Bidirectional Allosteric Communication between the ATP-Binding Site and the Regulatory PIF Pocket in PDK1 Protein Kinase

Highlights
- PS653 inhibits the binding of PIFtide by interaction with the ATP-binding site
- Adenosine binds at the ATP-binding site and enhances the binding of PIFtide
- Molecular dynamics simulations describe the conformational changes of PDK1
- Potent drugs to the ATP site of PDK1 have different effects on the PIF pocket

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In Brief
Schulze et al. show that compounds binding to the ATP-binding site of a kinase can produce profound effects on a distant site, enhancing or inhibiting the interaction with allosteric regulators. The principle can be exploited in drug discovery and development.

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Bidirectional Allosteric Communication between the ATP-Binding Site and the Regulatory PIF Pocket in PDK1 Protein Kinase

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SUMMARY

Allostery is a phenomenon observed in many proteins where binding of a macromolecular partner or a small-molecule ligand at one location leads to specific perturbations at a site not in direct contact with the region where the binding occurs. The list of proteins under allostERIC regulation includes AGC protein kinases. AGC kinases have a conserved allosteric site, the phosphoinositide-dependent protein kinase 1 (PDK1)-interacting fragment (PIF) pocket, which regulates protein ATP-binding, activity, and interaction with substrates. In this study, we identify small molecules that bind to the ATP-binding site and affect the PIF pocket of AGC kinase family members, PDK1 and Aurora kinase. We describe the mechanistic details and show that although PDK1 and Aurora kinase inhibitors bind to the conserved ATP-binding site, they differentially modulate physiological interactions at the PIF-pocket site. Our work outlines a strategy for developing bidirectional small-molecule allosteric modulators of protein kinases and other signaling proteins.

INTRODUCTION

Allostery is a fundamental and widespread mechanism of intramolecular signal transmission whereby local perturbations on a protein affect the structure and dynamics of specific distal regions (Changeux, 2012; Goodey and Benkovic, 2008; Nussinov and Tsai, 2013). The term “allostery,” which was once restricted to oligomeric proteins, is now also used to describe the conformational changes that intramolecularly link two given distant sites on a protein (e.g., an orthosteric site and an allosteric site). The transmission of signals across long distances relies on dynamic coupling between different structural motifs and can be described as a shift in the population or dynamics of conformers (Bray and Duke, 2004). The formulations for the allosteric conformational changes have been studied over the last 50 years (Fenton, 2008; Monod et al., 1965; Nussinov and Tsai, 2014; Weber, 1972). Allostery thus mediates the responses of regulatory proteins to different stimuli along the transduction of cellular signals. Although the allosteric process is intrinsically bidirectional, cell signaling most often uses allostery unidirectionally to transduce a signal downstream in a signaling pathway (Kuriyan and Eisenberg, 2007). Protein kinases as well as other regulatory proteins that participate in signal transduction have evolved stringent switches (allosteric sites) that control their activation, i.e., modulate the conformation of the ATP-binding site (orthosteric site) in response to the appropriate upstream signals. In recent years there is growing interest in the development of allosteric drugs (Conn et al., 2014; Fang et al., 2013; Gray and Fabbro, 2014). However, the development of allosteric drugs is hampered by the poor knowledge of the molecular details of the allosteric process and how this can be rationally modulated at will with small compounds. Here, we describe the “reverse” allosteric regulation by small compounds on the phosphoinositide-dependent protein kinase 1 (PDK1), i.e., how binding of molecules to the ATP-binding site (orthosteric site) affects a...
Figure 1. The Kinase Domain, the PIF Pocket Binding to PIFtide, and the Identification of Small Molecules that Displace PIFtide from the PIF Pocket
(A and B) Crystal structure of the catalytic domain of PDK1 in complex with ATP (yellow carbon atoms) binding at the active site and PS210 (orange carbon atoms) binding at the PIF-pocket allosteric site (PDB: 4AW1).
(C) Schematic representation of the AlphaScreen interaction assay employed for the screening of the library of small molecules.
regulatory allosteric site, reversing the direction in which the allosteric regulation between the two sites is observed in cell signaling.

The protein kinase domain is formed by two lobes with the ATP-binding site located in the cleft between the two (Figure 1A) (Zheng et al., 1993). It is currently proposed that dynamics is the underlying mechanism for allosteric regulation in protein kinases (Kornev and Taylor, 2015). In the prototype protein kinase A (PKA), the catalytic domain is constitutively active, and regulation of the enzymatic activity is provided by interaction with regulatory subunits (Taylor et al., 2012). In contrast, other members of the AGC group of protein kinases are not constitutively active and use a conserved hydrophobic pocket called the PDK1-interacting fragment (PIF) pocket as a key allosteric regulatory site. The PIF pocket was originally described in the PDK1 as a regulatory allosteric site that binds PIF, a polypeptide sequence derived from a conserved hydrophobic motif (HM) present in AGC kinases but has a phosphomimetic Asp residue instead of the Ser/Thr phosphorylation site. Phospho-HM polypeptides and PIFtide activate PDK1 and diverse AGC kinases (Biondi et al., 2000; Engel et al., 2006; Frodin et al., 2002; Yang et al., 2002) in vitro by interaction with the PIF pocket (Figures S1A and S1B). In PDK1, the PIF pocket plays an additional role in the specific recognition of a subgroup of its substrates. The HM of PDK1 substrates such as SGK, S6K, PKC, and RSK, but not protein kinase B (PKB)/Akt, require the docking of their HM to the PIF pocket of PDK1 to become efficiently phosphorylated (Bayscas, 2008; Biondi et al., 2001; Collins et al., 2003)(Figure S1C). We previously characterized the mechanism of activation of PDK1 upon binding of peptides or small molecules to the PIF pocket (Engel et al., 2006; Hindie et al., 2009; Sadowsky et al., 2011; Stockman et al., 2009; Wei et al., 2010) (Figures S1D and S1E). Crystal structures of PDK1 with the bound reversible activators PS48 (Hindie et al., 2009) and PS210 (Busschots et al., 2012) revealed structural aspects of the allosteric activation mechanism and the associated conformational changes. For instance, the crystal structure of PDK1 in complex with PS210 shows the complete closure of the ATP-binding site (Busschots et al., 2012).

The allosteric regulation of protein kinases mediated by the small lobe of the kinase domain is not restricted to AGC kinases. Aurora kinases are activated by the polypeptides of their interacting partners, which bind to a site equivalent to the PIF pocket (Bayliss et al., 2003). Furthermore, epidermal growth factor receptor kinases are activated by dimerization; an activating kinase interacts with a receiver kinase at a site equivalent to the PIF pocket (Zhang et al., 2006). Abi kinase is both allosterically inhibited and activated by intramolecular interaction with its SH2 domain (Wojcik et al., 2010), which, in the active state, docks at the top of the small lobe (Doiker et al., 2014; Nargar et al., 2003). However, except for the case of AGC kinases, these regulatory features mediated by the small lobe have not been investigated using small compounds.

Despite the great interest in drug development of protein kinases over the last 20 years, the allosteric effects of small compounds binding to the ATP-binding site have remained mostly unexplored. In the present work, we investigate the bidirectional allosteric regulation of PDK1 using small compounds that bind to the PIF pocket and ATP-binding site. The results show how different small compounds that bind at the ATP-binding site produce allosteric effects on the PIF-pocket regulatory site, enhancing or inhibiting the binding to PIFtide. This work highlights how the "old" concept of allostery provides new exciting opportunities for drug development of protein kinases.

RESULTS

Identification of Small Compounds that Bind to the ATP-Binding Site and Allosterically Affect the Binding of PIFtide to the PIF Pocket of PDK1

PDK1 interacts with the HM-polypeptide PIFtide with high affinity, and this interaction increases the specific activity of PDK1 in vitro as measured by its increased ability to phosphorylate T308tide, a polypeptide derived from the activation loop of PKB/Akt (Biondi et al., 2000)(Figure S1A). The interaction between His-PDK1 and biont–PIFtide can be measured by means of AlphaScreen technology (Figure 1C) (Busschots et al., 2012). Using this homogeneous assay, we screened a library of 14,400 small molecules (average molecular weight 320 Da) for their ability to affect the PDK1-PIFtide interaction. We identified small compounds that displaced the interaction, e.g., PSE10 and PS653 (Figures 1D and 1E). PSE10 (2-oxopropyl N-(4-chloro-phenyl)-[(2-chloro-6-fluorobenzoyl)amino]methanimidothioate) has two ring systems joined by a linker and a side chain, resembling compound 1 (Engel et al., 2006), PS48 (Hindie et al., 2009) and PS210 (Busschots et al., 2012), which we previously characterized as activators that bind to the PIF pocket of PDK1. In contrast, PS653 (1,6-dihydrodibenzo[c,d,g]indazol-6-one) presents a small, planar, anthrone-derived structure. We tested the ability of the newly identified compounds to affect the intrinsic kinase activity of PDK1 (Figure 1F). PSE10 activated PDK1 but did not affect the activity of PDK1 with mutations at the PIF pocket (Figures 1F–1J). The crystal structure of PSE10 in complex with PDK150–359 confirmed that PSE10 bound to the PIF pocket (Figure S2 and Table S1) in a manner similar to the binding modes of PS48 and PS210. In contrast, PS653 inhibited the activity of different PDK1 constructs (Figures 1G, 1H, and S3). We previously showed that PDK1 Leu155Glu, which contains a mutation at the center of the PIF pocket, is not activated by PIFtide (Biondi et al., 2000) or by small compounds that bind to the PIF pocket (Engel et al., 2006). Similarly, other
mutations in the PIF pocket also partially abolished the activating effect of PIFtide and small compounds (Engel et al., 2006). In contrast to PSE10, PS653 still inhibited PDK1 (Leu155Glu) and PDK1 (Val127Leu), which have mutations at different key residues in the PIF pocket (Figures 1I and 1J). These results were not compatible with PS653 binding to the PIF pocket; instead, they suggested that the binding site of PS653 was allosterically coupled to the PIF pocket.

The high-resolution crystal structure of PDK1<sub>50-359</sub> in complex with PS653 (Figures 2A and S2C; Table S1) revealed that PS653 binds at the ATP-binding site, thus confirming that the displacement of PIFtide by PS653 is mediated by a reverse allosteric effect and not due to a direct competition for the PIF-binding pocket. Like the adenosine moiety of ATP and most ATP-competitive inhibitors, PS653 forms two hydrogen bonds with the main chain atoms Ser160 CO and Ala162 N of the so-called linker region and is sandwiched between the hydrophobic side chains of Leu88, Val96, and Leu212. However, a major difference is that PS653 extends outside the usual ATP-binding cavity. Its outer ring is in close contact (3.25 and 3.0 Å, respectively) with the carbonyl groups of both Leu99 of the small lobe and Ala162 of the large lobe.

We then investigated whether adenosine or adenosine, which bind to the same site as PS653 (Figures 2B and 2C; Table S1), could produce similar allosteric effects. Surprisingly, adenosine enhanced the binding of PIFtide to PDK1, providing evidence of cooperativity, whereas adenosine did not produce any allosteric effect on the PIF pocket (Figure 2D). Interestingly, the enhancing effect was observed selectively with adenosine and not with adenosine, AMP, ADP, ATP, the non-hydrolyzable ATP analog thio-ATP, or cAMP (Figure S3B). The high-resolution crystal structure of PDK1 in complex with adenosine showed that the ribose moiety forms hydrogen bonds with Glu166 of the large lobe and an indirect hydrogen bond via a water molecule with Ser94 of the small lobe (Figure 2C). Thus, the presence of the sugar moiety between the lobes played a role in the allosteric enhancement of the binding of PIFtide to the PIF pocket. The differential effects caused by distinct compounds binding to the ATP-binding site showed that the resulting allosteric effects were highly selective.

The co-crystal structures revealed the precise binding mode of the allosteric and non-allosteric compounds under investigation. The crystal structures, on the other hand, did not reveal significant structural differences that could explain the observed allosteric effects.

**Molecular Dynamics Simulations Describe Changes to the Conformational Dynamics of PDK1 upon Ligand Binding**

To obtain more detailed information on the allosteric mechanisms, we analyzed the changes in the dynamics of PDK1 upon the binding of the different effectors using atomistic molecular dynamic (MD) simulations. We first performed long MD simulations to observe the effect of the different ligands on the sub-msec dynamics of PDK1. This is an unbiased approach to evaluate the whole dynamics of the protein. Interestingly, we identified an equilibrium between two populations of interconverting conformations, which differed in the stability of the small αB helix and could be appropriately quantified by the distance between the Cα atoms of Lys115 and Lys123 (end-to-end distance), shown as yellow spheres in Figures S4A and S4B.

To fully sample the conformational changes that in protein kinases can take place on timescales (μs to ms) not easily accessible to conventional MD (Saladino and Gervasio, 2012), and to reconstruct the conformational free energy landscape, we performed long multiple-replica parallel tempering (PT; Sugita and Okamoto, 1999) simulations in the well-tempered ensemble (WTE; Bonomi and Parrinello, 2010). Using this approach we clearly observed multiple transitions of PDK1 between the two conformations differing in the orientation and folding of helix linkers (Figures S5 and Table S1).
αB, with PS210 suppressing the population of the molecules with a more disordered helix. In Figure 3, we report the free energy projected along the variable describing the length of helix αB he-
lix (end-to-end distance) and the distance between the Gly-rich
loop (residues 90–94) and Asp205, which describes the kinase
hinge motion. It is clear that the two conformations observed in
the previous MD of the kinase with ATP that are well resolved
along the αB end-to-end distance variable are not observed in
the presence of PS210, the free energy surface (FES) of which
has a single minimum with a well-ordered
αB helix (Figure 3B).

The second minimum of the FES of PDK1 with ATP is also
compatible with a lower degree of
αB integrity and with our
observation that partial unfolding of the C-terminal end of the he-
lix takes place. Although the FES along the Gly-rich loop-Asp205
distance (hinge motion) shows a clear main minimum for ATP
and ATP + PS210, the morphology and position of the basins
suggests that the binding of PS210 restricts the kinase lobe dy-
namics, enforcing a more closed catalytic domain (Figure 3B), in
agreement with the crystal structure of the complex (Busschots
et al., 2012). Indeed, structures with a Gly-rich loop-Asp205 dis-
tance larger than 20 Å, appear to be relatively well populated
in the presence of ATP, but are rarely populated upon addition of
PS210. A highly conserved salt bridge between a Lys that posi-
tions the phosphate of ATP at the active site and a Glu from helix
αC (Lys111 and Glu130 in PDK1) is widely considered a hallmark
of active structures of protein kinases. The MD simulations show
that in the presence of ATP, the Lys111-Glu130 salt bridge is
present in closed structures. In agreement with being an acti-
vator, PS210 stabilizes conformations with a closed hinge and
well-formed
αB helix. The same conclusion can be drawn by
observing the projection of the FES along the first two eigenv-
ectors of the principal component analysis (Figure S4D). Different
orientations of Tyr126 were observed in our new simulations
for PDK1 with both ATP and PS210 (Figures S4C and S5A). Inter-
estingly, the same alternative conformation of Tyr126, with the
OH in close proximity to the terminal phosphates of ATP, was
observed independently in a new crystal form of PDK1 obtained
in complex with ATP and a phosphorylated HM polypeptide.
binding to the PIF pocket (Figure 3B and Table 1). In addition, this new crystal structure confirms the PIF pocket as the binding site of substrate HM polypeptides (Figure S5B). Overall, the PTWTE simulations confirmed the differences in the behavior of ATP- and ATP-PS48/PS210-bound PDK1 previously observed by MD and allowed better characterization of the dynamic and structural shifts arising from the binding of the allosteric effector. The MD simulations were in excellent agreement with the experimental data, and predicted Tyr126 movements that were independently confirmed in successive crystallography work. It should be noted that the simulations in the presence of ATP revealed that, in the open conformation, the PIF pocket is always well formed and stable, while the closed structure presents also a destabilized αB helix.

**Molecular Mechanism of the Reverse Allosteric Effects of PS653 and Adenosine on the PIF Pocket**

We next analyzed the molecular mechanism of the reverse allosteric effects of PS653 and adenosine on the PIF pocket. The free energy projections along the same variables used before are reported in Figure 3B. PS653 produced a marked enhancement of the PDK1 hinge/twist motions (Figures 3B and S4D). This, in turn, dramatically increases the population of more open structures (distances larger than 17 Å in Figure 3B). In contrast, adenosine enriched the most closed conformation (Figure 3B), suppressing the hinge motion, similar to what was observed in the PS210 FES. While both PS210 and PS653 rigidify the PIF pocket, the most populated conformations are quite different, and the two lobes appear more twisted in the structure with PS653 (Figures S6A and S6B). The additional hydrogen bonds formed by the ribose moiety of adenosine (Figure 2C) could trigger the hinge motion and tip the balance of the conformational equilibrium toward being more closed. Moreover, the changes observed in the conformational free energy landscape suggest that the stabilization of more closed structures by adenosine enhances the allosteric coupling between the two lobes and affects the binding of PIFtide to the PIF pocket by increasing the flexibility of the αB region. In contrast, PS653 has unfavorable close contacts in the crystal structure, and in the MD simulation induces the displacement of Leu88, hindering the full closure of the two lobes and breaking the catalytic spine (Kornev et al., 2006). We next compared the interaction of PDK1<sub>50-360</sub> with PS653 and adenosine using isothermal titration calorimetry. In both cases the results confirmed the existence of a single binding site for the compounds in solution, with dissociation constants in the low micromolar range (Figure S7 and Table 1). Notably, the interaction of PDK1<sub>50-360</sub> with PS653 was less enthalpic and more entropy driven (−1.2 kcal/mol; ΔH/ΔG = 15%) than the interaction with adenosine (−3.2 kcal/mol; ΔH/ΔG = 50%), in agreement with the above data showing that PS653 establishes less hydrogen bonds with residues in the enzyme active site, and that it promotes the hinge movement stabilizing the open conformation of the catalytic domain. Thus, the presence of PS653 tips the balance of the conformational equilibrium toward more open structures and in turn to a specific conformation of the PIF pocket that is less apt at binding PIFtide. This finding also suggests that the conformational flexibility of the αB region might be required for proper recognition of PIFtide and HM polypeptides of other physiological substrates that dock in the PIF pocket. Overall, the results indicated that the reverse allosteric effects that enhance the binding of PIFtide are transduced through the two lobes when the hinge motion brings them together in the closed conformation, while the decrease in binding to PIFtide is due to an induced opening of the hinge that interrupts the allosteric network.

The computational results point toward a central role played by the hinge and twist motions and an allosteric communication network that extends from one lobe to the other. Numerous residues increase interactions in the closed conformation, including residues from the αB and αC helices, the DFG motif, and the Gly-rich loop, as well as residues from the large lobe (Figure S6C). In addition, the MD simulations identified a series of salt bridges that were formed or broken with the hinge motion, e.g., Glu130-Lys111, Asp138-Lys199, Asp138-Lys144, and Lys163-Glu215. Of these, the salt bridge formed by Lys144 and Asp138 was only observed in the open form but not in the closed form, where it is replaced by the Lys199-Asp138, Glu130-Lys111, Asp138-Lys199, Asp138-Lys144, and Lys163-Glu215. Overall, the Lys144 mutants were normally activated by an excess of PIFtide (2 μM), reaching 93% and 89%, respectively, of the wild-type activity in the presence of PIFtide, indicating that they were active and overall well folded, suggesting that the mutation stabilized open-inactive forms of the kinase. Secondly, the Lys144 mutations importantly decreased the binding of PIFtide (Figure 4B). Together, we validated that Lys144 indeed played an important role in the hinge motion, stabilizing open conformations that have decreased ability to bind PIFtide. This is an important result and agreement with MD simulations of the Lys144Glu mutant. Starting from a structure where the original Asp138-Lys144 salt bridge was formed, the Lys144Glu mutation quickly leads to the formation of the alternative Lys199-Asp138, observed only in the open structures of the wild-type. Thus, the mutation actively shifts the population toward more open structures that, as previously demonstrated, are generally less prone to bind PIFtide than the mutant itself.

Table 1. Thermodynamic Parameters of the PDK1 Interaction with Low-Molecular-Weight Compounds Obtained by Isothermal Titration Calorimetry

<table>
<thead>
<tr>
<th>Compound</th>
<th>Temperature (K)</th>
<th>N</th>
<th>K&lt;sub&gt;d&lt;/sub&gt; (M&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>K&lt;sub&gt;d&lt;/sub&gt; (μM)</th>
<th>ΔH (kcal mol&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>ΔS (cal mol&lt;sup&gt;−1&lt;/sup&gt; deg&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>TΔS (kcal mol&lt;sup&gt;−1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>298</td>
<td>1.2 ± 0.2</td>
<td>(5 × 10&lt;sup&gt;−2&lt;/sup&gt;) ± (1 × 10&lt;sup&gt;−3&lt;/sup&gt;)</td>
<td>20</td>
<td>−3.2 ± 0.7</td>
<td>10.7</td>
<td>3.2</td>
</tr>
<tr>
<td>PS653</td>
<td>310</td>
<td>0.87 ± 0.02</td>
<td>(8 × 10&lt;sup&gt;−3&lt;/sup&gt;) ± (2 × 10&lt;sup&gt;−3&lt;/sup&gt;)</td>
<td>1.25</td>
<td>−1.20 ± 0.06</td>
<td>23.1</td>
<td>7.2</td>
</tr>
</tbody>
</table>

N is the stoichiometry of binding. See also Figure S7.
Glu144 does not appear to form any interaction and remains exposed to the solvent for the remainder of the simulation, suggesting that the effect of the mutation is not due to new interactions, but to the severing of important interactions in the existing allosteric network. In spite of the much lower affinity for PIFtide, we could perform a suitable interaction assay upon increasing the concentration of biotin-PIFtide in the assays (Figures 4D and 4E). Notably, the PDK1 Lys144 mutants increased the binding of PIFtide in the presence of adenosine (Figure 4D). In contrast to the lack of reverse allosteric effects by adenine on wild-type PDK1, adenine induced very high reverse allosteric induction of the binding of PIFtide in the PDK1 Lys144Glu mutant (Figure 4E). As the increased population of open structures is generally a consequence of the higher interlobe dynamics (as seen for PS653), the effect seen in the Lys144Glu PDK1 mutant could be due to adenine shifting back the equilibrium toward more closed structures to maximize the interactions within the binding site, de facto restoring the open-closed equilibrium of wild-type PDK1. We conclude that Lys144 plays a key role in the regulated hinge movement, which is central to the mechanism of allosteric coupling between the ATP-binding site and the PIF pocket.

**Drugs in Development Binding at the ATP-Binding Site and Allosterically Affecting the PIF Pocket**

We further investigated whether our findings can explain observed selective in-cell inhibition of downstream signaling by some PDK1 inhibitors. UCN01 is the 7’OH derivative of staurosporine, which binds to the ATP-binding site in PDK1 (Komander et al., 2003; Takahashi et al., 1989) and inhibits the phosphorylation of substrates. The recently developed GSK2334470 is a very potent ATP-competitive inhibitor of PDK1 (Axten et al., 2010) that shows high selectivity for PDK1. Intriguingly, GSK2334470 very efficiently inhibits S6K phosphorylation in cells but is a relatively poor inhibitor of the phosphorylation of another PDK1 substrate, PKB/Akt (Najafov et al., 2011). The reason for this differential inhibition of PDK1 substrates has remained elusive (Rettenmaier et al., 2014). Early studies described that the PIF pocket of PDK1 is required for the phosphorylation of S6K but not PKB/Akt (Biondi et al., 2001), and this was confirmed in knockin cells and tissues expressing PDK1 (Leu155Glu) (Arencibia et al., 2013; Bayascas, 2008; Collins et al., 2003) as well as pharmacologically using compounds binding to the PIF pocket of PDK1, which block the phosphorylation of S6K but not PKB/Akt (Busschots et al., 2012; Rettenmaier et al., 2014). Given that S6K but not PKB/Akt requires a docking interaction of its HM to the PIF pocket of PDK1 for its efficient phosphorylation, we hypothesized that the differential effect of GSK2334470 on S6K and PKB/Akt could be related to the identified reverse allosteric regulation effect. We performed a set of experiments with both GSK2334470 and UCN01 and observed that only the GSK compound displaced the binding of PIFtide from PDK1 (low nanomolar median inhibitory concentration), acting similar to PS653,
while UCN01 did not affect the binding of PIFtide even at high μM concentrations (Figures 5A and 5B). The results suggest a model in which the reverse allosteric effect induced by GSK2334470 enhances the inhibition of S6K phosphorylation, which requires binding to the PIF pocket. In this manner, GSK2334470 could inhibit S6K through a dual mechanism, inhibiting the intrinsic activity of PDK1 and additionally disturbing the docking interaction by reverse allostery, whereas PKB/Akt phosphorylation would be affected by the inhibition of PDK1 intrinsic activity but not by the reverse allosteric effect.

The above findings enable the development of substrate-selective inhibitors of kinases by targeting the ATP-binding site. In order to better illustrate possible uses of the described mechanism we tested the existence of such effects on the mitotic protein kinase Aurora A. The site equivalent to the PIF pocket in Aurora A binds to a protein partner, TPX2, a microtubule-associated protein which localizes the kinase in the centrosomes during mitosis. Aurora kinase also forms complexes with the oncoproteins N- and C-Myc, thereby stabilizing Myc and supporting tumor growth. There is interest in the identification of drugs that bind to Aurora kinase, disturb the complex, and destabilize the Myc oncogene (Brockmann et al., 2013; Gustafson et al., 2014). We have thus set up Aurora A-TPX2tide interaction assays, similar to those developed to test the interaction between PDK1 and PIFtide. Two different ATP-competitive drugs that have entered clinical trials were then tested on the reverse allosteric effect in Aurora kinase. Interestingly, while VX680 virtually did not affect the interaction (at μM concentrations), MLN8237 potently displaced the interaction (at low nM concentrations) (Figures 5C and 5D). The finding on Aurora kinase indicates that different compounds binding to the ATP-binding site can produce very different reverse allosteric effects on TPX2, an approach that can be exploited for the destabilization of N- and C-Myc as well as for the inactivation of Aurora kinase by delocalization.

**DISCUSSION**

The last decade has seen increasing interest in the development of allosteric drugs. However, the first examples of the rational modulation of allosteric transitions of proteins by small compounds have only recently started to emerge (Herbert et al., 2013). We describe small compounds that take advantage of the bidirectional nature of allosteric networks, enhancing or inhibiting the binding to a downstream signaling partner and further define the molecular mechanism for the reverse allostery between the active site and the PIF pocket in PDK1 (Figure 6). This knowledge can be exploited for protein kinase drug discovery and drug development.

We not only describe compounds that make full use of the bidirectional nature of allosteric communication by binding at the ATP-binding site and affecting the PIF pocket regulatory site, but we are also depicting the global molecular mechanism underlying the allosteric cross-regulation of the two sites. The hinge motion plays a pivotal role in allowing the allosteric communication. Orthosteric compounds (e.g., adenosine) that re-modulate the hinge motion and bring the lobes together promote the formation of an allosteric network connecting the ATP-binding site and the PIF pocket. Various residues pertaining to the catalytic and regulatory spines (C- and R-spines) are part of the network (Kornev et al., 2006), and two salt bridges alternatively formed by Lys144 act as a switching mechanism turning the communication on or off. Adenosine, by allosterically increasing the flexibility of aB and aC helices (Figure 4B), increases the ability of the PIF-pocket region to adapt to the binding PIFtide peptide. In stark contrast to adenosine, PS653 weakens the C-spine and favors an open-hinge conformation, breaking the allosteric communication network and locking the aB and aC helices lining the PIF pocket in a conformation less favorable to the binding of the PIFtide peptide. In this manner, PS653 disfavors the binding of the prototypical HM polypeptide that physiologically bind to the PIF pocket.

Our initial unbiased MD simulations clearly showed the existence of different conformations of PDK1, which fitted very well
Figure 6. Schematic Representation of the Bidirectional Allosteric Modulation of AGC Kinases by Small Compounds

(A) Representation of the hinge motion in equilibrium between open and closed structures of the protein kinase catalytic domain, showing the orthosteric ATP-binding site (active site), the HM polypeptide PIFtide, and the PIF pocket (allosteric site). The end-to-end distance variations of the αB helix are represented by the length of helices. The key residue Lys144 is represented as a red stick.

(B) Representation of the reverse allosteric mechanism by which PS653 and adenosine (Ado) affect the conformation of the PIF pocket and the binding of PIFtide. PS653 binds to the ATP-binding site, stabilizes open conformations, and rigidifies the PIF pocket, resulting in the inhibition of the binding of PIFtide. Adenosine binds to the ATP-binding site, favors the dynamic closure of the structure and disorders the PIF pocket in the closed conformation, resulting in the cooperative binding of PIFtide. Lys144, Asp138, and Lys199 (shown as stick) are represented in alternative salt bridge interactions formed in the closed and open conformations of PDK1.

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takes place for Akt/PKB, even if it is not the determinant of the phosphorylation of this kinase. During the writing of this article, the crystal structure of PDK1 in complex with a short version of PIFtide was published (Rettenmaier et al., 2014). From our results, it appears that PDK1 might require a limited increased flexibility of the PIF pocket to sample the appropriate conformation for PIFtide binding. According to this scenario, the disorder of the αC helix previously observed in solution (Hindie et al., 2009) and the disorder of αB helix predicted here by the MD simulations might provide a mechanism to facilitate the binding of different substrate HM polypeptides. This entropy-driven binding mechanism, also observed in ternary complexes of PKA (Masterson et al., 2011), may be an essential component of the ability of PDK1 to act as a “conformational sensor” (Biondi, 2004).

The initial crystal structures of PKA already depicted the versatility of the kinase domain revealing open, intermediate, and closed conformations of the ATP-binding site (Johnson et al., 2001). The hinge interlobe motion has subsequently been described by MD simulations of PKA (Masterson et al., 2011) and other kinases (Dolker et al., 2014). In PKA, it was revealed that the rate of hinge motion of the PKA catalytic domain (20 s⁻¹) correlated with the turnover of the enzyme (Kim et al., 2015; Srivastava et al., 2014). The turnover of PDK1 phosphorylation of different substrates, however, is approximately 1,000 times slower (0.6–1 min⁻¹) (Biondi et al., 2001), therefore indicating that most opening and closing motions of the PDK1 kinase domain are non-productive, not leading to the phosphorylation of substrates.

It is worth noting that a very important cellular molecule, such as adenosine, produces reverse allosteric effects on PDK1 in vitro. Adenosine is not only a metabolite from the synthesis of ATP, the substrate of PDK1, but is also a signaling molecule on its own (Borea et al., 2016). Our results suggest that an increase in adenosine could lead to the engagement of PDK1 in complexes with S6K, subtly regulating the identity of substrates of PDK1 to become phosphorylated. It is tempting to speculate that other protein kinases could also respond to the levels of ATP intermediates or other small physiological nucleotide molecules. This mechanism could indeed regulate cellular signaling by affecting the interactions of proteins with regulatory domains or regulatory subunits, formation of protein complexes, modulation of localization, or phosphorylation by upstream kinases, etc.

Previous data indicate that the full bidirectional allosteric regulation between the active site and the PIF pocket is functional in the regulation of AGC kinases, perhaps best exemplified in PRK2, where a pseudosubstrate inhibitory polypeptide, PLKtide binding to the active site, and PIFtide binding to the PIF pocket, are allosterically mutually excluded and contribute to the complex mechanism of regulation of PRKs (Bauer et al., 2012).

Although the mechanisms of allostery have been investigated for the last 50 years, it is still not possible to rationally design small molecules to modulate allosteric cellular switches at will. This work provides a deep understanding of the bidirectional allosteric coupling mechanism and suggests ways to exploit it. The use of MD simulations as described above enables the in silico testing of molecules before synthesis, and provides a technology to guide the design of variant compounds that produce a desired reverse allosteric effect. We expect that the results from this work will inspire the development of drugs with reverse allosteric effects on other protein kinases and, more generally, on other signaling proteins.

SIGNIFICANCE

Allostery is a fundamental and widespread regulation mechanism by which proteins transfer information between remote sites and functional sites in response to different stimuli. It is of interest to modulate allosteric processes for treatment of diseases and for future design of regulated molecular machines. Our proposed PDK1 model provides information on the conformational transitions of the catalytic domain and depicts for the first time, in atomistic detail, the bidirectional allosteric communication between the ATP-binding site and the regulatory PIF pocket, where compounds binding to the ATP-binding site can allosterically enhance or inhibit the interaction with a cellular partner. More generally, drugs directed to the ATP-binding site that have equal ability to inhibit a given kinase may have very different effects on the formation of protein complexes and cell signaling, different on-target side effects, and, overall, different degrees of success in the treatment of patients.

The PDK1/AGC kinase allosteric model system provides a rich example of the potential of small molecules to bidirectionally modulate the conformation of a protein kinase. By providing a validated computer simulation it will now be possible to use this tool to design allosteric drugs with a given allosteric effect. In turn, we expect that the perspective presented here will benefit drug development of members of the protein kinase family by providing a computer platform from which to rationally design compounds directed to the PIF pocket or ATP-binding site and produce desired secondary allosteric effects. Similarly, the above approach could also be applied more generally for drug discovery projects of other allosteric signaling proteins the functions of which could be modulated by molecules designed to produce desired conformational changes.

EXPERIMENTAL PROCEDURES

Materials
The polypeptide substrate of protein kinase PDK1 was T308tide (KTFC GTPYEALPEVRRT; >75% purity). Other polypeptides used were PIFtide (REPRILSEEEEOMFRFDFDY14WDWS), biotin-PIFtide (biotin-REPRILSEEEEOM FRFDFDY14WDWS) and biotin-TPX2tide (biotin-MSQVKSSYDAPSDFINFSSL DDEGDTQNDWSFEEKANLEN-NH2). Adenine and adenosine (≥99%) were from Sigma-Aldrich.

Crystal Structures
To obtain the crystal structure of PDK1 in complex with PS683, PSE10, adenine, and adenosine, PDK1 was expressed, purified, concentrated, crystallized, and soaked with compounds as previously described (Hindie et al., 2009). The crystal structure of PDK1 in the new crystal packing was obtained in a screening for new crystallization conditions in the presence of HM polypeptides. PDK1_120-359 (Y288Q,Q292A) was co-crystallized in solution (P)Y(K/DEPQGKPGQ/SA (underlined residues are conserved in the HM phosphorylation site of Akt/PKBs, SGKs, S6Ks, and PKCs).

PDK1 Expression, Purification, and In Vitro Activity Test
His-tagged PDK1_120-359 and His-tagged PDK1_120-359 employed in activity assays and in the AlphaScreen interaction assays were expressed in insect cells and
purified through Ni-NTA and gel-filtration chromatography as previously described (Busschots et al., 2012; Engel et al., 2006). His-tagged human Aurora A1-403 was recombinantly expressed in bacteria from pET28 plasmid and purified as described above for PDK1. The GST-fusion proteins were obtained from HEK293 cells after transient transfection of the corresponding pEBG2T plasmids and purified as previously described (Engel et al., 2006). The detailed conditions for the activity assay are described in the Supplemental Information.

**AlphaScreen Interaction-Displacement Assays**

The AlphaScreen assay was performed according to the standard protocol from the manufacturer (PerkinElmer). The setup of the interaction-displacement assay was previously described (Busschots et al., 2012). In short, the assay was performed in a final volume of 25 μL in white 384-well microtiter plates (Greiner Bio-One) with His-PDK1 50-359 (Tyr288Gly; Gln292Ala) (25 nM) and biotin-PiFtide (25 nM) in a buffer containing 50 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1 mM DTT, 0.01% (v/v) Tween20, and 0.1% (w/v) BSA, followed by the addition of 5 μL of beads (nickel chelate-coated acceptor beads and streptavidin-coated donor beads; 20 μg/mL final concentrations). The setup for Aurora A-TPX2tide interaction assay was the same, except that His-Aurora A (5 nM) and biotin-TPX2tide (10 nM) were employed.

**Molecular Dynamics**

We performed MD simulations using the GROMACS package (Hess et al., 2008) and the Amber99SB*-ILDN force field (Best and Hummer, 2009; Lindorf-Larsen et al., 2010). PT simulations were performed using PLUMED 2.1 (Trimbolo et al., 2014). We performed PT with five replicas in the 300–400 K range using the WTE (Bonomi and Parrinello, 2010).

**Isothermal Titration Calorimetry**

Calorimetric titrations were performed using the MicroCal iTC200 instrument (GE Healthcare) as previously described (Busschots et al., 2012) with the modifications indicated in the Supplemental Experimental Procedures.

**Small Molecules**

Compounds PS663 and PSE10 were commercially available from Maybridge. The commercial compounds were further analyzed by 1H-NMR, 13C-NMR, and ESI-MS (see Supplemental Experimental Procedures).

**ACCESSION NUMBERS**

The coordinates of the structures have been deposited in the PDB under accession codes PDB: SLVL (PS663), SLVM (adenine), SLVN (adenosine), SLVO (PSE10), and SLVP (HM-peptide).

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2016.06.017.

**AUTHOR CONTRIBUTIONS**

J.O.S. obtained and analyzed most of the crystallography data. V.H. solved initial PDK1 crystals. G.S. and F.L.G. designed the computational analysis. G.S. did the MD simulations. K.B. set up the screening assay and M.N.L. did biophysical assays. D.O. provided medicinal chemistry support. Screening and biochemical characterizations were performed by E.S., K.B., A.K.H., and S.N. under the supervision of R.M.B. S.Z. provided advice. P.M.A. supervised V.H. and analyzed data. R.M.B. supervised the whole research project. The manuscript was written by R.M.B. and F.L.G., supported by J.O.S., G.S., and P.M.A.

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