Tipping the Balance of the Protein Kinase PDK1 Allosteric Regulatory Mechanism by Low-Molecular-Weight Ligands

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Running Title: Bi-directional allostery in PDK1 using small compounds

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Summary

Here we describe compounds that bind to the active site of a protein kinase and influence the dynamics and function of a distant regulatory site. AGC protein kinases have a conserved allosteric site, the PIF-pocket, which regulates protein activity and interaction with substrates. The binding of substrate-derived docking peptides and small molecules to the PIF-pocket activates AGC kinases by allosterically affecting the ATP-binding site. In this study, we identify small compounds that bind to the ATP-binding site and affect the PIF-pocket of the kinase PDK1 and describe the molecular details of the mechanism whereby the compounds enhance or inhibit the function of the regulatory site. PDK1 and Aurora kinase inhibitors that bind to the ATP-binding site unintentionally differentially modulate physiological interactions at the PIF-pocket site. The rationale presented here has implications for the rational development of new allosteric drugs.

Introduction

Allostery is a fundamental and widespread mechanism of intra-molecular 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” that 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 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 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 (Fig. 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 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 PIF-pocket as a key allosteric regulatory site. The PIF-pocket was originally described in the PDK1 as a regulatory site that binds PIF (PDK1 Interacting Fragment), a polypeptide sequence derived from a PDK1 substrate (Biondi et al., 2000). The pocket is formed by the conserved helix αC, β-strands 4 and 5 and helix αB (Fig. 1B). The PIF sequence comprises an extended 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 (Fig. S1A,B). 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, RSK, but not PKB/Akt, require the docking of their HM to the PIF-pocket of PDK1 to become efficiently phosphorylated (Bayascas, 2008; Biondi et al., 2001; Collins et al., 2003) (Fig. 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) (Fig. S1D,E). 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, EGF-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). Abl kinase is both allosterically inhibited and activated by intramolecular interaction with its SH2 domain (Wojcik et al.), which, in the active state, docks at the top of the small lobe (Dolker et al., 2014; Nagar 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.

In spite of the large interest in drug development to 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 bi-directional 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 to 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) (Fig. S1A). The interaction between His-PDK1 and biotin-PIFtide can be measured by means of alphascreen technology (Fig. 1C) (Busschots et al., 2012). Using this homogeneous assay, we screened a library of 14400 small molecules (average mol. 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 (Fig. 1D,E). PSE10 ('2-oxopropyl N-(4-chlorophenyl)-[(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[cd, 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 (Fig. 1F). PSE10 activated PDK1 but did not affect the activity of PDK1 with mutations at the PIF-pocket (Fig. 1F-J). The crystal structure of PSE10 in complex with $PDK1_{50-359}$

confirmed that PSE10 bound to the PIF-pocket (Fig. 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 (Fig. 1G,H; Fig. 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 (Fig. 1I,J). 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 $PDK1_{50-359}$ in complex with PS653 (Fig. 2A; Fig. 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 adenine 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 in between the hydrophobic side chains of Leu88, Val96, and Leu212. However, a major difference is that PS653 extends outside of 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 adenine or adenosine, which bind to the same site as PS653 (Fig. 2B,C; Table S1), could produce similar allosteric effects. Surprisingly, adenosine *enhanced* the binding of PIFtide to PDK1, providing evidence of cooperativity, whereas adenine did not produce any allosteric effect on the PIF-pocket (Fig. 2D). Interestingly, the enhancing effect was observed selectively with adenosine and not with adenine, AMP, ADP,

ATP, the non-hydrolizable ATP analog thio-ATP or cAMP (Fig. 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 (Fig. 2C). Thus, the presence of the sugar moiety in 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-μs 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 figure S4A,B.

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 (Sugita and Okamoto, 1999) simulations in the Well Tempered Ensemble (PT-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 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 the helix αB (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, whose free energy surface (FES) has a single minimum with a well-ordered αB helix (Fig. 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 helix 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 dynamics, enforcing a more closed catalytic domain (Fig. 3B), in agreement with the crystal structure of the complex (Busschots et al., 2012). Indeed,structures with a Gly-rich loop-Asp205 distance 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 positions the phosphate of ATP at the active site and a Glu from the 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 activator, PS210 stabilizes conformations with a closed hinge and well-formed helix αB. The same conclusion can be drawn by observing the projection of the FES along the first two eigenvectors of the principal component analysis (PCA) (Fig. S4D). Different orientations of Tyr126 were observed in our new simulations for PDK1 with both ATP and PS210 (Fig. S4C and Fig. S5A). Interestingly, 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 (Fig. S4C and Table S1). In addition, this new crystal structure confirms the PIF-pocket as the binding site of substrate HM polypeptides (Fig. S5B). Overall, the PT-WTE 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. Together, the molecular dynamics simulations were in excellent agreement with the experimental data, and predicted Tyr126 movements that were independently confirmed in successive crystallography work. Noteworthy, 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 (Fig. 3B and Fig. S4D). This, in turn, dramatically increases the population of more open structures (distances larger that 17Å in Figure 3B). In contrast, adenosine enriched the most closed conformation (Fig. 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 (Fig. S6A,B). The additional hydrogen bonds formed by the ribose moiety of adenosine (Fig. 2C) could trigger the hinge motion and tip the balance of the conformational equilibrium towards 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

affect 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 $PDK1_{50-360}$ with PS653 and adenosine using isothermal titration calorimetry (ITC). The results confirmed in both cases the existence of a single binding site for the compounds in solution, with dissociation constants in the low micromolar range (Fig. S7 and Table 1). Notably, the interaction of $PDK1_{50-360}$ with PS653 was less enthalpic and more entropy driven (-1.2 kcal/mol; $\Delta H/\Delta G$= 15%) than the interaction with adenosine (-3.2 kcal/mol; $\Delta H/\Delta G$= 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 towards 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.

Together, the computational results point towards 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, the Gly-rich loop as well as residues from the large lobe (Fig. S6C). In addition, the molecular dynamics 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 closed form but not in the open form, where it is replaced by the Lys199-Asp138 interaction (Fig. 4A). A mutagenesis study based on the simulations confirmed a major role for Lys144. Firstly, we found that the purified Lys144Glu and Lys144Ala mutant proteins had slightly lower specific activities (approximately 30% decreased) than the control GST-PDK1 protein. In spite of lower basal activity, 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, and suggesting that the mutation stabilized open-inactive forms of the kinase. Secondly, the Lys144 mutations importantly decreased the binding of PIFtide (Fig. 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 in remarkable 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 towards more open structures that, as previously demonstrated, are generally less prone to bind PIFtide, as the mutant itself. The mutated Glu144 doesn't 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 (Fig. 4D,E). Notably, the PDK1 Lys144 mutants increased the binding of PIFtide in the presence of adenosine (Fig. 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 (Fig. 4E). As the increased population of open structures is generally a consequence of the higher inter-lobe dynamics (as seen for PS653), the effect seen in the Lys144Glu PDK1 mutant could be due to adenine shifting

back the equilibrium towards more closed structures to maximize the interactions within the binding site, *de-facto* restoring the open-closed equilibrium of wild type PDK1. On the whole, 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 knock-in 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 nM IC50), acting similar to PS653, while UCN01 did not affect the binding of PIFtide even at high μM concentrations (Fig. 5A,B). 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 oncogenes 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 effect the interaction (at μM concentrations), MLN8237 potently displaced the interaction (at low nM concentrations) (Fig. 5C,D). 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 here describe small compounds that take advantage of the bi-directional nature of allosteric networks, enhancing or inhibiting the binding to a downstream signaling partner and further defined the molecular mechanism for the reverse allostery between the active site and the PIF-pocket in PDK1 (Fig. 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 bi-directional 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 αB and αC 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 αB and αC 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 hydrophobic motif 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 to all previous knowledge on the biochemical and structural information on the activation of PDK1 by PIFtide and small compounds binding to the PIF-pocket. With current availability of computer resources it is not possible to extend

such unbiased simulations up to the high μs to ms time scales. However, this unbiased result prompted us to explore the dynamics of PDK1 by focusing on particular areas of the protein, by using PT-WTE, an effective enhanced-sampling algorithm. The detailed information from biochemical studies, structural work and molecular dynamics simulations (MD and PT-WTE) provided a clear mechanism of the bi-directional allosteric process in PDK1. We believe that a similar approach can be effectively used to investigate the mechanisms of allosteric regulation by occupancy of the site equivalent to the PIF-pocket in other protein kinases.

The previous characterization that GSK2334470 is a much stronger inhibitor of S6K than of PKB/Akt could be explained by the fact that this compound does not produce a double inhibitory effect on PKB/Akt, because this kinase does not require the HM-docking interaction with the PIF-pocket of PDK1 for its physiological phosphorylation. Thus, at concentrations where PDK1 is not fully inhibited by GSK2334470, its effects are similar to compounds binding to the PIF-pocket, which selectively inhibit S6K, without affecting PKB/Akt (Busschots et al., 2012; Rettenmaier et al., 2014). There is some precedence for paradoxical effects observed by drugs binding at the ATP-binding site of protein kinases. A reverse allosteric effect is probably responsible for the nucleotide pocket-induced activation of PKCε (Cameron et al., 2009) and the allosteric RNase activation of IRE1α (Wang et al., 2012). In addition, there is some evidence that a similar reverse allosteric modulation might be involved in the “paradoxical activation” of partially inhibited B-RAF dimers (Heidorn et al., 2010). Interestingly, the detailed characterization of the allosteric modulation by compounds binding to the ATP-binding site could inspire the rational development of drugs with add-on characteristics. A rich example to discuss the implications is that of Aurora kinase. Compounds that bind to the ATP-binding site and allosterically affect the interaction with TPX2 are expected to destabilize the oncogene Myc by destabilizing the Aurora-Myc complexes. In addition, since TPX2 provides proper localization to Aurora A, compounds that bind to the ATP-binding site and allosterically disrupt the interactions with TPX2, may render molecules functionally unable to phosphorylate their specific substrates, even after the

dissociation of the compound. Similarly, it can be expected that compounds that produce desired double effects, i.e blocking the active site and, in addition, disrupting or enhancing a given allosteric interaction may be desired. We must note that the current nomenclature to describe the mode of inhibition of kinases by small compounds (type I, type II and type III inhibitors) does not appropriately describe the allosteric effects of the compounds. A more appropriate nomenclature, in use for allosteric compounds acting on receptors, requires naming the identity of the receptor, the binding site of the small molecule, the site which is being allosterically affected and the kind of modulation. In one of the examples described here, GSK2334470 is a type I inhibitor but is also a negative allosteric modulator binding to the ATP-binding site and displacing the binding of PIFtide from the PIF-pocket of PDK1.

Here, we also provide the crystal structure of PDK1 in complex with the phosphorylated HM of Akt/PKB. The equivalent docking interaction is necessary for the phosphorylation and activation of most PDK1 substrates, including S6K and SGK. However, the PIF-pocket docking interaction is not considered to be required for the phosphorylation of Akt/PKB (Biondi et al., 2001; Busschots et al., 2012; Collins et al., 2003; Rettenmaier et al., 2014). Our structure suggests that the docking interaction still takes place for Akt/PKB, even if it is not the determinant of the phosphorylation of this kinase. While writing the current manuscript, 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 helix αC previously observed in solution (Hindie et al., 2009) and the disorder of helix αB predicted here by the molecular dynamics 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, was afterwards described by molecular dynamics 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 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 1000 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 it is also a signaling molecule on its own (Borea et al., 2016). Our results suggest that the 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 as well 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 indicates that the full bi-directional allosteric regulation between the active site and the PIF-pocket regulatory is functional in the regulation of AGC kinases, perhaps best exemplified in PRK2, where the a pseudosubstrate inhibitory polypeptide, PLKtide binding to the active site, and PIFtide binding to the PIF-pocket, are allosterically mutually excluded and contribute to PRKs complex mechanism of regulation (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. The present work provides a deep understanding of the bi-directional allosteric coupling mechanism and suggests ways to exploit it. The use of molecular dynamics 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 details, 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 success in the treatment of patients. The PDK1/AGC kinase allosteric model system provides a rich example of the potential of small molecules to bi-directionally 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 to members of the protein kinase family by providing a computer platform from where to rationally design compounds directed to the PIF-pocket or ATP-binding site and

producing desired secondary allosteric effects. Similarly, the above approach could also be applied more generally for drug discovery projects to other allosteric signaling proteins whose function could be modulated by molecules designed to produce desired conformational changes.

Experimental Procedures

Materials

The polypeptide substrate of protein kinase PDK1 was T308tide (KTFCGTPEYLAPEVRR; > 75% purity). Other polypeptides used were PIFtide (REPRILSEEEQEMFRDFDYIADWS), biotin-PIFtide (biotin-REPRILSEEEQEMFRDFDYIADWS) and biotin-TPX2tide (Biotin-MSQVKSSYSYDAPSDFINFSSLDDEGDTQNIDSWFEEKANLEN-NH2). Adenine and adenosine (≥ 99%) were from Sigma-Aldrich.

Crystal structures

To obtain the crystal structure of PDK1 in complex with PS653, 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. $PDK1_{50-359}$ [Y288G,Q292A] was co-crystallized in the presence of the phosphorylated peptide KGAGGGGFPQFS(P)YSA (underlined residues are conserved in the HM phosphorylation site of Akt/PKBs, SGKs, S6Ks, and PKCs). The coordinates of the structures have been deposited in the Protein Data Bank under accession codes XXX.

PDK1 expression, purification, and in vitro activity test

His-tagged $PDK1_{1-556}$ and His- tagged $PDK1_{50-359}$ 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 described (Busschots et al., 2012; Engel et al., 2006). His-tagged human Aurora A_{1-403} was recombinantly expressed in bacteria from pET28 plasmid and purified as described for PDK1 above. The GST-fusion proteins were obtained from HEK293 cells after transient transfection of the corresponding pEBG2T plasmids and purified as 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 (Perkin Elmer). The set-up 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) with His-$PDK1_{50-359}$ [Tyr288Gly; Gln292Ala] (25 nM) and Biotin-PIFtide (25 nM) in a buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM dithiotreitol, 0.01% (v/v) Tween-20 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 set-up 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 Molecular Dynamics (MD) simulations using the GROMACS 4.6 package (Hess et al., 2008) and the Amber99SB*-ILDN force field (Best and Hummer, 2009; Lindorff-Larsen et al., 2010). The system was minimized with 10000 steps of conjugated gradient and equilibrated in the NPT ensemble for 10 ns. A production run of 400 ns was then performed in the NVT ensemble, with a time step of 2 fs. Neighbor searching was performed every 5 steps. The PME algorithm was used for electrostatic interactions with a cut-off of 1.2 nm, while a single cut-off of 1.2 nm was used for Van der Waals interactions. Temperature coupling was done with the V-rescale algorithm (Bussi et al., 2007). The parallel-tempering

simulations were performed using Gromacs 4.6(Hess et al., 2008) combined with the PLUMED 2.1 plug-in (Tribello et al., 2014). We performed PT with 5 replicas in the 300K-400K range using the Well Tempered Ensemble (Bonomi and Parrinello, 2010).

Isothermal titration calorimetry

Calorimetric titrations were performed using the MicroCal iTC200 instrument (GE Healthcare Life Sciences) as previously described (Hindie et al., 2009) with the modifications indicated in the Supplemental Experimental Procedures. For the titrations of PDK1 with adenosine, the protein and the compound were prepared in 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM DTT and 1% v/v DMSO. For the titration of PDK1 with PS653 the protein and the compound were prepared in 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM DTT and 5% v/v DMSO. Errors on the thermodynamic parameter values in Table 1 are non linear least square fitting errors of the experimental binding isotherms using the Levenberg-Markardt iteration method (Freire et al., 2009).

Small molecules

Compounds **PS653** and **PSE10** were commercial available from Maybridge. The commercial compounds were further analyzed by ^{1}H-NMR, ^{13}C-NMR and ESI-MS (see Supplemental Experimental Procedures).

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Author contributions

JOS obtained and analyzed most of the crystallography data. VH solved initial PDK1 crystals. GS and FLG designed the computational analysis. GS did the MD simulations. KB set up the screening assay and MNL did biophysical assays. DO provided medicinal chemistry support. Screening and biochemical characterizations were performed by ES, KB, AKH and SN under the supervision of RMB. SZ provided advice. PMA supervised VH and analyzed data. RMB supervised the whole research project. The manuscript was written by RMB and FLG supported by JOS, GS and PMA.

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Figure Legends

Fig. 1. The kinase domain, the PIF-pocket binding to PIFtide, and the identification of small molecules that displace PIFtide from the PIF-pocket. (**A-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 ID: 4AW1). (**C**) Schematic representation of the alphascreen interaction assay employed for the screening of the library of small molecules. (**D**) Effect of PSE10 and PS653 on the interaction between His-PDK1 50-360 and biotin-PIFtide. (**E**) Structures of PSE10 and PS653 in comparison to previously described activators of PDK1, PS48 and PS210. (**F**) Schematic representation of the PDK1 *in vitro* activity assay, using the polypeptide T308tide as substrate of PDK1. (**G**) Effect of PSE10 and PS653 on the *in vitro* activity of His-PDK1 50-556; the inhibition of other PDK1 constructs is presented in figure S3. (**H-J**) Effect of PSE10, PS653 and PIFtide on the activity of GST-PDK1 1-556 and the PIF-pocket mutants Leu155Glu and Leu127Val. See also Figure S1-S2.

Fig. 2. PS653, adenine and adenosine bind to the ATP-binding site but produce distinct allosteric effects at the PIF-pocket. (**A-C**) Crystal structures of PDK1 in complex with PS653 (**A**), adenine (**B**) and adenosine (**C**). Only residues in direct contact with the compounds are shown as sticks. Hydrogen bonds are visualized as gray dotted lines. Close contacts of PS653 mentioned in the main text are indicated as red dotted lines. $|2F_o\text{-}F_c|$ electron density of the compounds is shown in blue and contoured at 1σ. (**D**) Effect of adenine and adenosine on the interaction between PDK1 and PIFtide. The interaction between His-$PDK1_{50\text{-}359}$ and biotin-PIFtide was measured using the alphascreen assay depicted in Figure 1C and the effect of adenine and adenosine quantified. See also Figure 1C and Table S1.

Fig. 3. Free energy calculation of PDK1 with compounds. (**A**) Schematic representation of parameters measured. Free energy surfaces as projected along the identified αB parameter

(end-to-end distance) and two distances describing the opening and rotation of the small lobe with respect to the large lobe. (**B**) Free energy calculation of PDK1 with ATP, PS210, PS653 and adenosine. PS653 clearly enhances the hinge motion and lobe rotation, while adenosine has the opposite effect, similar to what was observed for PS210. See also Figure S4; Figure S5.

Fig. 4. The reverse allosteric effect is mediated by Lys144 and is differentially affected by ATP-competitive PDK1 inhibitors. (**A**) Asp138 can form salt bridges with either Lys144 or Lys199. These residues are shown as sticks in a cartoon representation of a PDK1 crystal structure (PDB ID 3HRC). Asp138, Lys144 and Lys199 are shown. (**B-G**) The ability of GST-$PDK1_{1-359}$ wt and Lys144 mutants to bind biotin-PIFtide was studied in the alphascreen interaction assay. (**B**) Mutation of Lys144 to Ala or Glu affects the binding to PIFtide. (**C-E**) Effect of PS653 on the interaction between GST-PDK1proteins and biotin-PIFtide. See also Figure S6.

Fig. 5. Negative allosteric modulation of the binding to the PIF-pocket by drugs under development to the ATP-binding site of PDK1 and Aurora kinase. (**A-B**) Effect of UCN01 and GSK2334470 ATP-competitive inhibitors of PDK1 on the interaction of His-$PDK1_{50-359}$ and biotin-PIFtide. (**C-D**) Effect of VX680 and MLN8237 ATP-competitive inhibitors of Aurora kinase on the interaction of His-Aurora A and biotin-TPX2tide.

Fig. 6. Schematic representation of the bi-directional 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 helix αB 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 sticks) are represented in alternative salt bridge interactions formed in the closed and open conformations of PDK1.

Table 1. Thermodynamic parameters of PDK1 interaction with low-molecular-weight compounds obtained by ITC. See also Figure S7.

Compound	Temperature (K)	N	K_a (M^{-1})	K_d (μM)	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} deg^{-1})	TΔS (kcal mol^{-1})
Adenosine	298	1.2 ± 0.2	$5\ 10^4 \pm 1\ 10^4$	20	-3.2 ± 0.7	10.7	3.2
PS653	310	0.87 ± 0.02	$8\ 10^5 \pm 2\ 10^5$	1.25	-1.20 ± 0.06	23.1	7.2

N is the stoichiometry of binding.

Figure 1

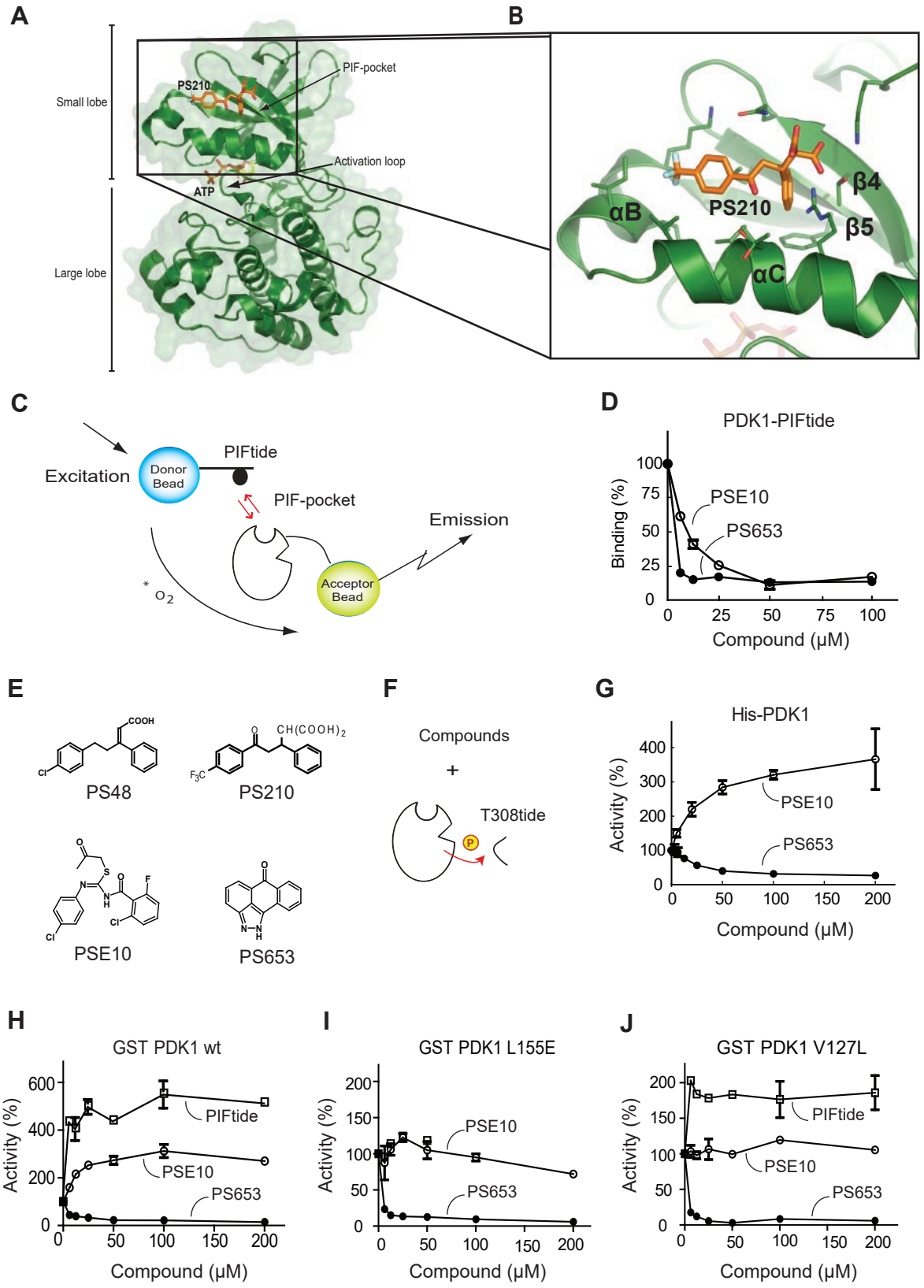

A
B
Small lobe
Large lobe
PS210
PIF-pocket
Activation loop
ATP
αB
β4
β5
αC
C
Excitation
Donor Bead
PIFtide
PIF-pocket
Emission
Acceptor Bead
D
PDK1-PIFtide
Binding (%)
PSE10
PS653
Compound (μM)
E
PS48
PS210
PSE10
PS653
F
Compounds
+
T308tide
G
His-PDK1
Activity (%)
PSE10
PS653
Compound (μM)
H
GST PDK1 wt
Activity (%)
PIFtide
PSE10
PS653
Compound (μM)
I
GST PDK1 L155E
Activity (%)
PSE10
PS653
Compound (μM)
J
GST PDK1 V127L
Activity (%)
PIFtide
PSE10
PS653
Compound (μM)

Figure 2

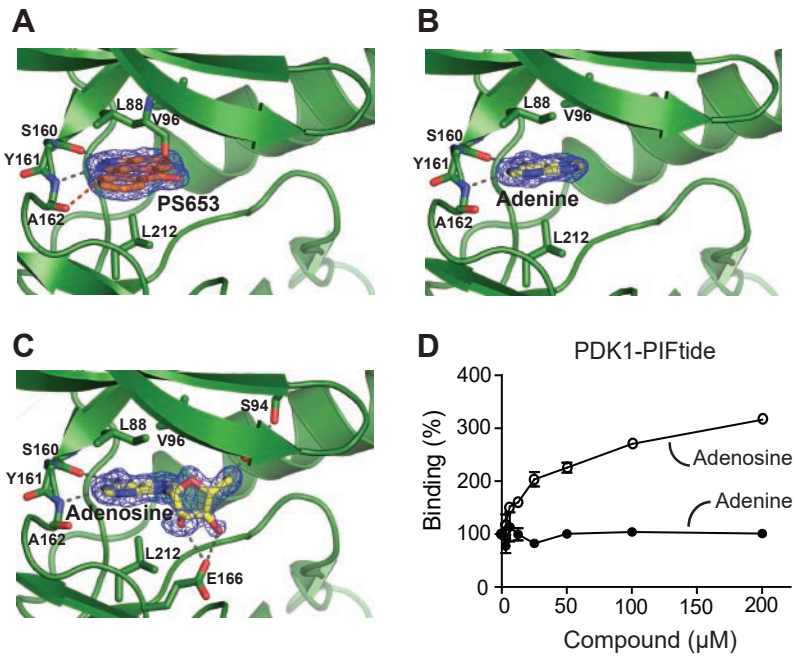

A
L88
V96
S160
Y161
PS653
A162
L212
B
L88
V96
S160
Y161
Adenine
A162
L212
C
S94
L88
V96
S160
Y161
Adenosine
A162
L212
E166
D
PDK1-PIFtide
Binding (%)
400
300
200
100
0
0
50
100
150
200
Compound (μM)
Adenosine
Adenine

Figure 3

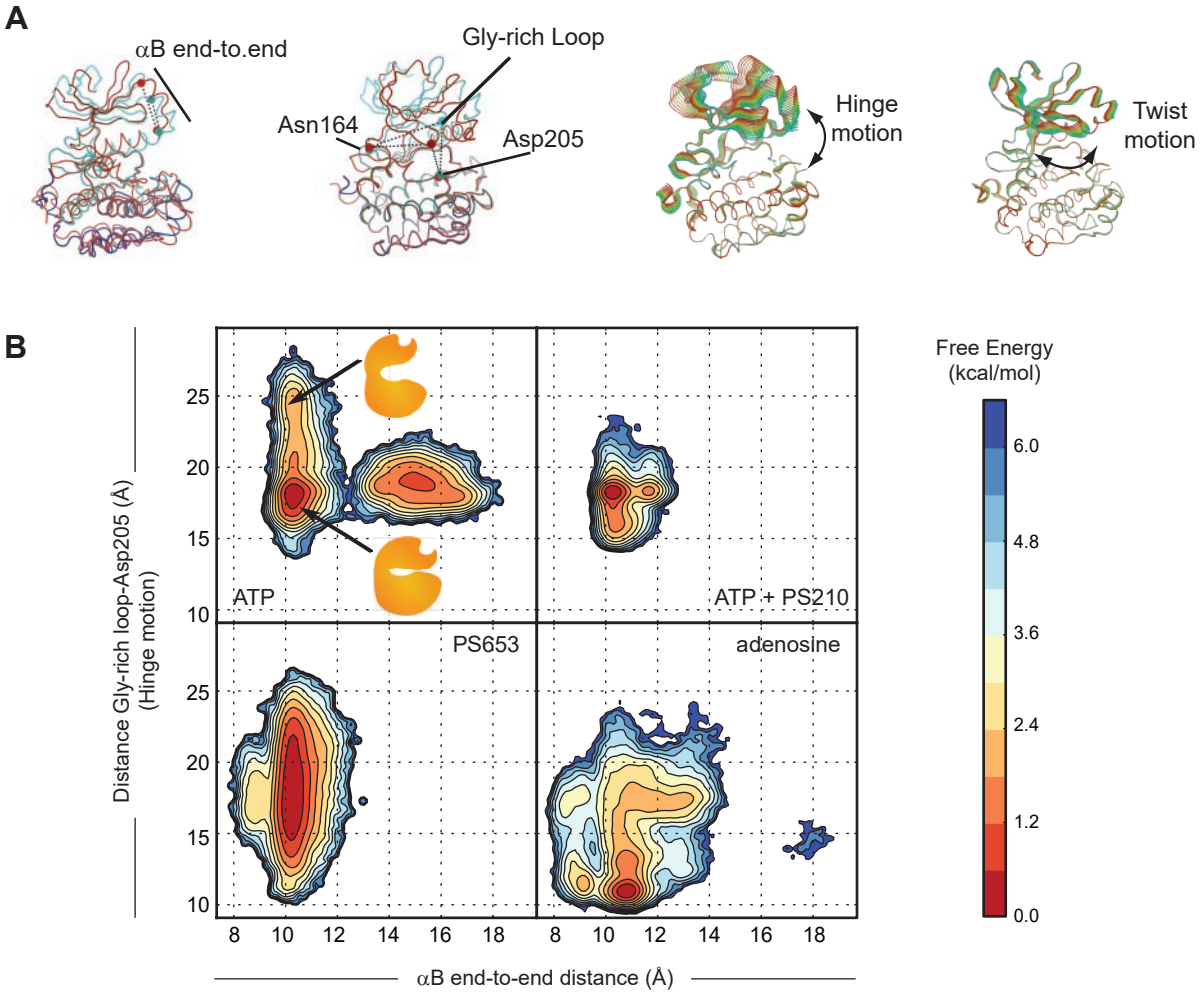

A
αB end-to.end
Gly-rich Loop
Asn164
Asp205
Hinge
motion
Twist
motion
B
Distance Gly-rich loop-Asp205 (Å)
(Hinge motion)
ATP
ATP + PS210
PS653
adenosine
25
20
15
10
8
10
12
14
16
18
αB end-to-end distance (Å)
Free Energy
(kcal/mol)
6.0
4.8
3.6
2.4
1.2
0.0

Figure 4

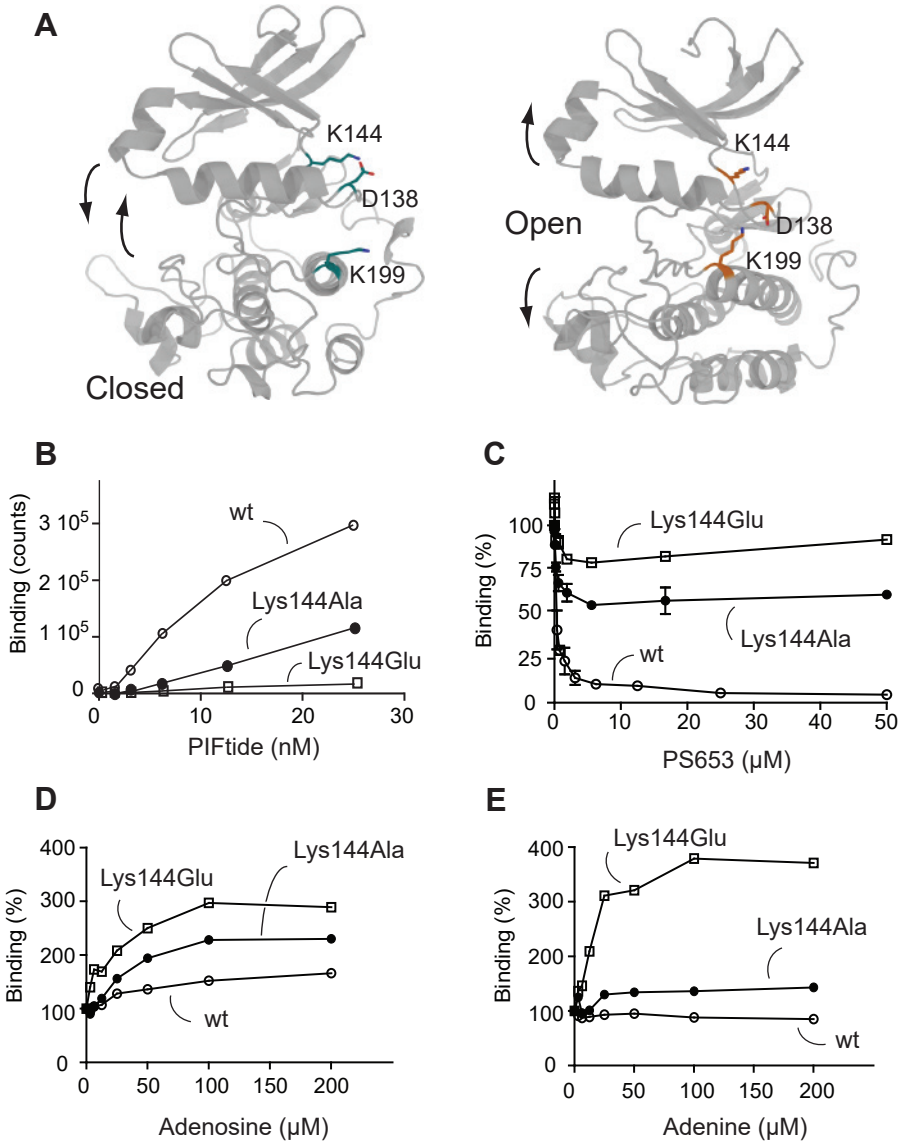

A
K144
D138
K199
Closed
Open
B
Binding (counts)
3 10^5
2 10^5
1 10^5
0
wt
Lys144Ala
Lys144Glu
0
10
20
30
PIFtide (nM)
C
Binding (%)
100
75
50
25
0
Lys144Glu
Lys144Ala
wt
0
10
20
30
40
50
PS653 (μM)
D
Binding (%)
400
300
200
100
0
Lys144Glu
Lys144Ala
wt
0
50
100
150
200
Adenosine (μM)
E
Binding (%)
400
300
200
100
0
Lys144Glu
Lys144Ala
wt
0
50
100
150
200
Adenine (μM)

Figure 5

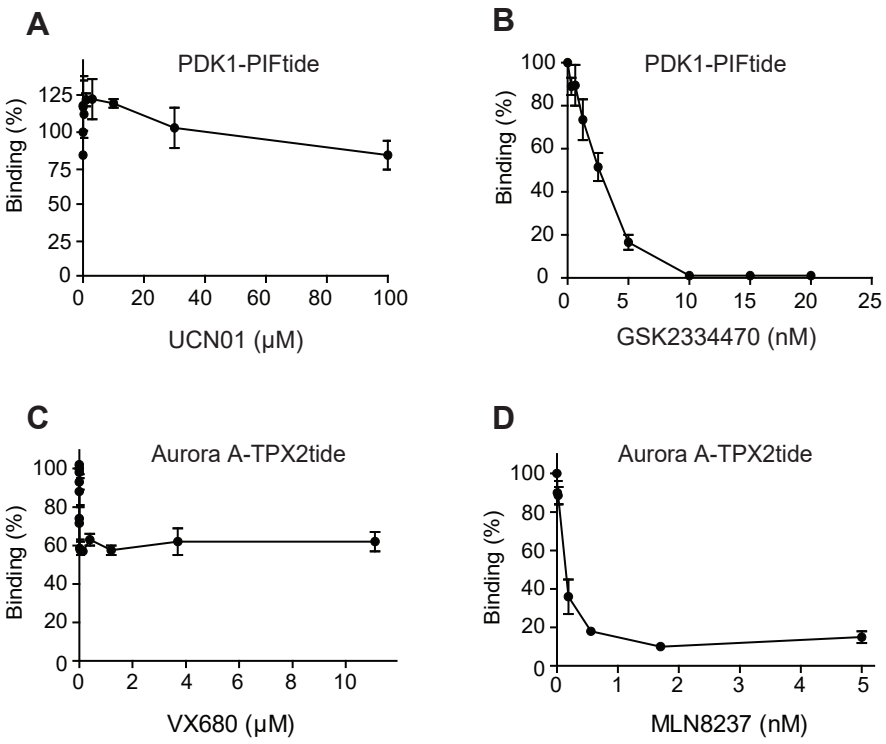
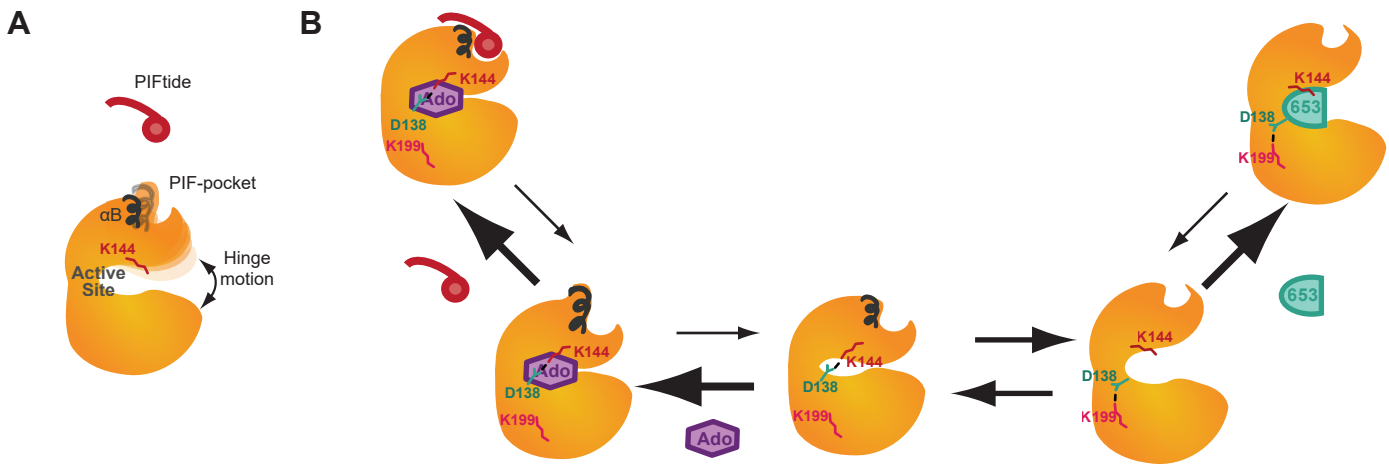

A
PDK1-PIFtide
Binding (%)
125
100
75
50
25
0
0
20
40
60
80
100
UCN01 (μM)
B
PDK1-PIFtide
Binding (%)
100
80
60
40
20
0
0
5
10
15
20
25
GSK2334470 (nM)
C
Aurora A-TPX2tide
Binding (%)
100
80
60
40
20
0
0
2
4
6
8
10
VX680 (μM)
D
Aurora A-TPX2tide
Binding (%)
100
80
60
40
20
0
0
1
2
3
4
5
MLN8237 (nM)

Figure 6


A
PIFtide
PIF-pocket
αB
K144
Active Site
Hinge motion
B
K144
Ado
D138
K199
653