An allosteric kinase inhibitor binds the p21-activated kinase autoregulatory domain covalently

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Abstract
Kinases are important therapeutic targets in oncology due to their frequent deregulation in cancer. Typical ATP-competitive kinase inhibitors, however, also inhibit off-target kinases that could lead to drug toxicity. Allosteric inhibitors represent an alternative approach to achieve greater kinase selectivity, although examples of such compounds are few. Here, we elucidate the mechanism of action of IPA-3, an allosteric inhibitor of Pak kinase activation. We show that IPA-3 binds covalently to the Pak1 regulatory domain and prevents binding to the upstream activator Cdc42. Preactivated Pak1, however, is neither inhibited nor bound significantly by IPA-3, demonstrating exquisite conformational specificity of the interaction. Using radiolabeled IPA-3, we show that inhibitor binding is specific and reversible in reducing environments. Finally, cell experiments using IPA-3 implicate Pak1 in phorbol-ester-stimulated membrane ruffling. This study reveals a novel allosteric mechanism for kinase inhibition through covalent targeting of a regulatory domain. [Mol Cancer Ther 2009;8(9):2559–65]

Introduction
Protein kinases have attracted much attention as therapeutic targets in oncology due to their frequent dysregulation in cancer and their tractability to small-molecule inhibition. A significant challenge in the development of kinase inhibitors is achieving kinase selectivity, due to the evolutionary conservation of the ATP-binding pocket among kinases. An attractive alternative approach would be allosteric inhibition by compounds binding at distant, less conserved sites.

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Materials and Methods
Materials
Cdc42-GTPγS, full-length Pak1, activated Pak2, GST-Rac1, GST-HR1 (from PRK1), myelin basic protein (MBP), and glutathione S-transferase (GST)-mini-N-WASP were prepared as described (11–14). GST-Pak1 kinase domain K299R (amino acids 248–545) and GST-RD (amino acids 67–150) were generated by PCR from templates provided by J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA) and were cloned into pGEX-6P1 and purified from Escherichia coli. Plasmids encoding HA-tagged human Pak1 (in pJ3) and myc-tagged Cdc42 (in pCMV6M) were generously provided by J. Chernoff.

Fluorescence Spectroscopy
Emission spectra (excitation, 280 nm) were recorded with a Cary Eclipse (Varian) in Kinase buffer [50 mmol/L HEPES (pH 7.5), 12.5 mmol/L NaCl, 0.625 mmol/L MgCl2, and 0.625 mmol/L MnCl2] at 22°C. A 0.5 μmol/L solution of each protein was titrated in parallel with IPA-3 or DMSO (solvent control), and after 5 min of incubation, tryptophan emission was monitored at 340 nm. Fluorescence changes observed in DMSO only titrations were subtracted to remove solvent effects on tryptophan fluorescence.
Kinase Assay

Pak1 (150 nmol/L final) was preincubated with MBP (8.3 μmol/L), indicated proteins, and IPA-3 or DMSO in Kinase buffer for 20 min at 4°C. Cdc42-GTPγS (3.2 μmol/L) was then added, and the reaction was pre-equilibrated 10 min at 30°C. Kinase reactions were started by the addition of ATP (to 30 μmol/L) containing [32P]ATP and were incubated 10 min and analyzed by SDS-PAGE and autoradiography.

Pak1-Cdc42 Binding Assays

GST (10 μg), GST-RD (20 μg), or GST-mini N-Wasp bound to Glutathione Sepharose 4B were incubated with IPA-3 or PIR3.5 for 10 min at room temperature and then 30 min on ice in 100 μL. Binding buffer [50 mmol/L Hepes (pH 7.5), 100 mmol/L NaCl, 10 mmol/L MgCl2, 1% Nonidet P40]. Cdc42-GTPγS (5 μg) was added for 30 min. Beads were washed with Binding buffer and resuspended in sample buffer for SDS-PAGE and Western blotting with anti-Cdc42 antibodies (Santa Cruz Biotechnology).

For cell studies, HEK293 cells were transfected with myc-Cdc42 or HA-Pak1 using Lipofectamine 2000 (Invitrogen), and the following day, serum was withdrawn from the culture medium. Forty-eight hours after transfection, Pak1 (150 nmol/L final) was preincubated with MBP (8.3 μmol/L), indicated proteins, and IPA-3 or DMSO in Kinase buffer for 10 min at 30°C. Pak1 or other proteins were incubated with [14C]-IPA-3 for 1 h (or as indicated) in Kinase buffer at 30°C. Covalently bound [14C]-IPA-3 was separated from free [14C]-IPA-3 by precipitation using 10 volumes of acetone and incubation for 1 h at 30°C. Precipitates were recovered by centrifugation, solubilized in 2% SDS, and analyzed by scintillation counting (Beckman LS6000SC). Efficiency of protein precipitation was >90% as assessed by silver staining of SDS-PAGE gels of precipitated protein from a parallel experiment using unlabeled IPA-3. A representative Coomassie-stained gel (top) and autoradiogram (middle) are shown as well as quantification of replicate experiments (bottom); columns, mean (n = 3); bars, SD.

Covalent binding of [14C]-IPA-3 was also assessed by their comigration in nonreducing SDS-PAGE. For Fig. 4B, 1.1 μg recombinant Pak1 were first added to serial dilutions of a high-speed supernatant of Xenopus egg cytoplasmic extract (7.2 mg/mL) prepared as described but without DTT (12), and then [14C]-IPA-3 was added to 20 μmol/L. After a 1 h incubation at 30°C, samples were analyzed as above.

For Fig. 4D, 15 μmol/L [14C]-IPA-3 was incubated with pure Pak1 for 1 h at 30°C in Kinase buffer and then partially proteolyzed for the indicated times on ice with 2.2 μg/mL chymotrypsin to generate chymotrypsin-resistant core fragments (15) and analyzed by 15% SDS-PAGE and autoradiography.

**Figure 1.** IPA-3 binds the Pak1 regulatory domain. A, a schematic diagram of the Pak1 dimer and the truncated constructs used here (CD, catalytic domain). B, the chemical structure of IPA-3. C, saturable binding of IPA-3 to the Pak1 RD. The indicated recombinant proteins were titrated with IPA-3 and intrinsic tryptophan fluorescence was monitored: excitation, 280 nm; emission, 340 nm. Data from three independent experiments were fitted to the equation ΔF = ΔFmax× ([IPA-3]) / ([IPA-3] + Kd); points, mean; bars, SD. D, excess Pak1 RD sequesters IPA-3 from full-length Pak1. Recombinant Pak1 was preincubated for 20 min on ice with MBP, in the absence or presence of 10 μmol/L IPA-3 and GST or GST-RD. Cdc42-GTPγS was then added, and the kinase reaction was started by adding radiolabeled ATP. A representative Coomassie-stained gel (top) and autoradiogram (middle) are shown as well as quantification of replicate experiments (bottom); columns, mean (n = 3); bars, SD.
Membrane Ruffling Assay

BS-C-1 cells were pretreated for 10 min with either DMSO, control compound PIR3.5, or IPA-3 (50 μmol/L). Cells were then stimulated for 15 min with 250 ng/mL PMA in culture medium and fixed in PBS containing 4% formaldehyde for 20 min at room temperature. For the washout experiment, cells were treated for 10 min with 50 μmol/L IPA-3 and then washed into media without IPA-3 but containing 500 μmol/L cycloheximide for 1 h before stimulation with PMA and fixation as above. After permeabilization, filamentous actin was stained with Alexa 488-phalloidin and 4',6-diamidino-2-phenylindole (Molecular Probes) and quantified as described (16). For Pak1 localization, BS-C-1 cells were transfected with HA-Pak1 using Lipofectamine 2000 (Invitrogen). Transfected Pak1 was localized in PMA-stimulated cells by indirect immunofluorescence using an anti-HA antibody (Covance).

Results

IPA-3 Binds the RD of Pak1

IPA-3 (Fig. 1B) inhibits the activation of Pak1 by small GTPases but does not inhibit the catalytic activity of pre-activated Pak1 (1). These observations suggest that IPA-3 might perturb conformational changes that normally accompany Pak1 activation. To determine whether IPA-3 interacts with the Pak1 RD, we titrated a solution of full-length Pak1, truncated Pak1 fragments or control proteins with IPA-3, and monitored changes in intrinsic tryptophan fluorescence. Three tryptophan residues are located within the kinase domain and one within the RD. Saturable binding of IPA-3 to full-length Pak1 was observed with an apparent dissociation constant of 1.92 ± 0.2 μmol/L, consistent with the reported IC50 of 2.5 μmol/L (Fig. 1C). IPA-3 bound the isolated RD with an even higher apparent affinity (0.1 ± 0.01 μmol/L), whereas the kinase domain showed a weaker interaction. As negative controls, GST alone and free tryptophan showed only weak perturbation of fluorescence. These observations indicate a direct binding of IPA-3 to the RD of Pak1. In addition, the higher apparent affinity for the isolated RD compared with full-length Pak1 suggests that IPA-3 may bind to a conformation of the RD distinct from that found in autoinhibited Pak1.

IPA-3 contains a disulfide bond, suggesting that it might act through covalent redox modification of Pak1. We previously showed that IPA-3 does not form mixed disulfides and by directly inhibiting Pak1 (15). Together, our results show direct binding of IPA-3 to the RD of Pak1.

IPA-3 Binding to the RD Inhibits Cdc42 Binding

Binding of small GTPases to the Pak1 RD initiates conformational changes leading to Pak1 activation. Because IPA-3 also binds to the RD, we tested whether IPA-3 inhibits Cdc42-RD binding. GST-RD immobilized on beads was used to precipitate soluble Cdc42 in the presence or absence of IPA-3. IPA-3 inhibited the interaction of Cdc42 with the Pak1 RD in a dose-dependent manner (Fig. 2A, lanes 3–5). As expected, the structurally related but inactive compound PIR3.5 had no effect (lanes 6–8). Moreover, IPA-3 has no effect on the binding of Cdc42 to the homologous RD from N-WASP (lanes 9–10), which shares 30% sequence identity to the RD of Pak1. Thus, IPA-3 selectively prevents Pak1 activation, in part, by preventing its interaction with small GTPase activators.

To confirm that IPA-3 could disrupt binding of Cdc42 to full-length Pak1 in the cellular context, we transfected HEK293 cells with Cdc42 and Pak and monitored their association by coimmunoprecipitation in the presence or absence of IPA-3 (Fig. 2B). Treatment with IPA-3 inhibited the binding of Pak1 to Cdc42 (compare lanes 2 and 3), supporting the in vitro results.

IPA-3 Binds Pak1 Covalently

IPA-3 inhibits Cdc42-GTPγS (hereafter simply called “Cdc42”) and [32P]-ATP were added to initiate the kinase assay. As expected, Cdc42 promoted Pak activation leading to higher levels of MBP phosphorylation, and this increased kinase activity was inhibited by IPA-3 (Fig. 1D; compare autoradiograms lanes 1–3). Inclusion of GST-RD protected Pak1 from IPA-3-mediated inhibition in a dose-dependent manner (lanes 4–5), whereas GST alone did not (lanes 6–9). This finding is particularly striking because GST-RD on its own inhibits Pak1 activation (compare lanes 6 and 7 with lane 2) by both sequestering Cdc42 and by directly inhibiting Pak1 (15).

IPA-3 inhibits Pak1-Cdc42 binding. A, IPA-3 inhibits Cdc42 binding to the Pak1 RD. Bead-bound GST-RD was incubated with soluble Cdc42-GTPγS in the absence (lanes 1–2) or presence (lanes 3–5) of IPA-3 or the control compound PIR3.5 (lanes 6–8). RD-bound Cdc42 was detected by anti-Cdc42 Western blotting. As a control, binding of Cdc42 to GST-mini N-WASP was also tested (lanes 9 and 10). B, IPA-3 inhibits binding of Cdc42 to full-length Pak1 in cells. HEK293 cells, transfected as indicated with myc-Cdc42 and/or HA-Pak1, were treated with solvent or 50 μmol/L IPA-3 before stimulation with 250 ng/mL phorbol myristate acetate for 15 min. Cell lysates were immunoprecipitated with anti-myc or anti-HA antibodies and the precipitates (top) and samples of the total lysates (bottom) were analyzed by Western blot with anti-myc and anti-HA. Arrowhead, HA-Pak1; *, heavy chain.
with surface-exposed cysteine residues of Pak1 (1). To test whether IPA-3 might covalently modify Pak1 at other sites, we synthesized IPA-3 using 8-[14C]-labeled 2-naphthol as a precursor to introduce the radiolabel into both naphthol ring systems (see Fig. 1B). Increasing amounts of recombinant Pak1 were incubated with [14C]-IPA-3 under conditions in which IPA-3 inhibits Pak1 activation by >90% and then acetone precipitated under denaturing conditions. Whereas [14C]-IPA-3 alone was freely soluble in acetone (data not shown), a small fraction of [14C]-IPA-3 precipitated in a Pak-dependent manner with a stoichiometry of ~2.5 mol IPA-3/mol Pak1 (Fig. 3A, ▲). Quantitatively similar binding was observed to the truncated constructs comprising the RD and kinase domains. No binding was detected to GST alone, however. Notably, the Pak1 RD lacks any cysteine residues, indicating that IPA-3 binding to this domain does not occur through the formation of a mixed disulfide. Time-dependent inhibition is a hallmark of covalent inhibitors, and kinetic experiments using this assay revealed slow binding of IPA-3 to Pak1 with a saturating stoichiometry of 2.6 ± 0.5 mol IPA-3/mol Pak1 (Fig. 3B).

To confirm that IPA-3 binding is covalent, Pak1 or GST were incubated with [14C]-IPA-3 and then analyzed by nonreducing SDS-PAGE and autoradiography. Comigration of the radiolabel with Pak1 confirmed covalent binding of IPA-3 to Pak1, whereas no binding of [14C]-IPA-3 to GST was detected (Fig. 3C, ▲). [14C]-IPA-3 also bound fragments of Pak1 corresponding to the RD and a catalytically dead form of the kinase domain, although with a higher apparent affinity to the RD (bottom).
To determine where IPA-3 bound in the context of the native homodimer, full-length Pak1 was incubated with $[^{14}C]$-IPA-3 followed by limited proteolysis with chymotrypsin, which produces protease-resistant fragments corresponding to the core of the regulatory and kinase domains (15). SDS-PAGE and autoradiography of the proteolytic digests showed radioactivity associated with both core domains (Fig. 3D). Phosphorimager analysis of the proteolytic digests showed $\sim 37\%$ of the Pak1-bound IPA-3 was associated with the stable catalytic domain (Fig. 3D, bottom), implying that the remaining radioactivity is present in low molecular weight peptide fragments that would include the RD. Thus, although IPA-3-RD binding prevents Cdc42-RD binding, we cannot rule out additional effects mediated by IPA-3 bound to the kinase domain. Although if important for Pak inhibition, this binding site must only transiently during activation because catalytically active Pak1 is not inhibited by IPA-3 (1).

We predicted that, if covalent, binding of IPA-3 to Pak1 should be temperature-dependent and irreversible under our in vitro conditions. To test this prediction, we preincubated $[^{14}C]$-IPA-3 with Pak1 and MBP at 4°, 15°, or 30°C and then added excess RD to sequester residual free IPA-3. The temperature of all reactions was then shifted to 30°C, Cdc42-GTPγS and $[^{32}P]$ATP were added, and Pak1 kinase activity was measured. Pak1 preincubated with IPA-3 at 4°C before RD addition showed robust kinase activity due to dose-dependent sequestration of IPA-3 by RD (Fig. 3E, compare lane 3 with lanes 4–5). Pak1 kinase activity was progressively inhibited after preincubation at 15°C or 30°C before RD addition (lanes 6–9). This result shows that inhibition by IPA-3 occurs in a temperature-dependent and irreversible manner during the preincubation step.

### IPA-3 Binding to Pak1 Is Selective

The low stoichiometry and saturability of IPA-3 binding to Pak1 and the lack of binding to GST suggested that the binding, although covalent, was highly specific. Indeed, IPA-3 exhibits significant kinase selectivity and also does not inhibit the catalytic activity of Pak1 that has been preactivated by Cdc42 (1). We therefore assessed the ability of IPA-3 to bind to Pak1 after preactivation by Cdc42. Whereas inactive Pak1 bound IPA-3 robustly, binding of IPA-3 to preactivated Pak1 was substantially reduced (Fig. 4A, compare lanes 1 and 2), consistent with the inability of IPA-3 to inhibit preactivated Pak1.

We also tested the ability of IPA-3 to bind covalently to a variety of other proteins. IPA-3 showed selectivity for inactive Pak1 or RD and did not bind significantly to other tested proteins including bovine serum albumin (Fig. 4A). To determine the selectivity of IPA-3 for Pak1 in a more complex protein mixture, recombinant Pak1 was added to serial dilutions of a cytosolic extract of *Xenopus laevis* eggs and $[^{14}C]$-IPA-3 was added, and the reaction was incubated for 1 hour. Mixtures were then analyzed by nonreducing SDS-PAGE and Phosphorimager analysis. In the most concentrated sample, Pak1 represented only 0.76% of total protein, yet IPA-3 binding to Pak1 was unaffected by the presence of excess *Xenopus* proteins and individual radiolabeled proteins other than Pak1 were not observed (Fig. 4B). Because of limitations in the sensitivity of detection of $[^{14}C]$-IPA-3, we cannot conclude that proteins other than Pak1 do not bind IPA-3. Nor would we expect to detect binding of IPA-3 to the low concentration of endogenous Pak1 protein. Nevertheless, the robust and specific labeling of added recombinant Pak1 in this complex mixture supports a selective binding of IPA-3 to Pak1. We thus conclude that the selective kinase inhibitory

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**Figure 4.** IPA-3 binding is highly selective for inactive Pak1. **A**, IPA-3 binds inactive Pak1 and the isolated RD but not preactivated Pak1 nor other control proteins. $[^{14}C]$-IPA-3 (10 pmol/L) was incubated with inactive Pak1, Pak1 preactivated by Cdc42, or the indicated recombinant proteins. Bound $[^{14}C]$-IPA-3 was determined by scintillation counting of acetone precipitates. Columns, mean (n = 3); bars, SD. **B**, selective binding of IPA-3 to Pak1 in the presence of a cytosolic extract. One microgram of Pak1 was added to 2-fold serial dilutions of *Xenopus laevis* egg cytoplasmic extract. $[^{14}C]$-IPA-3 (20 pmol/L) was added for 1 h, and reactions were analyzed by nonreducing SDS-PAGE and autoradiography (bottom). Parallel samples using unlabeled IPA-3 were analyzed by Coomassie staining (top). **C**, IPA-3 binding is reversed by DTT. Pak1 bound to $[^{14}C]$-IPA-3 (60 pmol) was incubated with the indicated concentration of DTT for 13 min. Remaining bound $[^{14}C]$-IPA-3 was quantified from acetone precipitates. Columns, mean (n = 3); bars, SD.
IPA-3 Binding and Inhibitory Activity Is Reversed by Reducing Environments In vitro and In vivo

The disulfide bond of IPA-3 is critical for inhibition of Pak1, and in vitro reduction by the reducing agent DTT abolishes Pak1 inhibition by IPA-3 (1). We therefore tested whether IPA-3 bound covalently to Pak1 would be released by DTT treatment. Pak1 was incubated with [14C]-IPA-3 followed by a range of DTT concentrations. Quantitation of Pak1-bound [14C]-IPA-3 from acetone precipitates revealed that DTT treatment released IPA-3 in a dose-dependent manner (Fig. 4C).

The cell cytoplasm is a reducing environment, which might result in reduction and inactivation of IPA-3 in cells. Nevertheless, IPA-3 treatment of cells inhibits Pak1 activation (1), most likely because of the large reservoir of freely exchanging IPA-3 present in the oxidizing environment of the cell culture medium. We therefore predicted that IPA-3 inhibition in cells might be reversed by the cellular redox environment on removal of IPA-3 from the culture medium. Pak1 activity is closely linked to dorsal membrane ruffling (17, 18) and stimulation of cells by the protein kinase C agonist PMA, activates Pak isoforms (19), and produces actin-rich ruffles containing Pak1 (Fig. 5A). PMA-induced ruffling is blocked by inhibitors of Rho GTPase signaling (16) consistent with a role for Pak1. IPA-3, but not the control compound PIR3.5, inhibited PMA-stimulated ruffling (Fig. 5B and C). Removal of IPA-3 from the culture media and addition of cyclohexamide to block new protein synthesis restored the ability of PMA-stimulated cells to ruffle (Fig. 5B and C). We therefore conclude that the effect of IPA-3 is reversible in live cells, most likely through reductive release of IPA-3.

Discussion

The present study has elucidated a novel mechanism for kinase inhibition by IPA-3, a non-ATP competitive inhibitor of Pak1 activation. The results have revealed three new and important mechanistic details of the mechanism of action of IPA-3. First, we show that IPA-3 binds Pak1 covalently, in a time- and temperature-dependent manner. Intriguingly, this interaction is highly selective, saturable, and of low stoichiometry, although the chemical basis for this selectivity...
and the precise nature of the adducts are, as yet, unknown. Second, we show that by binding Pak1, IPA-3 prevents binding of the Pak1 activator Cdc42, thus providing a mechanistic explanation for how IPA-3 inhibits Pak1 activation. Third, we show that IPA-3 binds directly to the Pak1 auto- 

D. Inhibition of kinases by selective covalent modification of active site residues is now well established (20–23). The findings with IPA-3 suggest that covalent modification of sites outside of the active site might offer additional opportunities for target inhibition. Indeed, covalent inhibitors of Polo-like kinase have been identified that target the functionally important Polo box domain rather than the kinase domain (24). Other examples of covalent allosteric inhibitors targeting nonkinase enzymes have also been described (25, 26). Kinase inhibitors acting by this mechanism may exhibit greater kinase selectivity and improved pharmacologic efficacy (27). Indeed, covalent posttranslational modifications occurring outside of active sites such as phosphorylation, ubiquitinylation, and SUMOylation are important regulators of enzyme activity in biological systems. This suggests that small-molecule inhibitors for other enzymes might be found that exploit the regulatory power of covalent modification distal to the active site.

Finally, our findings implicate Pak1 in the formation of actin-rich ruffles stimulated by PMA. Pak1 localized to these structures and ruffling was reversibly inhibited by IPA-3. Other proteins previously localized to dorsal membrane ruffles include cortactin and Arp2/3 complex, two known Pak1 substrates (28). Although the biological function of these structures and the signaling pathways that regulate them remain to be determined, they may play a role in of receptor internalization and/or extracellular matrix degradation (29). The results presented here suggest that Pak1 plays a central role in coordinating the underlying cytoskeletal organization.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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References

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