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An Isoform-Selective, Small-Molecule Inhibitor Targets the Autoregulatory Mechanism of p21-Activated Kinase

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SUMMARY

Autoregulatory domains found within kinases may provide more unique targets for chemical inhibitors than the conserved ATP-binding pocket targeted by most inhibitors. The kinase Pak1 contains an autoinhibitory domain that suppresses the catalytic activity of its kinase domain. Pak1 activators relieve this autoinhibition and initiate conformational rearrangements and autophosphorylation events leading to kinase activation. We developed a screen for allosteric *i*nhibitors targeting *P*ak1 *a*ctivation and identified the inhibitor IPA-3. Remarkably, preactivated Pak1 is resistant to IPA-3. IPA-3 also inhibits activation of related Pak isoforms regulated by autoinhibition, but not more distantly related Paks, nor >200 other kinases tested. Pak1 inhibition by IPA-3 in live cells supports a critical role for Pak in PDGF-stimulated Erk activation. These studies illustrate an alternative strategy for kinase inhibition and introduce a highly selective, cell-permeable chemical inhibitor of Pak.

INTRODUCTION

Protein kinases are important therapeutic targets and are considered highly druggable owing to their conserved ATP-binding pocket that can accommodate small molecules. However, because of the evolutionary conservation of this pocket across kinases, ATP-competitive inhibitors can inhibit large numbers of other kinases in addition to their intended targets (Bain et al., 2007; Karaman et al., 2008). Recently, it was demonstrated that ATP-competitive inhibitors such as imatinib (Gleevec) can achieve unusually high kinase selectivity by binding a less conserved region adjacent to the ATP-binding pocket (Nagar et al., 2002; Schindler et al., 2000), thus underscoring the idea that inhibitor interactions with less conserved regions of a kinase can provide opportunities for greater kinase selectivity. Indeed, many kinases contain nonconserved sequence elements outside the kinase domain that mediate important facets of their function such as localization, substrate recruitment, or the regulation of catalytic activity. Several kinases contain autoinhibitory domains that bind and inhibit the activity of the catalytic domain (Cheetham, 2004). We, and others, have proposed that proteins regulated by autoinhibition may be susceptible to inhibition by small molecules that perturb the conformational changes that accompany relief of autoinhibition (Cheetham, 2004; Liu and Gray, 2006; Peterson et al., 2004; Peterson and Golemis, 2004). The additional domains and conformational changes that mediate kinase autoregulation may, therefore, provide novel opportunities for more specific small-molecule inhibition than ATP-competitive compounds.

Members of the *p*21-*a*ctivated *k*inases (Paks) are one such family that is subject to autoregulation. Group I Paks (Paks 1–3) are regulated by autoinhibition that is relieved by binding to the 21 kDa GTP-binding proteins Rac and Cdc42. This distinct regulatory mechanism is not observed, however, in the more distantly related group II Paks (Paks 4–6). However, Pak5 may undergo autoinhibition mediated by an unrelated domain (Ching et al., 2003). Autoinhibition of Pak1 is mediated by the formation of an inactive homodimer in which the autoregulatory region of one monomer binds and inhibits the catalytic domain of its partner and vice versa (Lei et al., 2000; Parrini et al., 2002). One critical element of the autoregulatory region is the kinase-inhibitory segment, which binds in the active site cleft and sequesters the kinase activation loop in an inactive conformation (Lei et al., 2000). Pak1 activation involves the local unfolding of the autoinhibitory domain caused by binding of Rac/Cdc42 to a partially overlapping region, resulting in Pak1 monomer dissociation and displacement of the inhibitory segment. Subsequent autophosphorylation events at multiple sites along Pak1 stabilize the catalytically competent, monomeric conformation (Chong et al., 2001; Lei et al., 2000; Parrini et al., 2002). This multistep activation cascade may offer additional opportunities for small-molecule binding that could selectively inhibit group I Paks.

Increasing data implicate Pak1 in tumorigenesis and metastasis (reviewed in Kumar et al., 2006). Thus, inhibitors of Pak1 have been suggested as a novel oncologic therapy (Kumar et al., 2006; Nheu et al., 2002). Although no highly selective inhibitors of Pak1 have been reported, several compounds originally identified for their ability to target other kinases also inhibit Pak family members (Eswaran et al., 2007; Nheu et al., 2002; Porchia et al., 2007). Here we report the identification and characterization of a highly selective, non-ATP-competitive inhibitor that targets the

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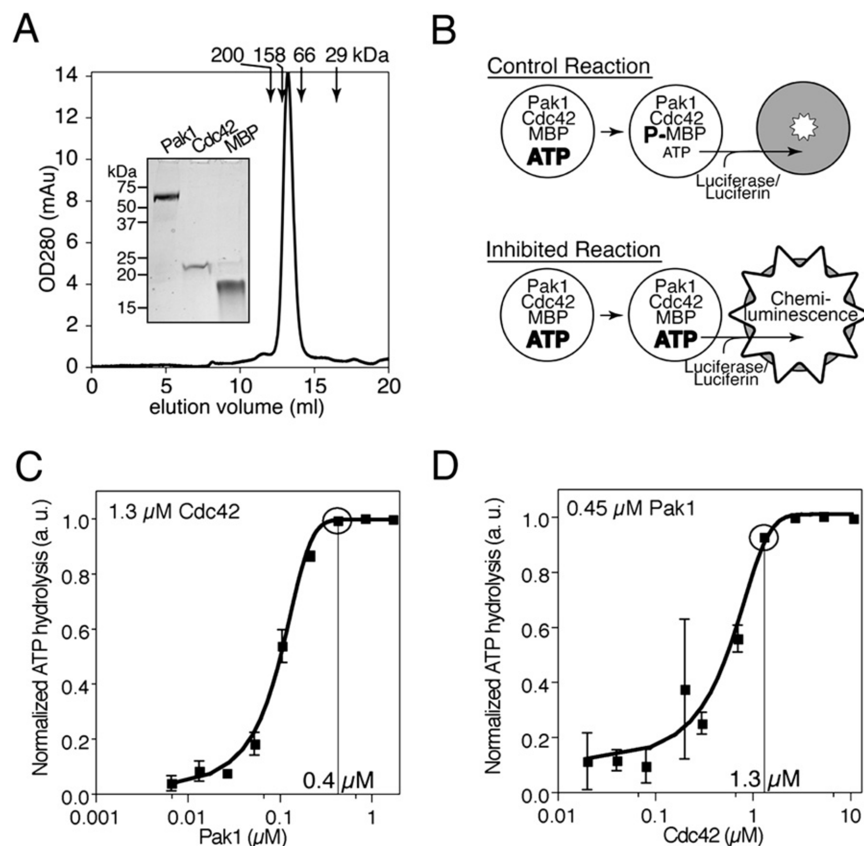

Figure 1. An ATP Depletion Assay Reports Cdc42-Dependent Pak1 Activation

(A) Recombinant Pak1 is a homodimer. The gel-filtration elution profile of Pak1 is shown. Elution volumes of standards are indicated. Inset: Coomassie-stained SDS-PAGE analysis of Pak1 and other proteins used in the screen.

(B) Pak1 assay scheme. Pak1 is incubated with its activator, GTPγS-charged Cdc42, and a substrate, myelin basic protein (MBP), in the presence of 10 μM ATP. Control kinase reactions (top) hydrolyze a substantial fraction of the starting ATP resulting in low final ATP concentrations, whereas inhibited reactions (bottom) do not. Residual ATP is enzymatically converted to chemiluminescence proportional to residual ATP.

(C) ATP hydrolysis is strictly Pak1-dependent. Cdc42 was incubated with MBP, ATP, and the indicated concentrations of Pak1. Residual ATP levels were measured as in (B). Results are expressed as ATP hydrolyzed in arbitrary units (normalized to the maximum ATP hydrolyzed) as a function of Pak1 concentration. Data points and error bars show mean and SEM of triplicate wells. Circled point indicates Pak1 concentration used in the screen.

(D) ATP hydrolysis by Pak1 is Cdc42-dependent. ATP depletion was monitored in reactions as in (C) except that the indicated concentrations of Cdc42 were used. Circled point indicates Cdc42 concentration used in the screen.

autoregulatory mechanism of group I Paks. This work illustrates how conformational rearrangements accompanying kinase activation can be exploited by compounds to achieve greater target specificity, and introduces a selective reagent for Pak inhibition.

RESULTS

A Chemical Screen Identifies IPA-3 as an Inhibitor of Pak1

To identify inhibitors of Pak1 activation, we developed a high-throughput assay measuring ATP hydrolysis as an indicator of Pak1 catalytic activity. Recombinant, full-length Pak1 exhibited an apparent molecular weight of ~130 kDa by gel-filtration chromatography (Figure 1A). SDS-PAGE analysis demonstrated the appropriate monomer molecular weight of ~60 kDa (Figure 1A, inset), as expected for the inactive Pak1 homodimer (Lei et al., 2000). Pak1 was incubated with individual compounds followed by addition of recombinant Cdc42-GTPγS (hereafter simply Cdc42) and myelin basic protein (MBP) as substrate (Figure 1A, inset) in the presence of 10 μM ATP (Figure 1B). Following incubation, nonhydrolyzed ATP was quantified using Kinase-Glo (Koresawa and Okabe, 2004). Titrations of both Pak1 (Figure 1C) and Cdc42 (Figure 1D) demonstrated that ATP hydrolysis was strictly dependent on both Pak1 and Cdc42. These results demonstrate that ATP hydrolysis measured in this assay is due to Pak1 kinase activity and confirm that the recombinant Pak1 utilized in the screen is autoinhibited yet activatable by Cdc42 as expected.

This assay was used to screen 33,000 structurally diverse small molecules in duplicate. Compounds inhibiting ATP hydrolysis by greater than three standard deviations below the mean of control reactions in both replicates were considered for further analysis. Approximately 1% of the compounds tested met this criterion. A secondary screen was then conducted on active compounds to identify those that were potentially non-ATP competitive. Individual compounds (10 μM) were incubated with Pak1, Cdc42, and MBP in the presence of [γ-^{32}P]ATP and were assayed for their ability to inhibit incorporation of [^{32}P]phosphate into MBP. To reduce the detection of undesired ATP-competitive inhibitors, reactions were conducted in the presence of 1 mM unlabeled ATP. Of the 342 compounds identified in the primary screen, 32 compounds continued to exhibit robust inhibition at 1 mM ATP. These compounds were ranked according to their relative potency and reproducibility of inhibition in subsequent assays, as well as their commercial availability. The cumulative results of these secondary assays led us to focus on one particular compound, 2,2′-dihydroxy-1,1′-dinaphthyldisulfide (Figure 2A; hereafter called IPA-3), that at 10 μM inhibited Pak1 activity by 95% ± 3%.

IPA-3 Is a Direct, Noncompetitive Inhibitor of Pak1

To determine the protein target of IPA-3, we performed Pak1 kinase assays in which MBP was omitted. Pak1 kinase activity was measured by monitoring Pak1 autophosphorylation using phospho-specific antibodies against threonine 423 within the activation loop (Thr423; Zenke et al., 1999). IPA-3 (10 μM) prevented Cdc42-stimulated Pak1 autophosphorylation on

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