

Synergistic Activation of p21-activated Kinase 1 by Phosphatidylinositol 4,5-Bisphosphate and Rho GTPases^{*[5]}

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Background: p21-activated kinase 1 (Pak1) is activated by Cdc42 as well as phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂).

Results: PI(4,5)P₂ and Cdc42 both contribute to Pak1 membrane recruitment and synergistically activate Pak1 but not another Cdc42 effector, Ack (activated Cdc42-associated kinase).

Conclusion: Pak1 is a coincidence detector regulated by GTPase and PI(4,5)P₂ binding.

Significance: Coincidence detection may allow for Pak1 activation independently from other Cdc42 effectors.

Autoinhibited p21-activated kinase 1 (Pak1) can be activated *in vitro* by the plasma membrane-bound Rho GTPases Rac1 and Cdc42 as well as by the lipid phosphatidylinositol (4,5)-bisphosphate (PIP₂). Activator binding is mediated by a GTPase-binding motif and an adjacent phosphoinositide-binding motif. Whether these two classes of activators play alternative, additive, or synergistic roles in Pak1 activation is unknown, as is their contributions to Pak1 activation *in vivo*. To address these questions, we developed a system to mimic the membrane anchoring of Rho GTPases by creating liposomes containing both PIP₂ and a Ni²⁺-NTA modified lipid capable of binding hexahistidine-tagged Cdc42. We find that among all biologically relevant phosphoinositides, only PIP₂ is able to synergistically activate Pak1 in concert with Cdc42. Membrane binding of the kinase was highly sensitive to the spatial density of PIP₂ and Pak1 demonstrated dramatically enhanced affinity for Cdc42 anchored in a PIP₂ environment. To validate these findings *in vivo*, we utilized an inducible recruitment system to drive the ectopic synthesis of PIP₂ on Golgi membranes, which normally have active Cdc42 but lack significant concentrations of PIP₂. Pak1 was recruited to PIP₂-containing membranes in a manner dependent on the ability of Pak1 to bind to both PIP₂ and Cdc42. These findings provide a mechanistic explanation for the essential role of both phosphoinositides and GTPases in Pak1 recruitment and activation. In contrast, Ack, another Cdc42 effector kinase that lacks an analogous phosphoinositide-binding motif, fails to show the same enhancement of membrane binding and activation by PIP₂, thus indicating that regulation by PIP₂ and Cdc42 could provide a combinatorial code for activation of different GTPase effectors in different subcellular locations.

It is well established that certain lipid species, particularly phosphoinositides, play active roles in cellular signaling events in addition to structural roles comprising cellular membranes (1–4). Phosphoinositides consist of phosphatidylinositol and seven other variously phosphorylated forms that localize to distinct membrane compartments and fulfill different biological functions (1, 2). For example, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂, or PIP₂)³ is predominantly found in the cytosolic leaflet of the plasma membrane where it plays numerous roles regulating endocytosis and the actin cytoskeleton (5–7), whereas phosphatidylinositol 4-phosphate (PI(4)P) is localized to and plays important roles at the Golgi membrane (1, 2). Signaling by phosphoinositides is mediated in part by their binding to a variety of phosphoinositide binding domains, including the pleckstrin homology (PH), phox homology (PX), and espin amino-terminal homology domains (1, 2), however, other proteins can bind these acidic phospholipids through so called “basic regions” (8–11), typically unstructured sequences enriched in positively charged amino acids.

Engagement of phosphoinositide-binding motifs can facilitate membrane translocation of signaling proteins (1). In some cases, phosphoinositide binding has been shown to cooperate with binding to other membrane features, including other lipids or GTP-binding proteins (GTPases) in a process referred to as “coincidence detection” (1, 12–14). For example, FAPP is recruited to the *trans*-Golgi network due to coordinated binding to both PI(4)P and the GTPase Arf1 (15), whereas the neuronal Wiskott-Aldrich syndrome protein (N-WASP) is targeted to the plasma membrane by coordinated binding to PIP₂ and the Rho family GTPase Cdc42 (9, 12).

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³ The abbreviations used are: PIP₂, phosphatidylinositol (4,5)-bisphosphate; Pak1, p21-activated kinase; PI(4)P, phosphatidylinositol 4-phosphate; PH, pleckstrin homology domain; PX, phox homology domain; N-WASP, neuronal Wiskott-Aldrich syndrome protein; PIP5K, phosphatidylinositol 5-phosphate kinase; iRap, 3-methylindole rapamycin; Grp1, general receptor for 3-phosphoinositides; FAPP, four-phosphate-adaptor protein; FRB, FKBP-rapamycin binding domain; FKBP, FK506-binding protein; Tgn38, *trans*-Golgi network protein 38; DGS-NTA(Ni), 1,2-dioleoyl-*sn*-glycero-3-[[N-(5-amino-1-carboxypentyl)iminodiacetic acid]succinyl]-nitrilotriacetic acid (nickel salt); Ack, activated Cdc42-associated kinase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

Synergistic Activation of Pak1

In addition to regulating membrane recruitment, coincident membrane features can also regulate the biological activity of signaling proteins. N-WASP adopts an autoinhibited conformation that negatively regulates its biochemical function: activation of actin filament assembly by the Arp2/3 complex (16, 17). Binding of N-WASP to PIP₂ and membrane-bound Cdc42 cooperatively disrupts this autoinhibited conformation and results in potent stimulation of Arp2/3 activity (9, 12, 18, 19). This mechanism allows for complex spatial and temporal regulation of Arp2/3 activity. It has been theorized that signaling information transmitted by phosphoinositides and GTPase-dependent pathways converge on N-WASP leading to Arp2/3 activation only when both upstream pathways are sufficiently active, thus suppressing inappropriate activation that could be triggered by stochastic activation of either pathway alone (9, 12, 19). Furthermore, dual inputs could provide a combinatorial code for N-WASP activation at specific subcellular locations. Although active, GTP-bound Cdc42 is present at both the plasma membrane and intracellular membranes (1), the primary localization of PIP₂ to the plasma membrane may ensure that this is predominantly where N-WASP activation occurs. Unfortunately, whereas biochemical aspects of N-WASP regulation by these activators have been examined exhaustively *in vitro*, the relative importance of these inputs regulating N-WASP or other Cdc42 effectors in cells remains poorly understood.

The serine/threonine kinase Pak1, which plays a fundamental role in cell morphology and proliferation control (20–23), is an effector of the Rho family GTPases Cdc42 and Rac1 (23, 24). Its dysregulation has recently been clearly linked to human cancers including melanoma (25, 26) and breast cancer (27–29). Like N-WASP, Pak1 exists in an autoinhibited conformation that is relieved by GTPase binding (30–33). A basic region was identified in Pak1 that binds to phosphoinositides, particularly PIP₂ (8). Pak1 is activated *in vitro* by binding of its basic region to liposomes containing PIP₂ and a Pak1 mutant lacking the basic region fails to be activated in cells stimulated by growth factors, suggesting that PIP₂ may be an important regulator of Pak1 *in vivo* (8). Here, using experimental modulations of phosphoinositide synthesizing enzymes in cells and the development of a novel *in vitro* system to recapitulate Cdc42 signaling, we demonstrate for the first time a direct role for PIP₂ as a regulator of Pak1 *in vivo* and reveal its mechanism of action. Thus, like N-WASP, Pak1 serves as an integrator of diverse upstream signaling cascades and is likely activated at the plasma membrane based on the concordance of signals mediated there by phosphoinositides and Rho family GTPases. Importantly, we find that another Cdc42 effector, the nonreceptor tyrosine kinase Ack (activated Cdc42-associated kinase) (34), which lacks an analogous basic region, shows no synergy between Cdc42 and PIP₂ for membrane recruitment or activation. Thus, more broadly, our findings, together with previous studies, suggest a specificity mechanism for controlling activation of specific effectors in particular subcellular contexts.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Recombinant proteins were prepared as previously described: Pak1 (8), 8T-Pak1 (8),

GST-Paktide (8), GST (8), GST-Grp1 (35), GST-dual adaptor of phosphotyrosine and 3-phosphoinositides (35), GST-PH^{PLC8} (35), and GST-sorting nexin-3 (36). Full-length, human Cdc42 Q61L (constitutively active (37)) was cloned into pET28a to append a C-terminal hexahistidine tag, expressed in *Escherichia coli* and purified according to the manufacturer's protocols (Qiagen). The peptide sequence GAKVIYDFIEKKKKG was fused in-frame to GST in the pGEX 6P-1 vector to create the GST-Acktide construct. It was expressed and purified according to the manufacturer's protocols (Qiagen).

Ack (residues 117–489) was expressed as a GST-tagged protein in Sf9 cells using the Bac-to-Bac system (Invitrogen). The pFastBac HTB vector (Invitrogen) was first modified to replace the His tag with a GST tag followed by a Pre-Scission protease site by cloning the GST tag and the Pre-Scission site from a pGEX 6P-1 vector (GE Healthcare) into the RsrII and NcoI sites of pFastBac HTB. DNA encoding residues 117 to 489 of human Ack was subsequently cloned into the EcoRI and NotI sites of the modified pFastBac vector. The resultant plasmid was transformed into DH10Bac cells (Invitrogen) and baculovirus was produced using the Bac-to-Bac system. Amplified virus was used to infect Sf9 cells and cells were harvested after 65 h. Cells were thawed and resuspended in 50 mM Tris, pH 7.5, 300 mM NaCl, 10% glycerol, 2 mM DTT, 1% Triton, 1 mM Na₃VO₄, and Complete-EDTA Free (Roche Applied Science), lysed by sonication, and debris was pelleted at 39,000 × *g* for 30 min. Soluble lysates were incubated with glutathione-Sepharose 4 Fast Flow (GE Healthcare) for 3 h at 4 °C. Beads were washed extensively with lysis buffer, then resuspended in a buffer containing 50 mM Tris, pH 8, 150 mM NaCl, 10% glycerol, 1 mM DTT, and 1 mM Na₃VO₄. To remove the GST tag, Pre-Scission protease was added, and beads were incubated overnight at 4 °C. Cleaved Ack was recovered from the supernatant by harvesting the beads at 3,000 × *g* for 10 min. The purified protein was concentrated, brought up to 50% glycerol, and stored at –80 °C.

Liposome Preparation—Stocks of phosphatidylcholine, phosphatidylethanolamine, DGS-NTA(Ni) (Ni²⁺ salt), phosphatidylinositol (4,5)-bisphosphate, and phosphatidylinositol (Avanti Polar Lipids) were stored in chloroform or chloroform:methanol (2:1) at –80 °C. Appropriate mole ratios of each were added in an amber bottle and dried under nitrogen gas. 2.5 mM liposome stock solutions were prepared by addition of the appropriate volume of lipid buffer (20 mM Hepes, pH 7.5, 300 mM NaCl, 200 mM sucrose, adapted from Ref. 38). Then bottles were sonicated at 4 °C for 20 min. Liposomes were stored at 4 °C and used within 10 days.

In Vitro Kinase Assays—Liposomes were incubated with Cdc42-His in kinase buffer (50 mM Hepes, pH 7.5, 12.5 mM NaCl, 650 μM MgCl₂, 650 μM MnCl₂ (8)) on ice for 30 min to allow binding to DGS-NTA(Ni). 0.4 μg of full-length WT or 8T-Pak1 or equimolar Ack was added along with excess GST-Paktide/Acktide and kinase buffer to a volume of 14 μl. Reactions were started by addition of ATP (1 μCi of [γ -³²P]ATP) to a final concentration of 30 μM. After incubation for 15 min at 30 °C, reactions were stopped by addition of loading buffer and heating to 95 °C for 10 min. Substrate and kinase were separated by SDS-PAGE. Gels were Coomassie-stained and dried. The degree of ³²P incorporation was determined by exposing

the dried gel to a PhosphorImager plate. Bands were visualized and quantitated using ImageGauge (version 4.0, FUJIFILM). The data were analyzed using GraphPad Prism to determine IC_{50} values. Liposomes used for kinase assays were always PC:PI:DGS-NTA(Ni) (equal molar PC, PI; 7% DGS-NTA(Ni) with or without 6% phosphoinositide). Concentrations of total lipid used in assays varied from 15 to 500 μM .

Liposome Sedimentation Assays—Liposomes were preincubated in lipid buffer (previously defined) containing 20 mM imidazole for 15 min on ice. Cdc42-His was then added. Complexes were allowed to form on ice for 15 min. Finally, 10 μg of Pak1 (or equimolar Ack), 300 μM ATP, and 5 μg of BSA were added to a volume of 40 μl (final buffer: 20 mM Tris, pH 8.5, 300 mM NaCl, 200 mM sucrose, 20 mM imidazole, adapted from Ref. 38). After 15 min on ice, reactions were spun at $100,000 \times g$ with a Beckman TLA 120.2 rotor at 4 °C. Supernatant was removed and pellets were resuspended with loading buffer. Pak1 was resolved by SDS-PAGE and visualized with silver stain or Western blot (α -Pak1, Invitrogen). Bands were quantitated with ImageJ and graphed with GraphPad Prism. Liposomes used for sedimentation assay were always PC:PE:DGS-NTA(Ni) (equal molar PC, PE; 3% DGS-NTA(Ni), with or without 12% phosphoinositide) and held at a constant 400 μM total lipid per reaction.

Pak1/Cdc42-His Pulldown Assays—10 μg of Pak1, 10 μg of 8T-Pak1 was incubated with Cdc42-His in a final buffer of 20 mM Tris, pH 8.5, 300 mM NaCl, 200 mM sucrose, 20 mM imidazole. Complexes were allowed to form during nutation at 4 °C for 1 h. 15 μl of Ni^{2+} -NTA-agarose (Qiagen) was added to each tube and allowed to mix for an additional 15 min. Resin was pelleted at $3,000 \times g$ and washed with excess buffer. Bound proteins were separated by SDS-PAGE and detected by Western blot with α -Pak1 (Invitrogen). Binding was quantified by ImageJ.

iRap-inducible PIP 5-Kinase Plasmids—The recruitable PIP5K constructs were kindly provided by Dr. Tamas Balla and were generated using the mouse PIP5KI α template and PCR amplifying a segment (1–461) using primers containing PvuI and KpnI sites in the 5' and 3' ends, respectively. This fragment was subcloned into the mRFP1 plasmid for determination of localization and catalytic activity and for verifying the sequence. This construct was then used to generate mutants where the Glu-61 residue was replaced with either a Met or Leu residue. These mutations comprise a Rac1 binding site and make the enzyme mostly cytoplasmic (39), although the constructs still bind to the PM at higher expression levels or longer transfections times (>16–20 h). The wild-type and E61L mutant enzymes were then subcloned into the mRFP-FKBP12 backbone (40) with PvuI/KpnI enzymes to generate the recruitable versions of the proteins.

iRap-inducible Recruitment Assays—HEK293 (ATCC) cells grown on coverslips were transfected with Tgn38-FRB-CFP (Golgi targeting plasmid) and FKBP-PIP5K-mRFP (PIP5 kinase) plasmids in addition to GFP-PH^{PLC δ} (40) or full-length HA-tagged WT-Pak1, 8T-Pak1, or LL-Pak1 (8). HA-tagged LL-Pak1 was kindly provided by Dr. Jonathan Chernoff. Transfections were performed with Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). 24 h following trans-

fection, dimerization was induced by the addition of 5 μM rapamycin or 3-methylindole rapamycin (iRap) for the designated time period. iRap was synthesized by the Organic Synthesis Facility at the Fox Chase Cancer Center. Coverslips were removed and cells were then fixed with formaldehyde and then immunostained with α -Pak1 (Invitrogen) and α -rabbit Alexa 488 (Invitrogen). Images were obtained with the Leica TCS SP5 spectral confocal system with a 68 \times PlanApo objective. Eighteen cells for each set of triply transfected cells were chosen at random. Using the coloc_2 plugin for Fiji/ImageJ,⁴ the outline of each cell was defined and a Pearson correlation coefficient was determined for both CFP/mRFP and CFP/Alexa 488 colocalization.

RESULTS

Selective Activation of Pak1 by PIP₂—Previous *in vitro* studies showed a weak potentiating effect of PIP₂-containing liposomes on catalytic activation of Pak1 by Rho GTPases (8). However, these studies were conducted with soluble, nonprenylated GTPase, whereas activated Rho GTPases in the cell are anchored via a C-terminal geranylgeranyl group to cellular membranes (43–45). In addition, not all of the naturally occurring phosphoinositides were tested for a role in Pak1 binding or activation (8). To exhaustively investigate the relative roles of phosphoinositides and GTPases in regulating Pak1 in a more physiologically relevant setting, we developed a novel experimental system in which synthetic liposomes of defined lipid composition are generated that incorporate lipids bearing a modified Ni^{2+} -NTA head group (DGS-NTA(Ni)). This modified lipid can bind to recombinant GTPases bearing a C-terminal hexahistidine tag, thus tethering these proteins to the liposome surface in a biologically relevant orientation (schematically represented in Fig. 1) (46–51). Importantly, lipid composition and GTPase density on the external leaflet of these liposomes can be easily and precisely controlled. Indeed, sedimentation assays confirmed approximately stoichiometric binding of Cdc42-His to Ni^{2+} -NTA lipid exposed on the outer face of these liposomes (supplemental Fig. S1). We then synthesized Ni^{2+} -NTA-containing liposomes incorporating each of the seven biologically relevant occurring phosphoinositide species and confirmed the proper presentation of incorporated phosphoinositides by documenting the binding of corresponding recombinant phosphoinositide-binding domains of known specificity in liposome sedimentation assays (35, 36) (supplemental Fig. S2).

Next, we comprehensively tested biologically relevant phosphoinositides for their ability to modulate Pak1 activation by liposome-bound Cdc42-His. Ni^{2+} -containing liposomes containing 6% (by molar ratio) of phosphoinositide, or control liposomes with no phosphoinositide, were charged with Cdc42-His and used in a [³²P]ATP *in vitro* kinase assay where Pak1 autophosphorylation was monitored by SDS-PAGE and digital autoradiography. The ability of each phosphoinositide to potentiate Pak1 activation by Cdc42-His was assessed by comparison with control liposomes bearing only GTPase. Strikingly, only liposomes with PI(4,5)P₂ showed significant

⁴ D. J. White, T. Kazimiers, and J. Schindelin, ImageJ software.

Synergistic Activation of Pak1

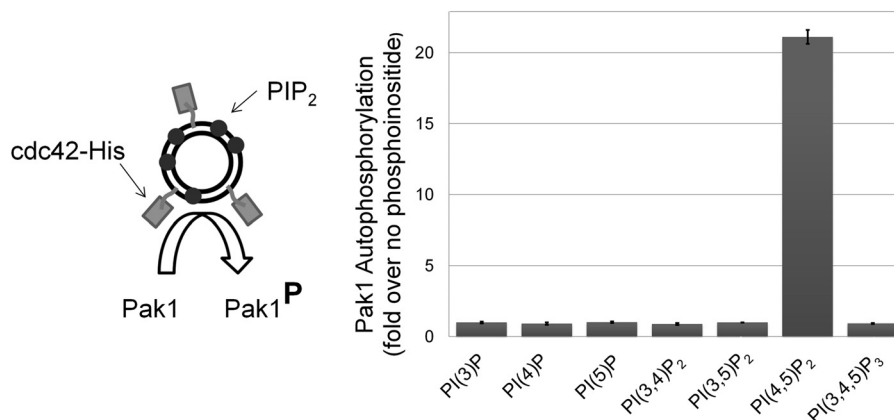


FIGURE 1. PI(4,5)P₂ selectively enhances Cdc42-dependent Pak1 activation. *Left*, the experimental system. Synthetic liposomes (double black circle) with bound Cdc42-His (rectangles) that either contained phosphoinositide (small dark circles) or not, were incubated with Pak1 and [γ -³²P]ATP to monitor phosphoinositide-dependent Pak1 autophosphorylation. *Right*, Pak1 autophosphorylation in kinase assays with Cdc42-His-bound liposomes (comprised of equimolar PC:PI and containing 7% (by mole) DGS-NTA(Ni) with or without 6% of the indicated phosphoinositides) is plotted normalized to control reactions with Cdc42-containing liposomes without the test phosphoinositide. Pak1 autophosphorylation was quantified by PhosphorImager analysis of dried electrophoretic gels of the reaction products. Mean results are shown from two independent replicates \pm S.D.

enhancement of Pak1 autophosphorylation compared with liposomes without phosphoinositide (Fig. 1). This experiment demonstrates that among biologically relevant phosphoinositides, PIP₂ uniquely promotes Pak1 activation by Cdc42. Furthermore, because PIP₂ is the most abundant plasma membrane phosphoinositide (11, 52), these results support a potentially predominant role for this phosphoinositide in Pak1 regulation in cells.

Pak1 Is Sensitive to the Spatial Density of Membrane PIP₂ in Vitro and in Vivo—The basic region of Pak1, which is both necessary and sufficient for PIP₂ binding (8), is a 25-amino acid region containing nine lysines and three arginines located just N-terminal to the Pak1 Cdc42-binding site (8). Similar positively charged phosphoinositide-binding motifs have been described in other proteins including other Rho GTPases effectors such as N-WASP (9–12). Previous work demonstrated that activation of N-WASP by PIP₂ is ultrasensitive with respect to the density of PIP₂ in the membrane (9, 12). The N-WASP basic motif contains 15 amino acids of which 10 are positively charged and binds PIP₂-containing liposomes of increasing PIP₂ density with sigmoidal dose dependence and a Hill coefficient of 3.1 (12). To ask if membrane binding of Pak1 was also sensitive to PIP₂ spatial density, we performed liposome sedimentation assays with full-length Pak1 and liposomes in which the mole percent of PIP₂ was increased from 0 to 20%. All reactions had a constant total lipid concentration (shown in Fig. 2). We found that Pak1 also bound to PIP₂ liposomes with a sigmoidal dependence on PIP₂ concentration and with an apparent Hill coefficient of 2.4 (Fig. 2). These data support cooperative, multivalent binding of the basic region of Pak1 to PIP₂ as has been observed for the basic region of N-WASP and other phosphoinositide-binding proteins.

Prior studies demonstrated an essential role for the basic region in Pak1 membrane recruitment and catalytic activation in cultured cells (8). Together with our data, these findings suggest that PIP₂ may regulate Pak1 membrane recruitment *in vivo*. To test this hypothesis directly, we utilized a rapamycin-based inducible recruitment system to artificially generate PIP₂ on membranes of the Golgi apparatus and assessed its impact

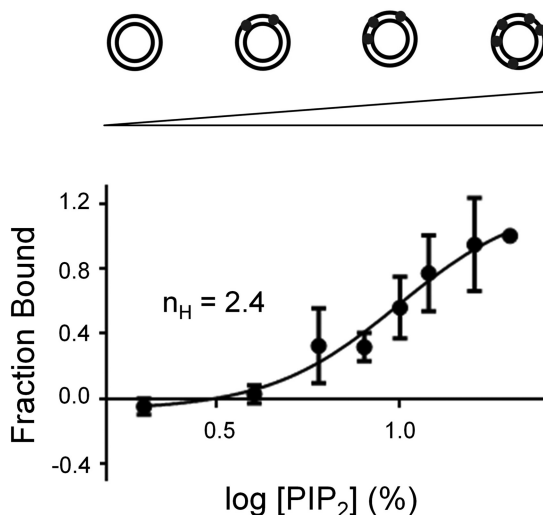


FIGURE 2. Pak1 binding is sensitive to the spatial density of PIP₂. Pak1 was incubated with 400 μ M PC:PE (equimolar) liposomes containing increasing mole fractions of PIP₂ (0–20%). Liposomes were sedimented and bound Pak1 was quantified by Western blotting. Results are shown for five replicate experiments (\pm S.D.) and the data were fit to a sigmoidal dose-response curve using GraphPad Prism to determine the Hill coefficient (n_H).

on membrane recruitment of Pak1. The Golgi apparatus is not thought to harbor a significant fraction of cellular PIP₂, although it is enriched in its biological precursor PI(4)P, which is phosphorylated by phosphatidylinositol 4-phosphate 5-kinase (PIP5K) to generate PIP₂. In addition, Golgi membranes are well known to contain GTP-bound Cdc42 on their cytoplasmic leaflet (53–59). Interestingly, however, this pool of activated Cdc42 has not been reported to recruit and activate Pak1, which is instead predominantly localized to the cytosol and plasma membrane (60, 61). Thus, we speculated that limiting PIP₂ concentrations in the Golgi membrane may prevent the recruitment of Pak1 by Golgi-localized Cdc42. We sought to test this idea by artificially generating PIP₂ at Golgi membranes and monitoring Pak1 recruitment and its dependence on PIP₂ and Cdc42 binding.

HEK293 cells were co-transfected with plasmids encoding the FRB domain of mammalian target of rapamycin fused to the

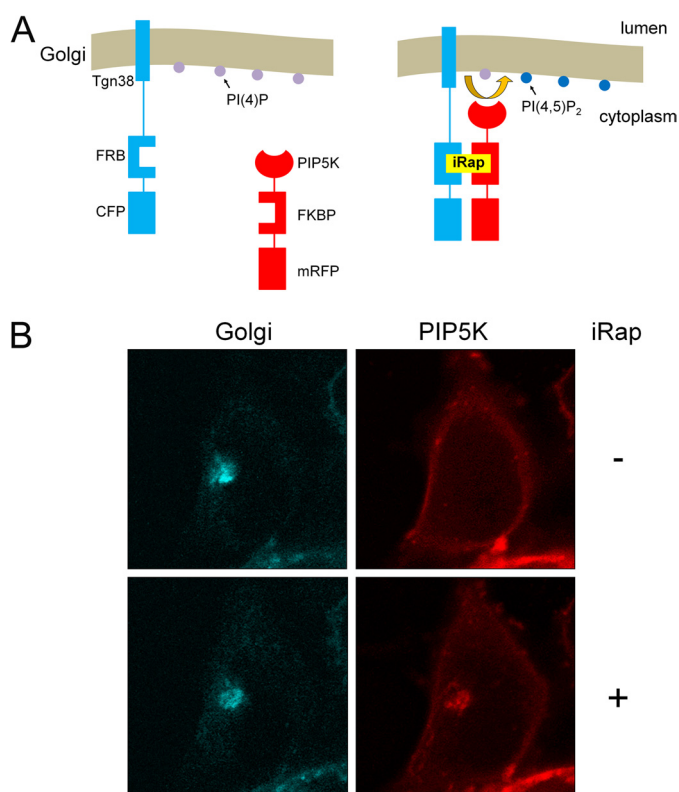


FIGURE 3. A system for rapamycin-inducible recruitment of PIP5K to the Golgi membrane for localized PIP₂ synthesis. *A*, schematic diagram: a fusion protein where the transmembrane region of Tgn38 anchors the FRB domain of mammalian target of rapamycin (labeled with cyan fluorescent protein, CFP) to the Golgi where PI(4)P (purple circles) is present. Rapamycin or its analog 3-methylindole rapamycin (iRap) triggers binding and recruitment of a cytosolic fusion protein containing the FKBP binding domain of FKBP12-rapamycin-associated protein (FRAP) fused to PIP5K (and labeled with monomeric red fluorescent protein, mRFP) to Golgi-localized Tgn38-FRB. Golgi recruitment of PIP5K allows for localized phosphorylation of PI(4)P to PIP₂ (blue circles). *B*, HEK293 cells were cotransfected with plasmids encoding Tgn38-FRB-CFP and PIP5K-FKB-mRFP. Transfected cells were stimulated with iRap for 5 min to induce dimerization. CFP and mRFP were visualized by fluorescence microscopy.

Golgi targeting sequence of the type 1 transmembrane Golgi-resident protein Tgn38 (42, 62) (cyan fluorescent protein labeled) and the FKBP domain of FKBP12-rapamycin-associated protein fused to PIP5K (red fluorescent protein labeled) (Fig. 3*A*). Tgn38-FRB exhibited perinuclear Golgi localization and FKBP-PIP5K was diffusely distributed in the cytoplasm before iRap treatment (Fig. 3*B*). Following rapamycin-induced dimerization for 5 min, however, PIP5K showed strong colocalization with FRB (Fig. 3*B*, right).

Next, we assessed whether Golgi-targeted PIP5K was capable of phosphorylating Golgi PI(4)P to PIP₂. We monitored PIP₂ production with a widely used PIP₂ biosensor, the pleckstrin homology domain of phospholipase C δ (GFP-PH^{PLC δ} , green fluorescent protein labeled). In addition, we utilized the rapamycin analog 3-methylindole rapamycin (iRap) with improved dimerization activity in cells. Triple transfected cells were stimulated with iRap and transfected proteins were visualized by confocal microscopy of fixed cells. As expected, stimulation with iRap triggered a dramatic relocalization of PIP5K that overlapped with FRB on perinuclear Golgi membranes (Fig. 4*A*). Importantly, the PIP₂ biosensor, GFP-PH^{PLC δ} , was also

strongly co-recruited to the Golgi. Quantitative analysis of the Pearson correlation coefficients for these proteins from multiple cells confirmed highly significant iRap-dependent synthesis of PIP₂ at the Golgi (Fig. 4*B*). Thus, this novel system allows for inducible PIP₂ generation at an endomembrane system that normally has low concentrations of this phosphoinositide.

Having validated the experimental system, we next repeated this experiment by replacing the PIP₂ reporter plasmid with wild-type and mutant forms of Pak1. Following iRap addition, Pearson correlation analysis revealed modest colocalization of wild-type Pak1 and the Golgi-anchored FRB, consistent with a direct role for PIP₂ in the membrane recruitment of Pak1 (Fig. 4, *C* and *D*). To confirm that this PIP₂-dependent recruitment was mediated by direct binding to the Pak1 basic region, we also tested 8T-Pak1, a mutant deficient in phosphoinositide binding in which 8 lysine residues in the Pak1 basic region are simultaneously mutated to threonine (8). As expected, 8T-Pak1 showed significantly weaker colocalization to the Golgi membranes ($p = 0.0148$). Finally, we tested LL-Pak1, a mutant that abolishes Pak1 binding to Rho GTPases (21). Strikingly, LL-Pak1 also exhibited significantly weaker Golgi recruitment following iRap treatment ($p = 0.0009$, Fig. 4, *C* and *D*). Taken together, these data support a specific role for PIP₂ in Pak1 membrane recruitment in live cells and, importantly, demonstrate that both PIP₂ binding and Rho GTPase binding are required to mediate full Pak1 recruitment.

Because the GTPase-binding domain of Pak1 and its basic region lie adjacent to one other (8), we next tested whether the binding of one enhanced the binding to the other. To do so we used liposome sedimentation assays to measure the binding of Pak1 to Cdc42-bound liposomes that either contained PIP₂ or did not. Although Pak1 bound Cdc42-bound liposomes without PIP₂ with an apparent affinity of 1.33 μM , PIP₂ enhanced the apparent affinity of liposome binding by approximately 1 order of magnitude to 0.16 μM (Fig. 5). This suggests that simultaneous binding of the basic region to PIP₂ and Cdc42 to the adjacent GTPase binding motif may enhance the affinity of Pak1 for such liposomes. We cannot rule out the alternative possibility, however, that PIP₂ in some way orients Cdc42 at the membrane to enhance its affinity for Pak1. Overall our results support a model in which PIP₂ plays a biologically important role together with Rho GTPases in membrane binding of Pak1.

Pak1 Is Synergistically Activated by Cdc42 and PIP₂—Our previous work established that PIP₂ can modestly potentiate catalytic activity *in vitro* by soluble Rac1 (8), however, the experiments reported here suggested highly synergistic binding interactions between Pak1 and PIP₂-containing liposomes bearing Cdc42-His (Figs. 2 and 5). In addition, we observed striking synergistic activation of Pak1 in this system (Fig. 1). Because Rho GTPases are found prenylated at the plasma membrane and our liposome system mimics those conditions, we sought to further explore the synergistic activation of Pak1. We first conducted dose-response experiments in which Cdc42-His was titrated into reactions containing a fixed concentration of PIP₂ liposomes (50 μM total lipid, 6% PIP₂) containing sufficient DGS-NTA(Ni) lipid to quantitatively bind all added Cdc42-His. These reactions were then incubated with Pak1, [³²P]ATP, and peptide substrate of Pak1 (GST-Paktide)

Synergistic Activation of Pak1

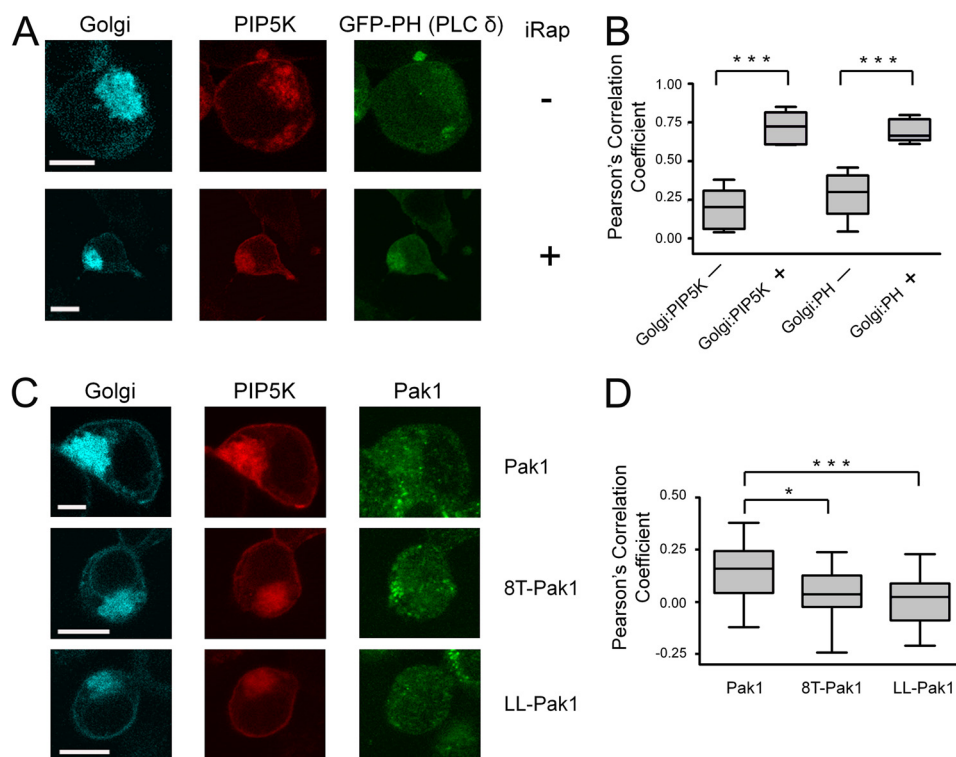


FIGURE 4. Pak1 recruitment to the Golgi membrane is enhanced by Golgi-localized PIP₂ and Cdc42. *A*, HEK293 cells were cotransfected with plasmids encoding Tgn38-FRB-CFP (*Golgi* targeting protein), PIP5K-FKBP-mRFP (*PIP5* kinase), and a GFP-tagged PIP₂-specific binding protein (*GFP-PH(PLCδ)*). Transfected cells were stimulated by iRap for 20 min to induce FRB-FKBP dimerization (*bottom panels*). The *white bars* indicate 10 μ m. *B*, Pearson correlation coefficients for the cellular colocalization of FRB and PIP5K were calculated for 5 randomly selected cells before (–) or after (+) 20 min of iRap treatment; colocalization of FRB and GFP-PH before (–) and after (+) iRap treatment was analyzed similarly. The iRap-stimulated colocalization of these proteins is highly significant for both FRB/PIP5K (***, $p = 0.0007$) and FRB/GFP-PH (***, $p = 0.0001$). The distribution of Pearson coefficients in each case are shown as a *box and whisker plot*, where *whiskers* represent the maximum and minimum values, the *box* represents the 25th to 75th percentiles, and the *central line* depicts the median. *C*, HEK293 cells were transfected with Tgn38-FRB-CFP, PIP5K-FKBP-mRFP, and HA-tagged Pak1, 8T-Pak1 (cannot bind PIP₂), or LL-Pak1 (cannot bind Cdc42). Cells were treated for 20 min with iRap and then immunostained for Pak1 using a total Pak1 antibody (Invitrogen). The *white bars* indicate 10 μ m. *D*, for each condition, 18 cells were randomly chosen and Pearson correlation coefficients were determined for colocalization of Pak1 and FRB. The distribution of Pearson correlation coefficients is shown as *box and whisker plots* as in *B*. *, $p = 0.0148$ and ***, $p = 0.0009$.

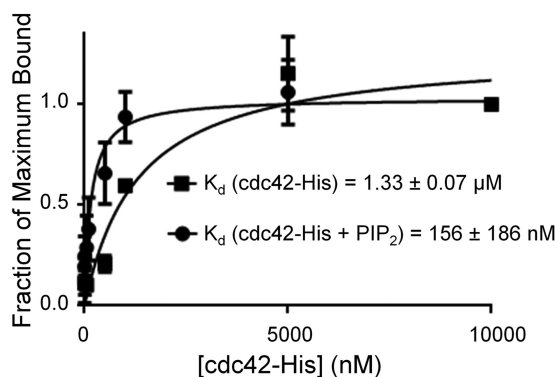


FIGURE 5. The presence of PIP₂ in the membrane increases the affinity of Pak1 for Cdc42 bound to liposomes. Cosedimentation of Pak1 with liposomes (equimolar PC:PE, 3% DGS-NTA(Ni)) was assessed in the presence or absence of 12% PIP₂ by titrating the reactions with increasing concentrations of Cdc42-His. The data were fit in GraphPad Prism to derive apparent affinities (shown). The average \pm S.D. of duplicates is shown.

to allow Pak1 to undergo autophosphorylation and phosphorylate GST-Paktide. Reaction products were analyzed by SDS-PAGE and digital autoradiography. As expected, the dose-response data revealed a steep dose dependence on Cdc42-His concentration for wild-type Pak1, consistent with a cooperative interaction of Pak1 by Cdc42-His and PIP₂ (*supplemental Fig. S3A*). To obtain further evidence of synergy, we next assessed

the individual contributions of Cdc42-His and PIP₂ to Pak1 activation. A combination of 3 μ M PIP₂ (50 μ M total lipid, 6% PIP₂ by mole percent) and 200 nM Cdc42-His led to maximum activation of Pak1 (*supplemental Fig. S3A*). We then compared this catalytic activity with the kinase incubated with either activator alone: (a) 3 μ M PIP₂ in Ni²⁺-NTA containing liposomes without Cdc42-His, (b) 200 nM Cdc42-His bound to control liposomes lacking PIP₂, or (c) 200 nM Cdc42-His bound to liposomes containing PIP₂. In each case, [³²P]ATP kinase assays were utilized to measure Pak1 autophosphorylation and substrate phosphorylation separately. Strikingly, Pak1 autophosphorylation was enhanced \sim 21-fold in the presence of both activators relative to the sum of each activator on its own. Similarly dramatic results were observed for substrate phosphorylation (*Fig. 6A*).

To confirm that this synergy was due to basic region-mediated binding to PIP₂, the same set of experiments was repeated using the 8T-Pak1 mutant. As expected, dose-response curves revealed less cooperativity than wild-type Pak1 (*supplemental Fig. S3B*). Maximal catalytic activity of 8T-Pak1 was observed in the combined presence of 30 μ M PIP₂ and 15 μ M Cdc42-His (*supplemental Fig. S3B*) and, unsurprisingly, the degree of catalytic activity observed for both inputs together was only slightly greater than the additive effect of each activator on its

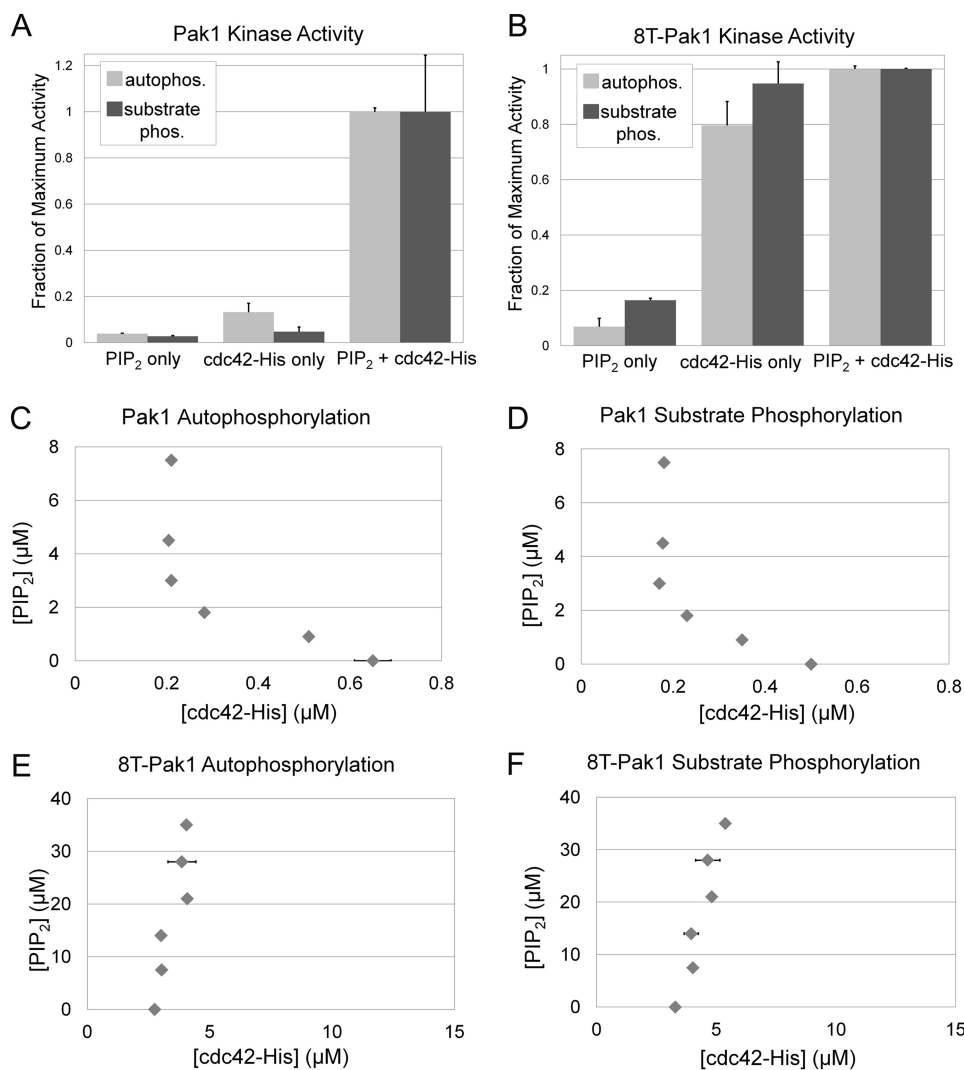


FIGURE 6. Pak1 catalytic activity is synergistically enhanced by Cdc42 and PIP₂. *A*, [γ -³²P]ATP kinase assays were conducted with Pak1 in the presence of the indicated activators and Pak1 catalytic activity was measured by either Pak1 autophosphorylation or Pak1 phosphorylation of a peptide substrate (GST-Paktide). Activators were either PIP₂-containing liposomes (PC:PI:PIP₂:DGS-NTA(Ni), 43.5:43.5:6:7 mole ratio) or control liposomes (PC:PI:DGS-NTA(Ni), 46.5:46.5:7 molar ratio) with 200 nM Cdc42-His or their combination. These activator concentrations were selected on the basis of their ability to maximally activate Pak1 when combined (supplemental Fig. S3A). Results are normalized to phosphorylation observed in the reaction containing both activators and are shown for two independent replicates \pm S.D. *B*, activation for 8T-Pak1 was measured as in *A*. Note that higher concentrations of PIP₂ (500 μ M total lipid and 15 μ M Cdc42-His) were required to achieve full activation of 8T-Pak1 (supplemental Fig. S3B). *C*, 50% activation contour for Pak1 autophosphorylation. The concentration of Cdc42-His required to achieve 50% activation of Pak1 was determined at each indicated PIP₂ concentration. Horizontal error bars (smaller than the data symbol in most cases) represent the S.D. calculated from two replicates. The curve that can be drawn through these points represents all combinations of PIP₂ and Cdc42-His that lead to 50% activation of Pak1. Table 1 presents the data used to generate the plots in *C–F*. *D*, Pak1 catalytic activity in the reactions in *C* was also measured by phosphorylation of a Pak1 substrate peptide included in the reaction and the results are presented as a 50% activation contour. The significant departure from linearity of the contour is indicative of synergistic activation. 8T-Pak1 autophosphorylation (*E*) and 8T-Pak1 substrate phosphorylation (*F*) is not enhanced by PIP₂. The 50% activation contours were determined as in *C* and *D* and the data are summarized in Table 1. The vertical nature of the 50% activation contour demonstrates a lack of sensitivity to PIP₂.

own, demonstrating that PIP₂ binding to the basic region is required for the synergistic activation of wild-type Pak1 by the combination of phosphoinositide and GTPase (Fig. 6B).

Surprisingly, 8T-Pak1 was significantly less sensitive to Cdc42-bound liposomes as a single agent compared with wild-type Pak1. Prior studies showed that 8T-Pak1 is activated comparably to wild-type Pak1 by soluble, nonprenylated Cdc42-His (8) and we confirmed equivalent binding of wild-type and 8T-Pak1 to soluble Cdc42-His (supplemental Fig. S4). By contrast, we consistently observed significantly weaker activation of 8T-Pak1 by liposome-bound Cdc42 than wild-type Pak1. This unexpected finding, together with the observed sensitivity

to PIP₂ spatial density (Figs. 4 and 5) suggests that the Pak1 basic region may also promote Pak1-Cdc42 binding independent of its role in PIP₂ binding in the physiological context of the membrane.

To comprehensively investigate the synergy between PIP₂ and Cdc42 in Pak1 activation, we conducted dose-response experiments in which Cdc42-His was titrated into Pak1 kinase assays in the presence of PIP₂/Ni²⁺-NTA-containing liposomes (see supplemental Fig. S3). The concentration of PIP₂ in those liposomes was systematically varied and the concentration of Cdc42-His required for 50% Pak1 activation at each PIP₂ concentration was determined. The experimentally deter-

Synergistic Activation of Pak1

TABLE 1

Quantitative analysis of Pak1 and 8T-Pak1 activation by PIP₂ and Cdc42-His

This table represents IC₅₀ values for Pak1 and 8T-Pak1 activation by Cdc42-His in the presence of the indicated concentration of PIP₂. PIP₂ concentrations were titrated by increasing total liposome concentration rather than the density of PIP₂ in individual liposomes. These data are presented graphically in Fig. 6, C-F. The single and double asterisks indicate data sets shown in full for illustrative purposes in supplemental Figs. 3, A and B, respectively.

Total lipid	IC ₅₀ (Cdc42-His)		
	PIP ₂	Pak1 autophosphorylation	GST-Paktide phosphorylation
μM	μM	μM	
Pak1			
0	0	0.65 ± 0.16	0.50 ± 0.02
15	0.9	0.51 ± 0.03	0.35 ± 0.01
30	1.8	0.282 ± 0.001	0.23 ± 0.01
50*	3	0.21 ± 0.02	0.17 ± 0.02
75	4.5	0.204 ± 0.004	0.1775 ± 0.0004
125	7.5	0.21 ± 0.01	0.18 ± 0.03
8T-Pak1			
0	0	2.75 ± 0.38	3.29 ± 0.26
125	7.5	3.04 ± 0.19	4.02 ± 0.27
200	12	3.01 ± 0.04	3.95 ± 0.59
300	18	4.08 ± 0.17	4.81 ± 0.12
400	24	3.86 ± 1.14	4.64 ± 1.01
500**	30	4.05 ± 0.26	5.38 ± 0.04

mined IC₅₀ values are listed in Table 1. To visualize the results, we plotted a 50% activation contour on a graph of PIP₂ concentration *versus* Cdc42-His IC₅₀ (12), in which each plotted point denotes a pair of concentrations of Cdc42-His and PIP₂ yielding 50% of maximal Pak1 activation. Separate contours were prepared quantifying Pak1 autophosphorylation (Fig. 6C) and substrate phosphorylation (Fig. 6D). The resulting contours reflect the relative importance of each activator alone in regulating Pak1 catalytic activity. Although an additive interaction would be expected to produce an approximately linear contour, the striking concavity of the contour is consistent with the synergy observed in Fig. 6A. Approximately 0.6 μM Cdc42-His is required for 50% Pak1 activation in the absence of PIP₂, but 3-fold less Cdc42-His is required in the presence of 3 μM PIP₂. Similarly, as the Cdc42-His concentration decreases, increasing PIP₂ must compensate to achieve similar levels of Pak1 activity. We were unable to extend this contour to very low concentrations of Cdc42-His because no concentration of PIP₂ was able to support 50% Pak1 activation below $\sim 0.2 \mu\text{M}$ Cdc42-His. Thus, PIP₂ serves as a potent modifier of Cdc42-dependent activation, enhancing Pak1 sensitivity to Cdc42, without a significant ability to activate Pak1 alone. The synergy we observe at moderate levels of each activator likely explains the requirement for both PIP₂ and GTPase for Pak1 recruitment to the Golgi in cells (Fig. 4).

A similar study was conducted with 8T-Pak1 (Fig. 6, E and F). As expected, these contours do not show similar concavity due to the insensitivity of this basic region mutant to PIP₂ and supports the loss of synergy observed in Fig. 6B. Instead, Cdc42-His concentrations required for 50% Pak1 activation are similar or even higher as PIP₂ concentrations increase. Thus, the synergistic activation observed for wild-type Pak1 is indeed due to PIP₂ binding to the Pak1 basic region.

Finally, to address whether synergy between PIP₂ and Cdc42 is relevant for other Cdc42 effectors or whether it could be a mechanism to selectively activate some Cdc42 effectors but not

others in PIP₂-enriched membrane contexts, we assessed the impact of PIP₂ on membrane recruitment and kinase activity of a different Cdc42 effector kinase, Ack. Unlike Pak1 and N-WASP, the amino acid sequence of Ack contains no analogous basic region adjacent to the Cdc42-binding motif. We prepared recombinant Ack protein comprising the kinase, SH3, and Cdc42-binding domains. Binding of Ack and Pak1 to liposomes with or without PIP₂ and Cdc42 was compared (Fig. 7A). Overall binding of Ack to both PIP₂ and Cdc42-His was weaker than Pak1 and Ack displayed additive binding to liposomes containing both PIP₂ and Cdc42-His. This was in striking contrast to the synergistic binding of Pak1 (Figs. 5 and 7A). Ack kinase activity was also measured in a radioactive kinase assay for its stimulation by Cdc42 and PIP₂ (Fig. 7B). Although Pak1 was synergistically activated by PIP₂ and Cdc42 (Fig. 6A), Ack activation was merely additive (Fig. 7B) and similar to the 8T mutant of Pak1 (Fig. 6B).

DISCUSSION

The Rho GTPases Rac1 and Cdc42 are found anchored to cellular membranes where they are able to activate downstream effector proteins (1). It was previously reported that PIP₂ has a weak, synergistic effect on Pak1 activation by Rac1, however, these experiments were performed with constitutively active GTPase and separate PIP₂-containing liposomes (8). Intriguingly, cellular studies suggest an absolutely essential role *in vivo* for PIP₂ binding on Pak1 activation (8). To reconcile these findings we developed an *in vitro* system in which Cdc42 was anchored in its native orientation on the surface of engineered liposomes and used it to measure the impact of phosphoinositides on Cdc42-dependent activation of Pak1 in a more physiologically relevant assay. Our most important discovery is that Pak1 is activated in a highly synergistic manner by PIP₂ and Cdc42. This finding provides a mechanistic basis for the *in vivo* dependence of kinase on PIP₂ for activation. Pak1 that is insensitive to PIP₂ (8T-Pak1) is unable to be activated in cells despite the presence of active GTPases (8). Furthermore, we validated the specific *in vivo* role for PIP₂ in Pak1 regulation by demonstrating that ectopic synthesis of PIP₂ at the Golgi membrane promotes Pak1 recruitment in a manner dependent on the ability of Pak1 to bind both PIP₂ and Cdc42. These data demonstrate for the first time an *in vivo* role for phosphoinositides in Pak1 activation, that PIP₂ is the only phosphoinositide able to fulfill this function, and that both PIP₂ and Cdc42 are required for Pak1 regulation. Thus, our results identify Pak1 as an integrator of information from phosphoinositide and GTPase-dependent signaling pathways.

We report here the development of two novel experimental systems with broad utility. In the first, we utilized DGS-NTA(Ni) functionalized lipids to immobilize Cdc42 in a signaling-competent manner on the surface of synthetic liposomes. Given the importance of phosphoinositides in the recruitment and regulation of many Ras superfamily GTPases (5), this highly controlled system could find broad use in the elucidation of membrane-associated GTPase signaling. Second, we employed a rapamycin-inducible recruitment system to drive the localization of PIP5K to the Golgi membrane for localized synthesis of PIP₂ where it is not normally enriched. To our knowledge, this

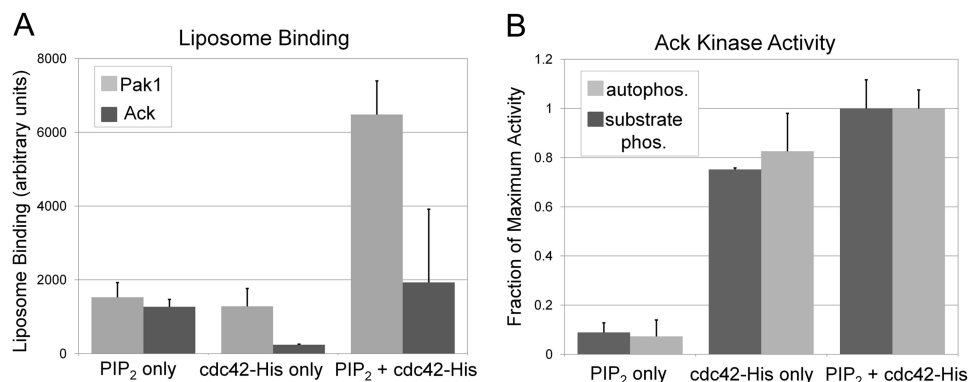


FIGURE 7. Ack binding and catalytic activity is not enhanced by PIP₂. *A*, cosedimentation of Pak1 and Ack with liposomes (equimolar PC:PE, 3% DGS-NTA(Ni)) was assessed in the presence or absence of 5 μ M Cdc42-His and 12% PIP₂. Equimolar amounts of Pak1 and Ack were used. *B*, [γ -³²P]ATP kinase assays were conducted with Ack in the presence of the indicated activators and Ack catalytic activity was measured by either Ack autophosphorylation or Ack phosphorylation of a peptide substrate (GST-Acktide). Activators were either PIP₂-containing liposomes (PC:PI:PIP₂:DGS-NTA(Ni), 43.5:43.5:6:7 mole ratio), or control liposomes (PC:PI:DGS-NTA(Ni), 46.5:46.5:7 molar ratio) with 300 nM Cdc42-His or their combination. These activator concentrations were selected on the basis of their ability to maximally activate Ack when combined. Results are normalized to phosphorylation observed in the reaction containing both activators and are shown for two independent replicates \pm S.D.

is the first example of an experimental system to probe PIP₂ function in the cellular context using such a gain of function strategy at the Golgi membrane. Here, we applied this tool to demonstrate a specific and functional *in vivo* role for PIP₂ in Pak1 localization.

The cooperative regulation of Pak1 kinase activity by phosphoinositides and GTPases is an evolutionarily ancient regulatory mechanism. Cla4, a *Saccharomyces cerevisiae* Pak1 orthologue, is coordinately regulated by Cdc42 and phosphoinositides, however, this interaction is mediated by a PH domain, rather than a basic-rich region and is specific for PI(4)P (13) rather than PIP₂. Similarly, *Schizosaccharomyces pombe* Pak2b contains tandem PH-p21 binding domain domains and both are essential for Pak2p function *in vivo* (41). Like Pak1, STE20 kinase from *Saccharomyces cerevisiae* contains a basic-rich region preceding its p21-protein binding domain and requires both domains for biological function, although there does not appear to be a specific phosphoinositide requirement because a variety of lipid binding domains can functionally substitute for the basic region (38). Here, we extend these prior studies in yeast to human Pak1 and elucidate the molecular details of these interactions. Importantly, human Pak2 and Pak3 also share similar domain organization with Pak1, suggesting that the regulatory mechanisms defined here may also apply to other Group I Paks (8).

Pak1 activation is mediated by trans-autophosphorylation of one dimer subunit by another (31). Such an autocatalytic, feed-forward type mechanism is potentially prone to dramatic amplification. The requirement for two distinct activators for kinase activation may provide a mechanism to prevent inappropriate triggering of a Pak1 activation cascade by transient, stochastic increases in PIP₂ or active GTPase levels. Indeed, noise dampening is an inherent feature of coincidence detectors in biological systems (19). Spatially coordinating phosphoinositide and GTPase signals to converge on Pak1 activation also ensures that the kinase is activated only in certain subcellular localizations at appropriate times.

Signaling pathways have evolved a variety of mechanisms to avoid inappropriate cross-talk between pathways that share

common components. GTPases like Cdc42 regulate many diverse downstream effector proteins and an important gap in our understanding is how specific effector pathways are activated when needed, whereas other effector pathways are suppressed. Regulation of Pak1 by PIP₂ and Cdc42 suggests one possible resolution for this problem through combinatorial control by multiple activators. According to this model, Pak1 recruitment and activation would be restricted to Rho GTPases active in a PIP₂-rich environment, such as the plasma membrane, whereas GTPases localized to the Golgi membrane, normally lacking in large concentrations of PIP₂, would selectively activate other downstream effectors. Such a specificity mechanism would allow for selective activation of discrete GTPase effectors in distinct spatial or temporal contexts. Indeed, this concept is strongly supported by our finding that recruitment and activation of Ack, another Cdc42 kinase effector, does not show PIP₂-mediated synergy. The fact that effectors of other GTPases, such as FAPP, oxysterol binding protein, and the adaptor complex AP-1, also require both GTPases as well as certain phosphoinositides (1), suggests that this mechanism is a common regulatory theme in GTPase signaling.

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