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Biochemical Suppression of Small-Molecule Inhibitors: A Strategy to Identify Inhibitor Targets and Signaling Pathway Components

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Summary

Identification of small-molecule targets remains an important challenge for chemical genetics. We report an approach for target identification and protein discovery based on functional suppression of chemical inhibition in vitro. We discovered pirl1, an inhibitor of actin assembly, in a screen conducted with cytoplasmic extracts. Pirl1 was used to partially inhibit actin assembly in the same assay, and concentrated biochemical fractions of cytoplasmic extracts were added to find activities that suppressed pirl1 inhibition. Two activities were detected, separately purified, and identified as Arp2/3 complex and Cdc42/RhoGDI complex, both known regulators of actin assembly. We show that pirl1 directly inhibits activation of Cdc42/RhoGDI, but that Arp2/3 complex represents a downstream suppressor. This work introduces a general method for using low-micromolar chemical inhibitors to identify both inhibitor targets and other components of a signaling pathway.

Introduction

Phenotypic screens for small-molecule inhibitors are a powerful method by which to probe biological pathways, but they require subsequent identification of the inhibitor target. When used as a protein discovery tool, these screens have been called "forward chemical genetics," based on the analogy to traditional forward genetics in which random mutants are screened for a phenotype of interest and the mutated genes are subsequently identified [1]. Whereas genetics offers general approaches for the identification of mutated genes, such as complementation, the identification of the targets of small-molecule inhibitors, particularly low-micromolar "hits" often identified in phenotypic screens, can be challenging [2, 3]. Consequently, diverse new approaches to small-molecule target identification are greatly needed.

In addition to identifying mutated genes, traditional genetics can also be used to discover other components in a biological pathway of interest, for example, through high-copy suppressor screens. In this approach, individual genes from cDNA libraries are overexpressed to identify clones that suppress the original mutation, restoring the wild-type phenotype. This can be accomplished by overexpressing a wild-type copy of the mutated gene but also by overexpressing other pathway components downstream of the inactivated gene.

In the context of chemical genetics, high-copy suppressor screens have been used successfully to identify genes that, when overexpressed, confer resistance to small molecules with antimicrobial and anticancer activities [4, 5], but this strategy is limited to compounds that function in genetically tractable organisms. Phenotypic screens for small-molecule inhibitors, however, are now increasingly being conducted in cytoplasmic extracts and other complex systems that are not amenable to subsequent genetic screens [6, 7]. Inspired by the power of genetic high-copy suppressor screens to identify multiple components of a biological pathway in vivo, we developed an analogous biochemical approach to identify components of pathways that can be recapitulated in vitro. We use chemical inhibitors as "mutations" and the addition of partially purified protein mixtures as a means of "overexpressing" potential suppressors.

This biochemical suppression approach is conceptually related to the classic work of Rothman and colleagues in which the sulfhydryl alkylating agent N-ethylmaleimide (NEM) was used to inactivate proteins required for Golgi membrane fusion in a cell-free system [8]. The inactivated preparations were then functionally complemented by the addition of partially purified protein fractions to allow an activity-based purification of the proteins inactivated by NEM. This approach relied on the relatively nonspecific and covalent modification of any functionally relevant protein by NEM. By contrast, the method we introduce here uses a noncovalent and titratable small-molecule inhibitor, *specifically* identified in a phenotypic screen, to *partially* inactivate its protein target. Specific and partial inhibition of a protein in a signaling pathway allowed for the identification not only of the target of the inhibitor, but also of a downstream component in the same pathway.

We used the biochemical suppression approach to study a signaling pathway regulating actin polymerization in cytoplasmic extracts of *Xenopus laevis* eggs (*Xenopus* egg extracts) [9–11]. Liposomes containing phosphatidylinositol 4,5-bisphosphate (PIP_2 liposomes) stimulate actin filament nucleation and polymerization in the extract by activating the Rho family GTPase Cdc42. Cdc42 then binds to two effectors, the transducer of Cdc42-dependent actin assembly (Toca-1) and the neural-Wiskott-Aldrich syndrome protein (N-WASP), the latter of which directly activates Arp2/3 complex [11]. Arp2/3 complex is a seven polypeptide protein assembly that directly nucleates new actin filaments [12]. The components of this pathway are evolutionarily conserved and are thought to play central roles in cell motility and membrane trafficking [12].

In a screen for small-molecule inhibitors of PIP_2-induced actin polymerization [13, 14], we identified pirl1.

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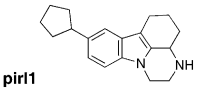
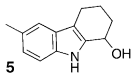
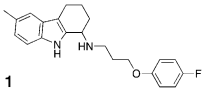
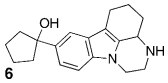
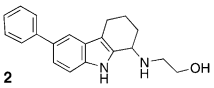
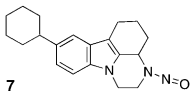
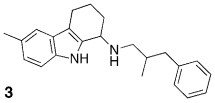
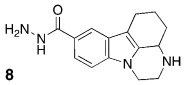
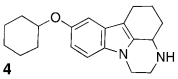
Using pirl1 to inhibit signaling through this pathway, we then characterized and purified two activities that suppressed inhibition by pirl1. These activities correspond to Arp2/3 complex and Cdc42/RhoGDI (Rho guanine nucleotide dissociation inhibitor) complex, two of the protein complexes that mediate signaling from PIP_2 to actin. We present evidence that pirl1 acts by inhibiting guanine nucleotide exchange on Cdc42, indicating that the biochemical suppression strategy identified a direct target of pirl1. In addition, we show that Arp2/3 complex is not directly inhibited by pirl1, demonstrating that this strategy also identified a downstream effector of the pathway. These results illustrate a novel approach to target identification that applies the power of genetic high-copy suppressor screens to low-affinity chemical inhibitors obtained in phenotypic high-throughput screens conducted in vitro.

Results

Identification of Pirl1 and Characterization of "Suppressor of Pirl1" Activity

A high-throughput screen for small-molecule inhibitors of PIP_2-induced actin polymerization [13, 14] identified pirl1, a tetracyclic indole structurally similar to the monoamine oxidase inhibitor pirlindole (Table 1). Pirl1 inhibited actin assembly induced by 10 μM PIP_2 liposomes in *Xenopus* egg extracts with an IC_{50} (dose required to inhibit the maximum polymerization rate by 50%) of 3 μM. Table 1 also presents structural derivatives of pirl1 and their potency in this assay.

Table 1. Structure of Pirl1 and Related Compounds and Corresponding IC_{50} in PIP_2-Stimulated Actin Polymerization Assays

Structure	IC_{50} (μM)	Structure	IC_{50} (μM)
 pirl1	3	 5	39
 1	2	 6	82
 2	2	 7	99
 3	4	 8	>100
 4	12		

Using this screen, we previously reported the identification and characterization of wiskostatin, another small-molecule inhibitor of PIP_2-induced actin assembly [13]. N-WASP was identified as the target of wiskostatin by testing candidate proteins in in vitro reactions containing purified proteins that reconstitute portions of the PIP_2-induced actin assembly pathway downstream of Cdc42. In similar experiments, pirl1 failed to inhibit in vitro reactions at doses that inhibit PIP_2-induced actin assembly in extracts. Consequently, we sought an alternative, less biased approach to identify the target of pirl1 in *Xenopus* egg extract.

Affinity-based methods for small-molecule target identification are most likely to be successful with small-molecule target affinities higher than the weak binding implied by the low-micromolar IC_{50} observed with pirl1. Furthermore, such methods as affinity labeling and affinity chromatography generally favor abundant targets [3]. We therefore approached the problem by looking for proteins that *functionally* rescue the inhibited pathway.

Genetic high-copy suppressor screens can identify the wild-type allele of the mutated gene, but they can also identify other components of the pathway that, when overexpressed, overcome the phenotypic defect. To adapt this concept to an in vitro assay, we considered that partially inhibiting a protein in a signaling pathway with a small molecule is analogous to generating a hypomorphic allele. Next, we reasoned that adding concentrated protein fractions to introduce suppressor activities is analogous to overexpressing proteins genetically. These two steps form the basis for the activity-based biochemical purification of suppressor activities through iterative rounds of protein fractionation and activity assays (Figure 1A).

We tested the feasibility of this approach in PIP_2-induced actin polymerization reactions inhibited by pirl1. First, we partially inhibited *Xenopus* egg extract by adding 5 μM pirl1 (Figure 1B; red trace), an inhibitor concentration that provides a wide dynamic range for measuring suppression of inhibition while maintaining sensitivity. Separately, we fractionated uninhibited extract by cation exchange chromatography (SP Sepharose), concentrated the fractions, and added them to aliquots of pirl1-inhibited extract. Fraction 9 potently stimulated actin assembly despite pirl1 (Figure 1C).

We considered two trivial reasons why this fraction might suppress inhibition by pirl1. First, an abundant protein that binds the small molecule nonspecifically might titrate pirl1 away from its relevant target. Alternatively, a factor might appear to suppress pirl1 inhibition by stimulating actin assembly through a PIP_2-independent mechanism. To address the first possibility, fraction 9 or buffer was added to extract in the absence of inhibitor, and actin assembly was induced by the addition of PIP_2 liposomes. If the suppressor activity was due to nonspecific titration of the inhibitor, it should not affect actin polymerization kinetics in the absence of pirl1. However, fraction 9 significantly enhanced actin polymerization kinetics in the absence of pirl1 (Figure 1D; compare blue and red traces), indicating that this fraction acts in a positive manner to promote actin assembly rather than simply titrating pirl1 away from its target.

To test if the suppressor activity in fraction 9 stimulated actin assembly independently of the PIP_2 pathway,

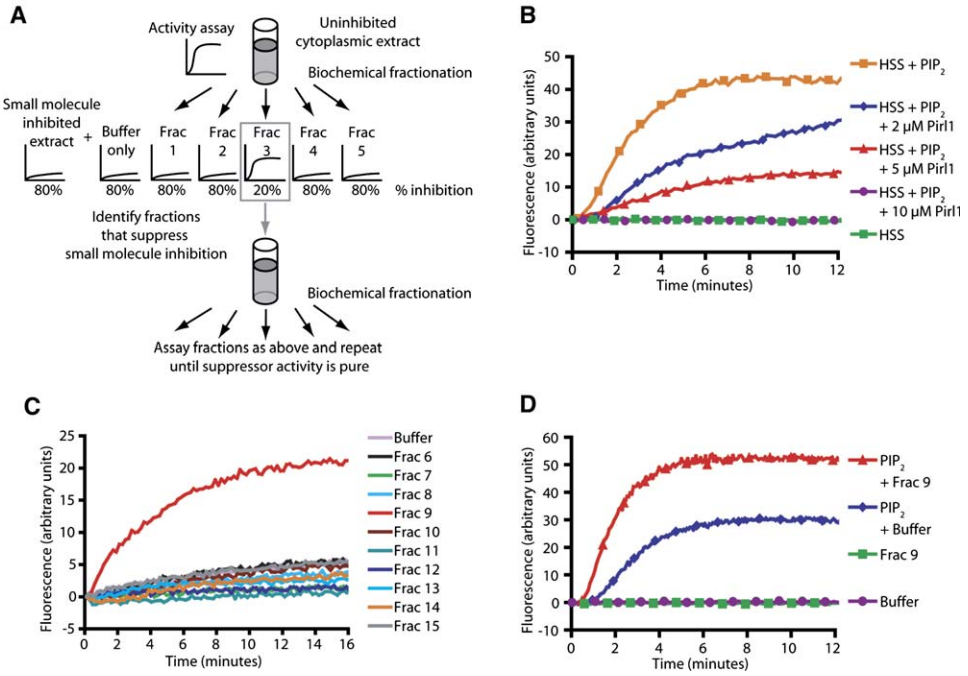


Figure 1. The Biochemical Suppression Approach and Initial Characterization of Suppressor Activity

(A) Biochemical suppression of small-molecule inhibition. A small molecule is added to cytoplasmic extract to partially inhibit the activity of interest. Separately, uninhibited extract is fractionated, and individual fractions are added to the inhibited extract. Fractions that suppress compound inhibition in the activity assay are then fractionated further. The suppressor activity is purified by iterative rounds of fractionation and activity assays.

(B) Pirl1 inhibits PIP_2-induced actin polymerization in *Xenopus* egg extract. Extracts supplemented with pyrene-actin (HSS) were preincubated with the indicated concentrations of pirl1 or DMSO vehicle, and 10 μM PIP_2 liposomes were added (as indicated) to stimulate actin filament nucleation. Actin polymerization was detected by the fluorescence increase of pyrene-actin upon incorporation into filaments.

(C) Assay for suppressor activity. The indicated concentrated fractions from uninhibited extract fractionated by cation exchange chromatography were mixed with complete extract containing pirl1 (5 μM final concentration), and 10 μM PIP_2 liposomes were added to induce actin polymerization.

(D) The suppressor activity does not titrate pirl1 nonspecifically and is PIP_2-dependent. Actin polymerization was monitored in uninhibited extracts to which fraction 9, PIP_2 liposomes, or both were added.

fraction 9 was added to a quiescent extract without PIP_2 stimulation. No polymerization of actin due to fraction 9 was observed in the absence of PIP_2 liposomes (Figure 1D; compare purple and green traces), demonstrating that the activity in fraction 9 was strictly dependent on PIP_2 to stimulate actin polymerization. Thus, fraction 9 appeared to contain a bona fide component of the PIP_2-dependent actin assembly pathway capable of suppressing inhibition by pirl1. We therefore named this activity SOP (suppressor of pirl1) and conducted a large-scale biochemical purification to identify the protein or proteins responsible for the SOP activity.

Purification and Identification of SOP1 as Arp2/3 Complex and SOP2 as Cdc42/RhoGDI Complex

Xenopus egg extract was fractionated over SP Sepharose, and fractions containing SOP activity were further fractionated by Mono Q chromatography (Figure 2A). Interestingly, two independent, nonoverlapping peaks of SOP activity were observed in fractions eluting from the Mono Q column at 95 and 150 mM NaCl (data not shown). We called these activities SOP1 and SOP2, respectively, and we purified them independently.

SOP1-containing fractions from the Mono Q elution were applied to a gel filtration column (Superdex 200), and the elution profile revealed a major $Abs_{280\ nm}$ peak migrating at ~240 kDa. SDS-PAGE analysis of the corresponding fractions showed seven perfectly cofractionating bands that correlated with SOP activity and had molecular weights from 18 to 50 kDa, highly suggestive of pure Arp2/3 complex (Figure 2B). Indeed, the identity of these proteins as intact Arp2/3 complex was confirmed by their comigration with purified bovine brain Arp2/3 complex (Figure 2C), by Western blotting with antibodies to the Arp2 subunit (Figure 2C), and by in vitro functional assays demonstrating that the SOP1-containing Superdex 200 fractions nucleate actin assembly when stimulated by a peptide corresponding to the C-terminal VCA domain of N-WASP (not shown), a unique property of Arp2/3 complex [12]. Finally, native Arp2/3 complex purified from bovine brain (shown in Figure 3C) also exhibited SOP activity (Figure S1 in the Supplemental Data available with this article online), demonstrating that *Xenopus* Arp2/3 complex, and not a minor contaminating protein, is responsible for SOP1 activity.

The identification of Arp2/3 complex as the SOP1 activity suggested that Arp2/3 complex might be directly inhibited by pirl1. However, pirl1 had no effect on VCA-stimulated actin polymerization in *Xenopus* egg extract (Figure 2D; compare red and blue traces) under conditions in which PIP_2-stimulated actin polymerization

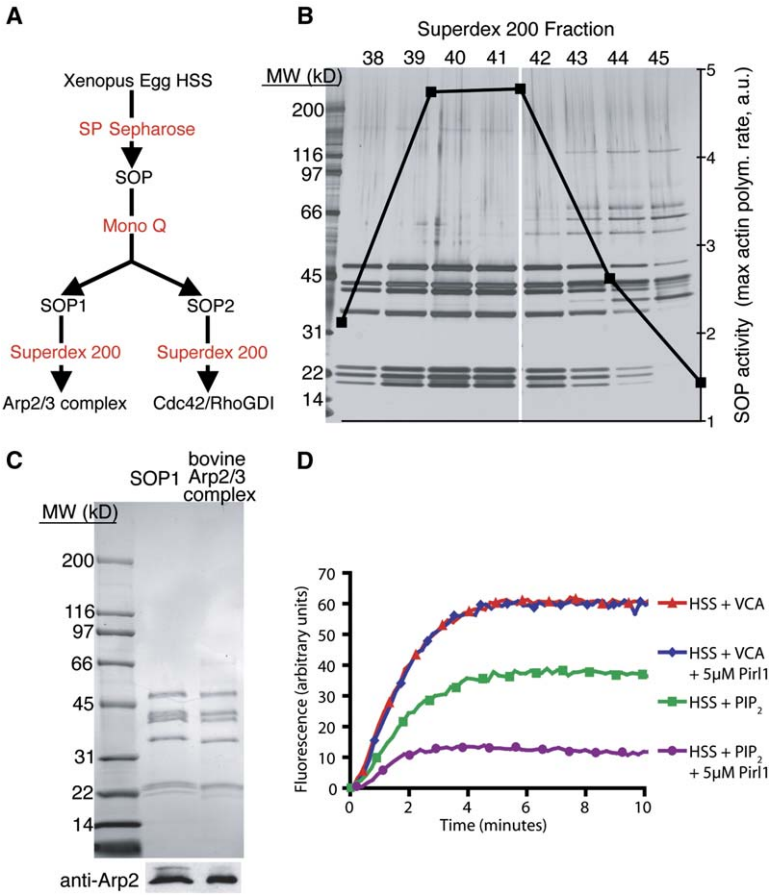


Figure 2. Purification and Identification of SOP1 as Arp2/3 Complex

(A) Purification scheme.

(B) Silver-stained SDS-PAGE of SOP1-containing Superdex 200 fractions and corresponding maximal actin polymerization rates from pirl1-inhibited, PIP_2-induced pyrene-actin assays supplemented with pooled pairs of adjacent column fractions (e.g., 37/38, 39/40), as in Figure 1C.

(C) Coomassie-stained SDS-PAGE of SOP1-containing Superdex 200 fractions (pooled fractions 38–42) and native bovine Arp2/3 complex. The lower panel shows a corresponding Western blot for the Arp2 subunit of Arp2/3 complex.

(D) Pirl1 does not directly inhibit Arp2/3 complex. *Xenopus* egg extracts supplemented with pyrene-actin were treated with 5 μM pirl1 or DMSO vehicle and then stimulated with either 10 μM PIP_2 liposomes or 300 nM GST-VCA to directly activate Arp2/3 complex.

was inhibited (Figure 2D; compare green and purple traces). Furthermore, the actin nucleation activity of neither purified *Xenopus* Arp2/3 complex nor bovine brain Arp2/3 complex was affected by 25 μM pirl1 in purified protein assays stimulated by the VCA polypeptide (data not shown), suggesting, instead, that Arp2/3 complex suppresses pirl1 inhibition by overcoming an upstream inhibited step through increased levels of a downstream component. Yet, addition of excess Arp2/3 complex to unstimulated extract did not spontaneously induce actin nucleation (Figure S1), indicating that the activity of Arp2/3 complex still requires stimulation by PIP_2.

To purify the second SOP activity, SOP2-containing fractions eluting from the Mono Q column were fractionated by gel filtration and assayed for SOP activity (Figure 3A). Five silver-stained protein bands cofractionated with SOP activity and eluted at ~50 kDa from the Superdex 200 column (Figure 3A, asterisks). Mass spectrometry analysis of tryptic digests of these five bands, from highest to lowest apparent molecular weight (MW), identified the following *Xenopus laevis* proteins: arginyl aminopeptidase (MW = 70 kDa, 11 peptides, 15% coverage); protein phosphatase 5 (MW = 56 kDa, 5 peptides, 11% coverage); a mixture of eukaryotic translation initiation factor 4H (MW = 26 kDa, 3 peptides, 22% coverage) and RAN binding protein 1 (MW = 24 kDa, 7 peptides, 24% coverage); Rho GDP-dissociation inhibitor (RhoGDI) (MW = 23 kDa, four peptides, 30% coverage); Cdc42 (MW = 21 kDa, 2 peptides, 13% coverage) (Figure 3B shows sequence coverage for RhoGDI and Cdc42). Quantitation by densitometry of the bands corresponding to Cdc42 and RhoGDI in the fractions from the Superdex 200 elution showed a striking correlation between the abundance of these proteins and SOP activity (Figure 3C), and Cdc42-specific Western blot analysis of the SOP2 peak fraction revealed a strongly reactive band that comigrated with recombinant and bovine Cdc42 (Figure 3D), confirming the presence of Cdc42 in the SOP2-containing fractions.

The small GTPase Cdc42 is posttranslationally modified by a hydrophobic isoprenoid group and is kept soluble in the cytoplasm by binding to RhoGDI [15]. RhoGDI inhibits Cdc42 signaling by preventing guanine nucleotide exchange on Cdc42 and precluding interactions with Cdc42 effectors [15]. Given the well established role of Cdc42 in mediating PIP_2-induced actin assembly [9–11], the presence of both Cdc42 and RhoGDI in the SOP2 peak fractions eluting from the gel filtration column strongly suggested that the complex of these two proteins might be responsible for suppressing pirl1 inhibition of actin assembly in the extracts. Indeed, recombinant Cdc42/RhoGDI complex suppressed pirl1 inhibition of PIP_2-induced actin nucleation in a dose-dependent manner (Figure 3E), demonstrating that Cdc42/RhoGDI exhibits SOP activity. As for Arp2/3 complex, the actin polymerization-promoting activity of recombinant Cdc42/RhoGDI (as well as that of SOP2-containing fractions) depended strictly on stimulation by PIP_2 liposomes (Figure S2).

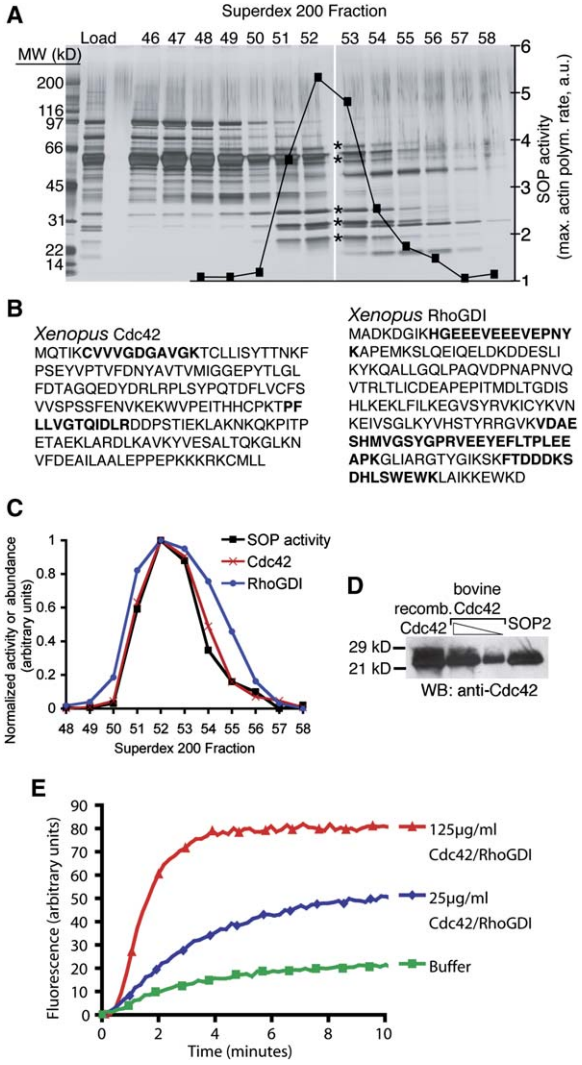


Figure 3. SOP2 Is the Native Complex of Cdc42/RhoGDI

(A) Silver-stained SDS-PAGE of SOP2-containing Superdex 200 fractions and corresponding maximal actin polymerization rates from pirl1-inhibited, PIP_2-induced pyrene-actin assays supplemented with each fraction. Asterisks indicate proteins with elution profiles correlating with SOP activity.

(B) Amino acid sequences of *Xenopus* Cdc42 and RhoGDI are shown, and sequences of tryptic peptides identified by mass spectrometry in SOP2-containing fractions (51–54) are indicated in bold.

(C) Graph of SOP activity and densitometry of bands corresponding to Cdc42 and RhoGDI in each Superdex 200 fraction (shown in [A]).

(D) Cdc42 Western blot analysis of recombinant Cdc42, two concentrations of partially purified, native bovine Cdc42/RhoGDI complex, and SOP2-containing fraction 52.

(E) Recombinant Cdc42/RhoGDI complex exhibits SOP activity. *Xenopus* egg extracts supplemented with pyrene-actin were preincubated with 5 μM pirl1 and the indicated concentrations of recombinant Cdc42/RhoGDI complex, and PIP_2 liposomes were added to induce actin polymerization.

Pirl1 Inhibits Nucleotide Exchange on Cdc42

Since Cdc42 is inhibited by its interaction with RhoGDI [15], activation of Cdc42/RhoGDI complex is required for this complex to promote actin assembly. A necessary event for activation is the exchange of GDP for GTP on Cdc42 catalyzed by a guanine nucleotide exchange factor (GEF) [16]. However, which GEF is involved in PIP_2-stimulated actin assembly in *Xenopus* egg extract is not known.

To test if nucleotide exchange on Cdc42 is prevented by pirl1, pirl1-inhibited extracts were stimulated by PIP_2 liposomes in the presence of the nonhydrolyzable GTP analog GTPγS to trap GTP bound Cdc42, which can be captured by affinity isolation by using the p21 binding domain of Pak kinase [17]. PIP_2 stimulation caused a dramatic activation of endogenous Cdc42 that was inhibited by pirl1 (Figure 4A). Importantly, the ability of eight other pirl1 derivatives to inhibit PIP_2-induced Cdc42 activation perfectly correlated with their ability to inhibit PIP_2-induced actin assembly, implying that inhibition of Cdc42 activation is responsible for inhibition of actin assembly.

Inhibition of nucleotide exchange on Cdc42/RhoGDI complex by pirl1 could be direct or due to inhibition of an upstream step in the pathway initiated by PIP_2. To discriminate between these possibilities, we conducted in vitro guanine nucleotide exchange assays by using recombinant Cdc42/RhoGDI complex, excess free RhoGDI to minimize the basal rate of nucleotide exchange on Cdc42, and the isolated DH-PH domain of Dbs, a well-characterized GEF for Cdc42 [18]. In this assay, Dbs catalyzed nucleotide exchange onto Cdc42 only upon PIP_2 stimulation [19] (Figure 4B). Pirl1, but not the inactive derivative **6**, inhibited this nucleotide exchange (Figure 4B).

Nucleotide exchange on Cdc42 can be artificially induced in vitro by chelating magnesium with EDTA [20]. Because nucleotide release from Cdc42 requires dissociation from RhoGDI, the kinetics of EDTA-mediated nucleotide exchange on Cdc42/RhoGDI complex are likely dictated by the dissociation rate of Cdc42/RhoGDI complex itself. EDTA-mediated exchange was strictly PIP_2 liposome dependent, suggesting that PIP_2 liposomes may promote dissociation of the complex [19] (Figure 4C). Importantly, pirl1, but not the inactive derivative **6**, inhibited EDTA-mediated nucleotide exchange on Cdc42 (Figures 4C and 4D), indicating that pirl1 directly affects Cdc42/RhoGDI complex or its interaction with PIP_2 liposomes. Pirl1 did not affect EDTA-mediated nucleotide exchange on nonprenylated Cdc42, which does not form a complex with RhoGDI (Figure 4E), indicating that pirl1 does not inhibit nucleotide release from or binding to Cdc42 per se.

Thus, direct inhibition of PIP_2-mediated guanine nucleotide exchange on Cdc42/RhoGDI by pirl1 accounts for the inhibition of actin polymerization in PIP_2-stimulated *Xenopus* egg extracts. These results also establish the ability of the biochemical suppression approach to identify signaling pathway components directly targeted by small-molecule inhibitors as well as other components in the inhibited pathway.

Pirl1 Reversibly Inhibits Phorbol Ester-Induced Membrane Ruffling

To test if pirl1 is cell permeable and perturbs actin dynamics in living cells, BS-C-1 cells were stimulated with phorbol myristate acetate (PMA) to induce actin-dependent membrane ruffling in the presence of DMSO, pirl1, compound **1**, or the inactive control compounds **5**, **6**, **7**, or **8** (Figure 5; control compounds **5**, **7**, and **8** are not shown). Consistent with their activities in

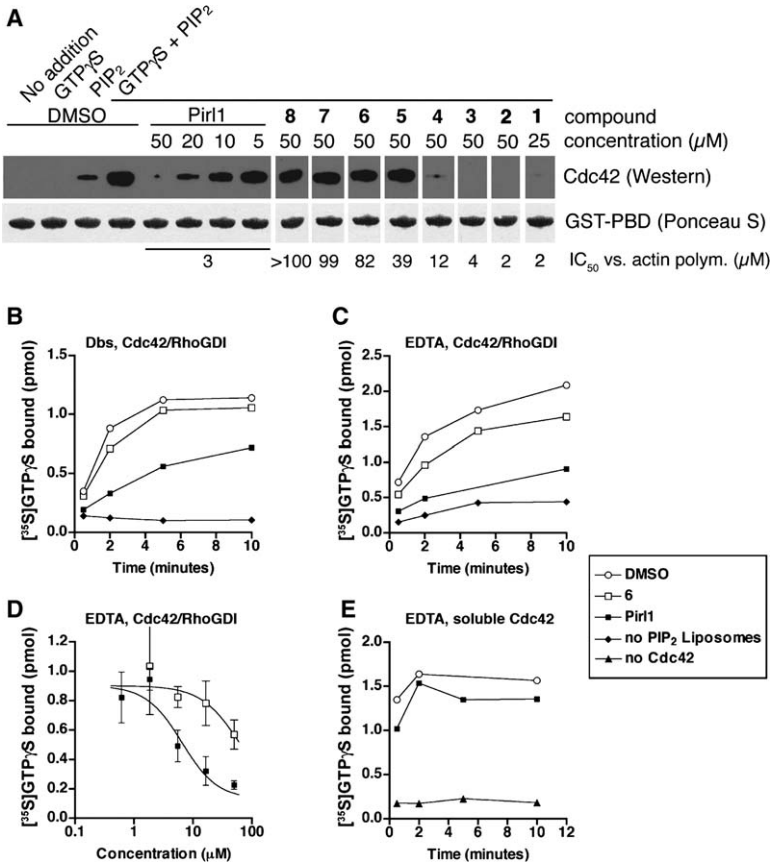


Figure 4. Pirl1 Inhibits Activation of Cdc42 in *Xenopus* Egg Extract and Guanine Nucleotide Exchange on Cdc42/RhoGDI Complex in Purified Protein Assays

(A) PIP_2-mediated activation of Cdc42 in *Xenopus* egg extract is inhibited by pirl1. Extracts were pretreated with different concentrations of pirl1, related compounds (**1–8**, see Table 1), or DMSO vehicle as indicated. After PIP_2 liposome stimulation in the presence of GTPγS, activated Cdc42 was coprecipitated by using the p21 binding domain of Pak kinase (GST-PBD) bound to glutathione-agarose beads. Cdc42 was detected by Western blotting.

(B) Pirl1 inhibits Dbs-mediated nucleotide exchange on purified recombinant Cdc42/RhoGDI complex. Cdc42/RhoGDI complex (0.5 μM) was incubated with 1 μM RhoGDI; 30 nM DH-PH domain of Dbs; [^{35}S]GTPγS; 100 μM PIP_2 liposomes; and either 25 μM pirl1, compound **6**, or DMSO vehicle as indicated. At each time point, protein bound [^{35}S]GTPγS was captured by filtration and quantitated by scintillation counting.

(C) Pirl1 inhibits EDTA-mediated nucleotide exchange on purified recombinant Cdc42/RhoGDI. Assays were conducted as in (B), except that 4 mM EDTA was used instead of Dbs.

(D) Dose dependence of pirl1 and compound **6** inhibition of EDTA-mediated nucleotide exchange on purified Cdc42/RhoGDI complex. Reactions like those in (C) were conducted with the indicated concentrations of pirl1 or compound **6**, and protein bound GTPγS was quantified after 3 min of incubation. Error bars indicate standard error (n = 3).

(E) Pirl1 does not inhibit nucleotide exchange on nonprenylated Cdc42. Assays were conducted as in (C), except that soluble, nonprenylated Cdc42 was used instead of Cdc42/RhoGDI complex.

Xenopus egg extracts, pirl1 and compound **1**, but not the inactive control compounds, prevented the formation of actin-rich ruffles on the dorsal cell surface, whereas actin stress fibers, normally disrupted by PMA treatment (Figure 5A, "PMA"), remained intact. Quantitation of this effect is shown in Figure 5B. Remarkably, the inhibitory effect of pirl1 did not require preincubation of the cells with pirl1 prior to PMA stimulation, and removal of pirl1 from the media restored the ability of treated cells to respond to PMA (Figure 5, "Pirl1 washout"). These results indicate that pirl1 is cell permeable, and that it can reversibly inhibit PMA-induced membrane ruffling.

Discussion

The work presented here applies, for the first time, the conceptual principles of a genetic high-copy suppressor screen to an in vitro biochemical reaction partially inhibited by a small molecule. Using iterative rounds of activity assays and biochemical fractionation, we identified two distinct protein complexes that, when added at increased concentrations, suppress inhibition by pirl1, a novel chemical inhibitor of the PIP_2-dependent actin assembly pathway. SOP1 was identified as the native *Xenopus* Arp2/3 complex, and SOP2 was identified as the native *Xenopus* Cdc42/RhoGDI complex. Both protein complexes are known mediators of signaling from PIP_2 to actin, validating this method for the discovery of proteins mediating signaling pathways. Pirl1 did not directly inhibit Arp2/3 complex function, suggesting that it was identified as a SOP activity by virtue of acting downstream of the inhibited component of the signaling pathway. In contrast, pirl1 potently inhibited guanine nucleotide exchange on Cdc42/RhoGDI complex, both in extracts and in purified protein assays, indicating that this complex is the inhibited target in the pathway.

Consistent with its ability to perturb actin filament nucleation in *Xenopus* egg extract, pirl1 inhibited membrane ruffling induced by PMA in live cells in a reversible manner. Caution must be exercised, however, when interpreting phenotypes induced by pirl1 in live cells until its specificity and effectiveness at inhibiting Cdc42 are established in future work. Other members of the Rho family of small GTPases, for example, Rac and Rho, also form complexes with and are regulated by RhoGDI, and their activation could conceivably also be inhibited by pirl1. Potential inhibition of these other GTPases, however, does not conflict with identification of Cdc42/RhoGDI as the relevant target of pirl1 by biochemical suppression. In fact, one advantage of the biochemical suppression strategy lies in its ability to identify targets that are active in the pathway being studied, even if the inhibitor also targets other proteins that are not functionally relevant.

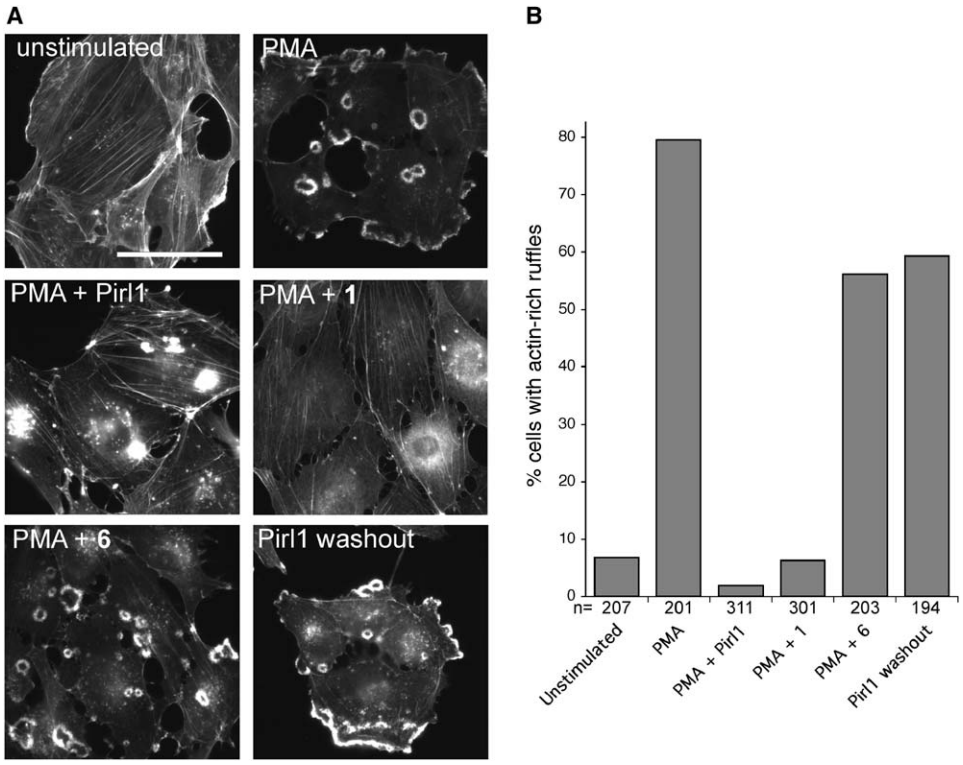


Figure 5. Pirl1 Reversibly Inhibits the Formation of Actin-Rich Membrane Ruffles in PMA-Stimulated Cells

(A) BS-C-1 cells were either fixed directly (unstimulated) or simultaneously treated for 15 min with 250 ng/ml phorbol myristate acetate (PMA) and either DMSO vehicle (labeled "PMA"), 50 μM pirl1, compound **1**, or inactive control compound **6**. Cells labeled "Pirl1 washout" were treated for 15 min with 50 μM pirl1 alone, and then the media was removed and replaced with media lacking pirl1 for 1 hr prior to stimulation with PMA as described above. All cells were fixed and stained with Alexa 488-labeled phalloidin to visualize the filamentous actin cytoskeleton. The scale bar is 50 μm.

(B) Quantitative analysis of the experiment shown in (A). The percentage of cells exhibiting actin-rich membrane ruffles is shown for each condition. The number of cells counted for each condition is shown beneath each bar.

We cannot presently conclude that Cdc42 is the relevant GTPase mediating inhibition of PMA-induced membrane ruffling in BS-C-1 cells because the mechanisms mediating PMA-induced ruffling are still not completely understood and may be cell-type specific. Although experiments involving expression of dominant-negative GTPases in a murine macrophage cell line suggest that PMA-induced ruffling requires Cdc42 but not Rac1 [21], similar experiments in Swiss 3T3 cells suggest that PMA-induced ruffling does require Rac1 [22, 23]. Notwithstanding the issue of specificity, our studies introduce a novel, reversible reagent for probing actin dynamics in living cells. These types of compounds have historically played an important role in the study of the cytoskeleton [24].

An important distinction between the biochemical suppression strategy described here and traditional genetic high-copy suppressor screens is that biochemical suppression is mediated by completely native protein forms as opposed to individual gene products. Indeed, it is unlikely that overexpression of individual subunits of either Arp2/3 complex or Cdc42/RhoGDI complex would suppress pirl1 inhibition because of the importance of the integrity of the complexes for their function. Thus, biochemical suppression offers the distinct advantage of identifying physiologically relevant protein complexes that constitute suppressor activities and that are typically inaccessible to genetic approaches.

Our approach is also distinguished from the classic work of Rothman and colleagues, who inactivated factors required for Golgi membrane fusion with the alkylating agent NEM to allow activity-based purification of those factors by complementation [8]. NEM treatment covalently modifies many free sulfhydryl groups, and, consequently, it would be difficult to control the reaction to only partially modify a particular target. Thus, in NEM-treated cytosol, NEM-sensitive factors are *completely* inactivated. By contrast, pirl1 is a noncovalent and reversible inhibitor, and it could be carefully titrated to only *partially* inhibit Cdc42/RhoGDI in *Xenopus* egg extract. The importance of this distinction is established by the fact that partial inhibition of Cdc42/RhoGDI complex by pirl1 enabled the identification of Arp2/3 complex as an SOP activity. The ability of Arp2/3 complex to suppress inhibition by pirl1 is strictly dependent on the presence of some residual Cdc42 activity owing to only *partial* inhibition by pirl1. For instance, addition of Arp2/3 complex to unstimulated extracts (where Cdc42 activity is completely absent) does not induce actin assembly, even in the absence of pirl1. Thus, noncovalent, titratable small-molecule inhibitors can be used to alter rate-limiting steps in signaling pathways rather than

completely inhibit them, allowing for the identification of nontarget suppressor activities.

Biochemical suppression is particularly suited for the relatively low-affinity inhibitors typically identified in phenotypic screens. For example, in order to achieve the partial inhibition essential for detecting nontarget suppressors, a high-affinity (e.g., low nanomolar IC_{50}) inhibitor would have to be used at such a low concentration that it would likely be much more readily titrated away nonspecifically by abundant proteins in biochemical suppression assays. In this context, it is interesting to note that neither of the two suppressor activities we identified titrated away the inhibitor nonspecifically or stimulated actin assembly by an unrelated mechanism.

Because the strategy requires only the ability to introduce partially purified protein fractions into an assay of interest, it should be widely applicable to phenotypic screens conducted in extracts, complex mixtures, or permeabilized cells [6, 7]. Such unbiased screens for small-molecule inhibitors that disrupt a biological process of interest are powerful discovery tools because, having multiple potential targets, they allow those components of a pathway that are most susceptible to chemical inhibition to reveal themselves. In addition, cytoplasmic extracts can generally be prepared in large scale at relatively low cost compared to the production and purification of recombinant proteins for targeted screening. Furthermore, because the purification of suppressors is based on biochemical activity, even proteins present at low abundance, but exhibiting measurable biological activity, can be identified. By contrast, purification of protein targets on immobilized small-molecule matrices is substantially biased by protein abundance.

Finally, our results broadly suggest a strategy for choosing targets for drug discovery. Rather than investing substantial time and effort in choosing and validating a specific protein target in advance of high-throughput screening, primary phenotypic screens can be used in conjunction with target identification strategies such as the biochemical suppression approach both to identify critical components of a signaling pathway of interest in an unbiased way and to produce initial lead inhibitors. A second phase of targeted screening in vitro with the purified target protein can then be used to identify compounds of greater potency.

Significance

Identification of the targets of low-affinity small-molecule inhibitors discovered in phenotypic screens is a major challenge for chemical genetics. We present a novel approach to target identification, inspired by genetic high-copy suppressor screens, in which suppressor activities are introduced as biochemical fractions into partially inhibited in vitro reactions. We call this strategy "biochemical suppression." Using pirl1, a novel compound identified by a screen for inhibitors of a phosphoinositide (PIP_2)-dependent signaling pathway regulating actin assembly, we identified two distinct suppressor activities and purified them by iterative rounds of biochemical fractionation and activity assays. One of these activities was the native complex of Cdc42 bound to RhoGDI, and the second was Arp2/3 complex. Both are known components of the PIP_2-dependent actin assembly pathway, thus validating the approach. In vitro experiments established that Cdc42/RhoGDI complex is a direct target of pirl1, whereas Arp2/3 complex is a downstream component of the pathway capable of relieving upstream inhibition of Cdc42/RhoGDI when added at high concentrations. Thus, biochemical suppression, like genetic high-copy suppressor screens, allows for the identification of not only the functionally perturbed protein in a biological pathway, but also of other components of the pathway. This approach can therefore be used as a protein discovery tool by which to identify multiple components of a signaling pathway mediating a biological process of interest. Importantly, the suppressor activities are introduced as native protein forms rather than individual gene products, allowing for the identification of suppressor activities composed of complexes of multiple proteins. Furthermore, the biochemical suppression strategy can, in principle, be used in any assay to which partially purified biochemical fractions can be added and, consequently, may be of broad utility. Finally, we establish that pirl1 reversibly perturbs actin-dependent membrane ruffling in live cells, and that it may therefore be a versatile reagent with which to study this complex process.

Experimental Procedures

Reagents

Pirl1 (8-Cyclopentyl-2,3,3a,4,5,6-hexahydro-1*H*-pyrazino[3,2,1-*jk*]carbazole), compounds **1**, **2**, **3**, and **6** were purchased from Chembridge (San Diego, CA). Compounds **5**, **7**, and **8** were purchased from Chemnavigator (San Diego, CA). The identity and purity (>95%) of pirl1 were confirmed by liquid chromatography/mass spectrometry (LC/MS) and NMR. All compounds were solubilized in DMSO and stored at −20ºC. Anti-Cdc42 antibodies were purchased from Transduction Labs, and anti-Arp2 antibodies were purchased from Santa Cruz Biotechnology. Recombinant Cdc42/RhoGDI complex was kindly provided by Drs. Greg Hoffman and Richard Cerione. For guanine nucleotide exchange experiments, Cdc42/RhoGDI complex was prepared as previously described [15]. A plasmid encoding the His6-tagged DH-PH domain of Dbs was kindly provided by Dr. John Sondek (UNC, Chapel Hill).

Actin Polymerization Assays in *Xenopus* Egg Extracts

PIP_2 liposomes (4:48:48 PI[4,5]P_2:phosphatidylcholine:phosphatidylinositol) were added to *Xenopus* egg extracts containing 2 μM pyrene-actin and DMSO vehicle (1% final concentration) or small molecule as previously described [14]. Pyrene fluorescence (excitation 347 nm, emission 386 nm) was measured at 22ºC in a fluorescence spectrophotometer (Varian Cary Eclipse). Maximum polymerization rates were determined as reported [13], by using a 1 min sliding window across the entire time course. For IC_{50} determination, maximum polymerization rates were plotted as a function of small-molecule concentration and fit to a sigmoidal dose-response curve with variable slope (by using Prism 4.0). IC_{50} is defined as the compound dose required to inhibit the maximum polymerization rate by 50%. For assays of column fractions, the high-speed supernatant of *Xenopus* egg extracts [14] (~8 mg/ml) containing 10 μM pirl1 and pyrene-actin was diluted with an equal volume of 0.2 mM ATP/CSF-XB (100 mM KCl, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM potassium HEPES [pH 7.7], 5 mM EGTA) ± each protein fraction (previously dialyzed into CSF-XB) and then stimulated with PIP_2 liposomes.

Purification of Suppressor of Pirl1 Activities

For preliminary characterization of the SOP activity, 4 ml high-speed supernatant of *Xenopus* egg extract [14] (~8 mg/ml) was dialyzed into 20 mM Tris (pH 7.6), 20 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT. Immediately prior to fractionation, the pH was adjusted to 6.1 by the addition of 0.1 volumes of 0.5 M PIPES (pH 6.1), and the high-speed supernatant was fractionated over SP Sepharose (GE Healthcare). Bound proteins were eluted over 8 column volumes in buffer S (20 mM PIPES [pH 6.1], 20 mM NaCl, 1 mM DTT) with a linear NaCl gradient from 20 mM to 1 M. Fractions were neutralized with 0.05 volumes of 1 M HEPES (pH 7.75) and were concentrated ~11-fold by centrifugal ultrafiltration prior to dialysis against CSF-XB/1 mM DTT. Fraction volumes were normalized by the addition of CSF-XB/1 mM DTT to the more concentrated fractions.

For large-scale purification of SOP1 and SOP2, low-speed *Xenopus* egg extract [25] from 70 frogs was diluted 1:4 with 10 mM KCl, 0.1 mM $CaCl_2$, 10 mM HEPES (pH 7.7), 5 mM EGTA before high-speed centrifugation (3 hr at 40,000 rpm in a Beckman Type 45 Ti rotor, followed by 4 hr at 28,000 rpm in a Beckman SW28 rotor). A total of 1.25 g protein obtained in the supernatant (~200 ml) was acidified by the addition of 0.1 volumes of 0.5 M PIPES (pH 6.1) and recentrifuged for 30 min at 40,000 rpm in a Beckman Type 45 Ti rotor. This supernatant was applied to a 150 ml SP Sepharose HP column and eluted over 2 liter in buffer S with a linear NaCl gradient from 20 mM to 1 M. Fractions (20 ml) were collected and neutralized with 0.05 volumes of 1 M HEPES (pH 7.85). Samples of pooled, adjacent fractions were concentrated ~6.5-fold and dialyzed against 0.1 mM ATP/CSF-XB/1 mM DTT, and fraction volumes were normalized as described above. Fractions containing SOP activity (21 mg total protein, eluting at ~200 mM NaCl) were pooled and dialyzed against 20 mM Tris (pH 7.7), 40 mM NaCl, 1 mM DTT and applied to a Mono Q HR 5/5 column (GE Healthcare) and eluted over 20 ml in 20 mM Tris (pH 7.7), 20 mM NaCl, 1 mM DTT with a linear NaCl gradient from 20 mM to 1 M. Samples of each fraction were assayed for SOP activity directly. Fractions containing SOP1 activity (1.17 mg total protein, eluting at 95 mM NaCl) and SOP2 activity (2.1 mg total protein, eluting at 150 mM NaCl) were pooled separately and fractionated independently over a calibrated Superdex 200 16/60 column (GE Healthcare) preequilibrated into CSF-XB/1 mM DTT.

Purified Arp2/3 Complex and Cdc42/RhoGDI Complex

Native bovine brain Arp2/3 complex was purified as described [13]. Recombinant Cdc42/RhoGDI complex and the GST fusion protein with the VCA domain of N-WASP (GST-VCA) were expressed and purified as described in [15] and [10], respectively.

Mass Spectrometry

Protein bands cofractionating with SOP2 activity were cut from SDS-PAGE gels and subjected to in-gel trypsin digestion, and peptides were identified by microcapillary LC/MS/MS analysis by the Taplin Biological Mass Spectrometry Facility (Harvard Medical School).

Cdc42 Activation Assays in *Xenopus* Egg Extract

A total of 200 μl *Xenopus* egg high-speed extract (8 mg/ml) was brought to 0.2 mM ATP, 10 μM latrunculin B (to prevent actin polymerization), and compound or DMSO vehicle (1% final concentration) was added at the indicated concentration. Extracts were stimulated by the addition of 20 μM GTPγS and/or 20 μM PIP_2 liposomes and incubated 10 min at room temperature as described [19]. GTP/GTPγS bound Cdc42 was recovered by the addition of 18 μg recombinant GST-p21 binding domain of Pak kinase (GST-PBD) [17], incubation for an additional 10 min, followed by addition of lysis buffer (100 mM Tris [pH 7.5], 2 mM $MgCl_2$, 0.4 M NaCl, 2% NP40, 10% glycerol) and glutathione-agarose beads. After washes in 25 mM Tris (pH 7.5), 1 mM DTT, 30 mM $MgCl_2$, 40 mM NaCl, 1% NP40, and the same buffer lacking NP40, beads were analyzed by SDS-PAGE and Western blotting with Cdc42-specific antibodies.

In Vitro Nucleotide Exchange Assays

Nucleotide exchange assays were performed as described [19]. Briefly, 10 μM [^{35}S]GTPγS (Perkin Elmer, 2,000 dpm/pmol) was added (at t = 0) to the mixture of pure components at the indicated concentrations in reaction buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT) at 25ºC. The reaction received compound or an equivalent volume of DMSO (1%). At each time point, 15 μl aliquots of each reaction were diluted into 2 ml 4ºC termination buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 10 mM $MgCl_2$) and filtered through nitrocellulose. The nitrocellulose was washed twice with termination buffer, dried, and scintillation counted.

PMA-Induced Membrane Ruffling Assays

BS-C-1 cells were stimulated for 15 min by the addition of complete media (10% fetal bovine serum in DMEM) containing 250 ng/ml phorbol myristate actetate (PMA) and either 50 μM pirl1, compounds **1**, **5**, **6**, **7**, or **8**, or DMSO vehicle. Unstimulated cells were treated with an equivalent volume of DMSO (0.7% final concentration) only. Cells were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature and then rinsed in PBS. For the washout experiment, cells were treated for 15 min with 50 μM pirl1 and then washed into media without pirl1 for 1 hr prior to stimulation with PMA and fixation as described above (Pirl1 washout, Figure 5). After 10 min of permeabilization with 0.1% Triton X-100 in PBS (PBS-T), cells were rinsed and blocked for 10 min in AbDil (PBS-T containing 2% bovine serum albumin and 0.1% sodium azide). The filamentous actin cytoskeleton was stained for 20 min with Alexa 488-phalloidin (Molecular Probes) at 1 μg/ml in AbDil. Epifluorescence images were captured with identical exposure times for each sample on a CoolSnap ES camera (Photometrics) by using a Nikon TE2000 microscope and a 60× oil immersion objective. The percentage of cells exhibiting actin-rich membrane ruffles in the experiment shown in Figure 5A was determined by a blinded observer by counting total numbers of cells by using phase contrast imaging prior to counting cells with prominent phalloidin-stained ruffles visualized by epifluorescence.

Supplemental Data

Supplemental Data demonstrating that purified Arp2/3 complex exhibits SOP activity and that Cdc42/RhoGDI complex requires PIP_2 liposomes to activate actin polymerization in *Xenopus* egg extracts are available at http://www.chembiol.com/cgi/content/full/13/4/443/DC1/.

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