation. A: MBP was used as a substrate to detect PAK1 phosphorylation in an in vitro kinase assay (top panel). Western blots of the immunoprecipitates were probed with 9e10 to detect myc-tagged PAK1 (middle panel). Western blots of cell lysates were probed with 12Ca5 to detect HA-tagged proteins (bottom panel). pRK7 plasmid was used as a negative vector control (−ve). B: Stimulation of PAK1 activity assayed following treatment with 200 nM PMA is not blocked by dominant-negative Akt. C: Graph depicting activation of mycPAK1 in COS7 cells.

4. Discussion

PAKs are involved in the regulation of a wide range of cellular activities and abnormalities of PAK function are thus likely to contribute to various pathologies. A few examples of diseases where aberrant PAK expression and/or activation may play a role are inflammatory diseases involving enhanced leukocyte influx and activation, cancers involving high levels of metastasis, defects in neuronal formation or degeneration, and autoimmune disorders, developmental abnormalities, and cancers in which defective apoptotic cell death plays an etiologic role [15]. Two important endpoints of PAK signaling have emerged: nuclear events that influence gene expression, and cytoskeletal events that impact upon

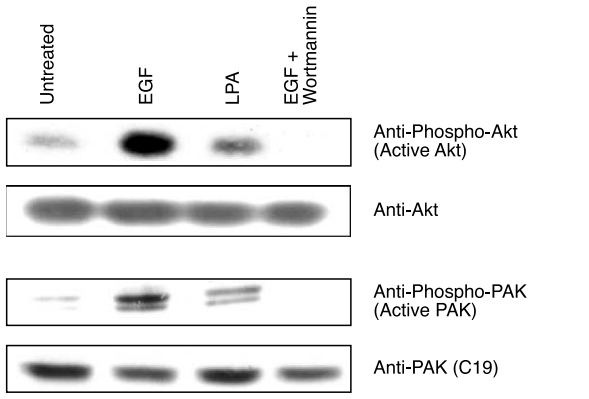


Fig. 5. Agonist activation of endogenous Akt and PAK1. COS7 cells were treated with 10 μM LPA for 15 min, or 100 nM EGF for 5 min (with or without a 30 min pretreatment with 200 nM wortmannin) and then whole cell extracts were prepared [29]. The samples were run in parallel for Western blotting for both active and total populations of Akt and PAK1, as indicated.

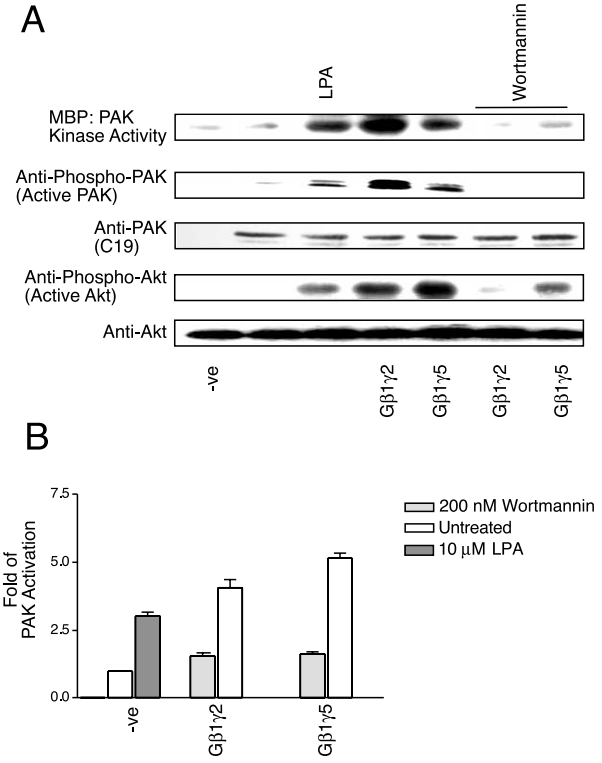


Fig. 6. Activation of endogenous PAK1 by Gβγ occurs through PI3-kinase. Overexpression of Gβ1γ2 or Gβ1γ5 activates endogenous PAK1 in COS7 cells. This activity is inhibited by wortmannin. Control immunoprecipitations (−ve) were performed with a rabbit anti-mouse antibody in place of the N-20 anti-PAK antibody. A: MBP was used as a substrate to detect PAK1 phosphorylation in an in vitro kinase assay (top panel). Western blots of the immunoprecipitates were probed first with C19 anti-PAK to detect endogenous PAK1 (middle panel), then stripped and reprobed with a Phospho-PAK1 (Thr-423)/PAK2 (Thr-402) antibody (second panel from top). Ten percent of the lysates used for the immunoprecipitations were instead subjected to TCA precipitation [30] and then processed for Western blotting for active (phosphoSer-473) Akt (second panel from bottom). These blots were stripped and reprobed for total Akt (bottom panel). B: Graph depicting activation of PAK1 as observed by the assay of MBP phosphorylation.

cellular dynamics. While these biological endpoints provide interesting possibilities regarding cellular signaling by Cdc42 and Rac, they also raise questions regarding the nature of PAK regulation and the identity of downstream targets [36]. In recent years there has been a great interest in upstream components that lead to PAK1 activation. In the present study we show that PAK1 activation in COS7 transfectants can occur through a Cdc42/Rac1-independent manner via PI3-kinase and Akt.

PI3-kinases have emerged as important constituents of signaling pathways. In mammalian cells, three distinct classes of PI3-kinases have been discovered, characterized, cloned, and found to differ in their activation mechanisms [35]. Members of the PI3-kinase family have also been considered as potential participants in the oncogenic process because they control cell cycle progression, differentiation, survival, invasion and metastasis and angiogenesis [37–39]. Receptor-regulated class Ib p110γ/p101 PI3Kγ is activated by Gβγ on stimulation of GPCRs. Gβγ is considered to be the principal, direct stimulus of GPCR-induced PI3Kγ enzymatic activity. Convincing evidence for this has come from reconstitution of purified proteins, demonstrating that in vitro the enzymatic activity of monomeric and heterodimeric PI3Kγ is significantly stimulated by Gβγ [40–44]. Several lines of evidence indicate that specific combinations of G protein α, β, and γ subunits are required for different receptors or receptor–effector networks, and that a higher degree of specificity for Gα and Gβγ is observed in intact systems than reported in vitro [45]. There is also evidence that PI3Kβ is activated by Gβγ subunits in NIH3T3 fibroblasts and that this activated PI3Kβ can further stimulate Akt activity [46]. PI3Kα and PI3Kβ are expressed in COS7 cells [47]. It has also been observed that PI3-kinase activity in COS7 cell can be inhibited by wortmannin [1,48], and that p110β rather than p110γ is involved in the production of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 induced by LPA [49,50].

Akt is a downstream effector of PI3-kinase that is recruited to the membrane by PtdIns(3,4)P2 and PtdIns(3,4,5)P3. Akt is then phosphorylated at Thr-308 by phosphoinositide-dependent kinase 1 (PDK-1) [51]. Subsequent to membrane localization, other residues of Akt, such as Ser-473, are also phosphorylated to contribute to its activation [52]. PAK1 can be activated by Rac1/Cdc42 [15–17,32] and also by Akt [53,54]. PI3-kinase activation of PAK1 has been reported earlier [55–57], and we are able to show that in our system both PI3-kinase and Akt are required for PAK1 activation by Gβγ. Akt has previously been reported to activate PAK1 [47,53,58], in one case as an intermediate between Ras and PAK1 activation [53]. Further connections between PI3-kinase and PAK1 are supported by the observations that heregulin regulates cytoskeletal reorganization and cell migration through PAK1 via PI3-kinase [59], that PAK1 is phosphorylated and activated by PDK-1 [60], and that exposure of opossum kidney cells to opioid agonists results in alterations of actin polymerization through PI3-kinase activation of PAK1 that were independent of both Rac1/Cdc42 and Akt [55].

Our results show that expression of a constitutively activated Akt (myrAkt.HA) is sufficient to activate PAK1, and that Akt activation is necessary for Gβγ-dependent PAK1 activation through a mechanism that is independent of Rac1/Cdc42. Our results also show, however, that this role for Akt is not ubiquitous for all instances of PAK1 activation. Thus EGF activation of PAK1, which does require PI3-kinase activity and is associated with Akt activation, is still dependent on Rac1/Cdc42 activity [31]. Further, the G protein-coupled agonist LPA can activate both Akt and PAK1, but the effects are separable as the former is inhibited by wortmannin and the latter is not [31]. One factor that may need to be considered in the interpretation of these data may be the fact that PAK1 is also regulated by its recruitment to specific subcellular locales, for example by the adaptor protein Nck [61–63].

This investigation of the role of Gβγ subunits in PAK1 activation stems from the role of Gβγ activation of Ste20 in yeast [18]. The PAK1 homologue Ste20 is involved in transmitting the mating-pheromone signal from the Gβγ subunits of a heterotrimeric G protein to a downstream MAPK cascade in budding yeast [19,64]. Binding of Gβγ with the non-catalytic C-terminal region of Ste20 is essential for the activation of the MAPK cascade signaling from the mating factor receptor [18,65]. In the current study, co-transfections with Gβ1γ5, Gβ1γ2 plus mycPAK1 demonstrated that PAK1 is activated by the Gβγ subunits. It has been reported by others in an in vitro assay that PAK1 is inhibited by Gβ1γ2 subunits [66]. In that particular study PAK1 (232–544) was incubated with either 150 nM Gαz or 200 nM myelin basic protein (MBP) and decreasing concentrations of Gβγ. The authors did note that Gβγ slightly but reproducibly stimulated PAK1 autophosphorylation in the presence or absence of added substrate, indicating that Gβγ binds directly to PAK1 to alter its function [66]. In our study we overexpressed the Gβγ subunits in COS7 cells. We conclude from our work that the activation of PAK1, whether endogenous or myc-tagged, is through PI3-kinase. Thus whether direct binding of Gβγ to PAK1 is required for this activation has not been addressed, although such association has recently been reported [67].

It is also significant that the mechanism used by Gβγ subunits to activate PAK1 (through PI3-kinase/Akt but independent of Rac1/Cdc42) is distinct from two other pathways that are stimulated by the G protein-coupled agonists that we have studied. We have previously shown that LPA stimulates a Rho-dependent pathway to activate PAK1 in COS7 cells [31], whereas the majority of the signaling from muscarinic receptors to PAK1 is transduced through transactivation of the EGF receptor [31]. Both of these G protein-coupled receptor pathways to PAK1 activation are dependent on the activity of Rac1/Cdc42. Thus a receptor system in COS7 cells that operates through this newly described Gβγ-dependent mechanism is yet to be described, although chemoattractant receptor signaling in neutrophils has recently been shown to utilize Gβγ to activate PAK1 [67].

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