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Substrate-Selective Inhibition of Protein Kinase PDK1 by Small Compounds that Bind to the PIF-Pocket Allosteric Docking Site

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SUMMARY

The PIF-pocket of AGC protein kinases participates in the physiologic mechanism of regulation by acting as a docking site for substrates and as a switch for the transduction of the conformational changes needed for activation or inhibition. We describe the effects of compounds that bind to the PIF-pocket of PDK1. In vitro, PS210 is a potent activator of PDK1, and the crystal structure of the PDK1-ATP-PS210 complex shows that PS210 stimulates the closure of the kinase domain. However, in cells, the prodrug of PS210 (PS423) acts as a substrate-selective inhibitor of PDK1, inhibiting the phosphorylation and activation of S6K, which requires docking to the PIF-pocket, but not affecting PKB/Akt. This work describes a tool to study the dynamics of PDK1 activity and a potential approach for drug discovery.

INTRODUCTION

Cells use protein phosphorylation to transduce information and intracellular signals (Johnson and Lewis, 2001). Almost every cellular signaling pathway is known to be regulated by phosphorylation (Pawson and Scott, 2005). Therefore, the action of protein kinases and protein phosphatases is essential for multiple physiologic responses. The uncontrolled action of protein kinases can result in diseases, such as cancer, diabetes, and neurologic disorders, making protein kinases a highly sought-after drug target class in the pharmaceutical industry (Cohen, 2002).

The activation of protein kinases often involves phosphorylation, regulation by N- or C-terminal domains or regulation by interacting partners. The cAMP-dependent protein kinase PKA is a prototype for the structural studies on the catalytic domain of protein kinases. A number of crystal structures show the catalytic subunit in "open," "closed," and "intermediate" conformations that reflect the relative motions between the small and the large lobes of the protein. The transition to the close-active conformation of PKA is induced by ATP and peptide binding to the substrate binding sites (Johnson et al., 2001; Knighton et al., 1991a, 1991b). In PKA, as well as in other kinases from the AGC group, the Phe residues within the C-terminal sequence (Phe-Xaa-Xaa-Phe-COOH) bind to the small lobe of the kinase domain in a hydrophobic pocket termed the "PIF-pocket." These Phe residues are necessary for activity in PKA (Batkin et al., 2000; Etchebehere et al., 1997). However, neither the opening-closing of the domains nor the Phe-Xaa-Xaa-Phe-COOH motif appears to play a central role in the stimulus-induced regulation of PKA activity. In contrast, other AGC kinases possess an extended hydrophobic motif (HM) comprising the equivalent Phe residues followed by a phosphorylation site (Phe-Xaa-Xaa-Phe-Ser/Thr-Tyr, where the phosphorylated amino acid is underlined), which participates in the stimulus-induced regulation of the activity of AGC kinases by interacting with the PIF-pocket in a phosphorylation-dependent manner (Biondi, 2004; Frödin et al., 2002; Pearce et al., 2010; Pearl and Barford, 2002; Yang et al., 2002). In atypical PKCs, the PIF-pocket mediates the inhibition by the N-terminal domain (Lopez-Garcia et al., 2011). Thus, even if the mechanism of regulation of the different AGC kinases is different, they seem to converge on the PIF-pocket.

In target cells, insulin induces the activation of the phosphoinositide 3-kinase (PI3-kinase), which catalyzes the synthesis of the second messenger phosphatidylinositol (3,4,5)-triphosphate (PIP_3) at the plasma membrane (Figure 1). In the presence of PIP_3, the phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase B (PKB, also termed Akt) rapidly colocalize at the plasma membrane where PDK1 phosphorylates the activation loop of PKB/Akt, activating this enzyme. Active PKB/Akt then transduces a number of insulin effects, such as those leading to glucose uptake and the synthesis of glycogen in muscles (Vanhaesebroeck and Alessi, 2000).

With a slower time course following insulin stimulation, PDK1 also phosphorylates the activation loop of the serum- and glucocorticoid-stimulated kinase (SGK), p70 ribosomal S6 kinase (S6K), protein kinase C-related kinase (PRK), and p90 ribosomal S6 kinase (RSK) isoforms (Vanhaesebroeck and Alessi, 2000). Other substrates of PDK1, like classical protein kinase C (PKCs), appear to be phosphorylated by PDK1 constitutively

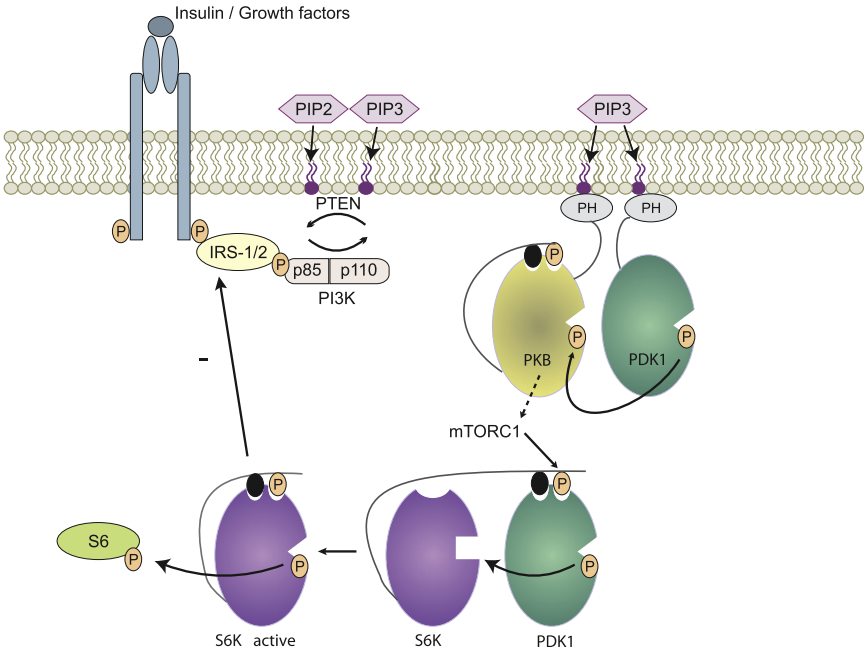

Figure 1. Role of PDK1 in the Insulin Signaling Pathway

The binding of insulin or growth factors to the insulin receptor results in the autophosphorylation of a number of tyrosine residues that are present in the receptor and recognized by members of the insulin receptor substrate (IRS) family. The insulin receptor also phosphorylates key tyrosine residues on IRS proteins, some of which are recognized by the SH2 domain of the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K). The catalytic subunit of PI3K, p110, then phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2), leading to the formation of phosphatidylinositol (3,4,5)-triphosphate (PIP_3) at the plasma membrane. This phosphorylation allows for the recruitment of PDK1 and PKB to the plasma membrane via their PH domains. PDK1 then activates PKB by phosphorylating T308 in its activation loop. PKB plays a key role in mediating the glucose uptake and glycogen synthesis after insulin stimulation in skeletal muscles.

With a slower time course following insulin stimulation, PDK1 phosphorylates and activates S6K. The interaction and subsequent activation is triggered by the docking of the phosphorylated hydrophobic motif (HM) of S6K in the PIF-binding pocket and an associated phosphate-binding site of PDK1. Active S6K can phosphorylate several substrates, including the ribosomal S6 protein. S6K can also desensitize the insulin receptor by phosphorylating several serines in IRS-1, creating a negative feedback loop that inhibits PI3K downstream signaling.

(Newton, 2003). The difference in the timing of substrate phosphorylation can be explained by the different molecular mechanisms of recognition and interaction between PDK1 and its substrates (Biondi et al., 2001; Leslie et al., 2001). In contrast to PKB/Akt, all other AGC kinase substrates of PDK1 described so far require docking of their HM to the PIF-binding pocket of PDK1 to become phosphorylated (Biondi, 2004; Biondi et al., 2001). Biochemical studies showed that PIFtide and phosphorylated HM polypeptides derived from substrates of PDK1 bound to the PIF-binding pocket of PDK1 and activated PDK1 in vitro (Biondi et al., 2000; Engel et al., 2006; Frödin et al., 2000). Furthermore, crystallography studies identified a sulfate-binding site contiguous to the hydrophobic PIF-binding pocket (Biondi et al., 2002), and biochemical evidence confirmed that the residues that form part of this site participated in the binding to the phosphorylated HM (Biondi et al., 2002; Frödin et al., 2002). The phosphorylation of the HM of SGK, RSK and S6K triggers the interaction of the substrate kinase with the PIF-binding pocket and the associated phosphate-binding site on PDK1 (Biondi, 2004; Pearce et al., 2010), although the sequence of events is contested in the case of S6K (Keshwani et al., 2011). This model suggests that the requirement for previous phosphorylations could be at the heart of the different timing of phosphorylation of PDK1 substrates. As a consequence of the docking of the HM of substrates to the PIF-binding pocket of PDK1, a conformational change in PDK1 is triggered, stimulating its activity and subsequent phosphorylation of the protein kinase substrate at the activation loop. Elegant knock-in experiments where the endogenous PDK1 was replaced with a PDK1 protein mutated at the center of the PIF-binding pocket (PDK1 Leu155Glu) or at the phosphate-binding site, confirmed that the PIF-binding pocket of PDK1 and its associated phosphate-binding site are necessary for the phosphorylation of S6K but not PKB/Akt in vivo (Collins et al., 2003, 2005). Thus, in vitro and in vivo studies using mutants of the PIF-binding pocket suggested that the integrity of the pocket is required for the docking of several substrates of PDK1.

Following insulin stimulation, activated S6K phosphorylates its substrates, including the ribosomal S6 protein and elongation factor 2-kinase that support insulin-stimulated protein synthesis. In adipocytes and muscle cells, S6K also phosphorylates IRS1, which is an adaptor protein that links the insulin receptor to the p85 regulatory subunit of PI3-kinase. Phosphorylation of IRS1 by S6K acts as a negative feedback loop, inhibiting PI3-kinase downstream signaling (Tremblay et al., 2007; Um et al., 2004). Constitutive activation of this negative feedback loop may be at the center of insulin resistance and diabetes. Indeed, increased S6K1 activity is associated with impaired glucose tolerance in humans (Barbour et al., 2011). Collectively, the knowledge about the S6K-IRS1 negative feedback loop highlighted S6K as a possible target to sensitize organisms to insulin action for the treatment of type II diabetes (Boura-Halfon and Zick, 2009; Harrington et al., 2004). In agreement with this possibility, S6K knock-out mice are protected against obesity induced by age or diet and have an increased sensitivity to insulin (Um et al., 2004, 2006).

In previous work, we developed low molecular weight compounds that bind in the PIF-binding pocket of PDK1 and activate this kinase in vitro (Engel et al., 2006; Hindie et al., 2009; Stroba et al., 2009). Additional structural and biochemical experiments indicated that the binding of activator compounds to the PIF-binding pocket transduces allosteric effects to the ATP-binding site (Hindie et al., 2009), although the full molecular details of the activation process remain unknown. Recently,

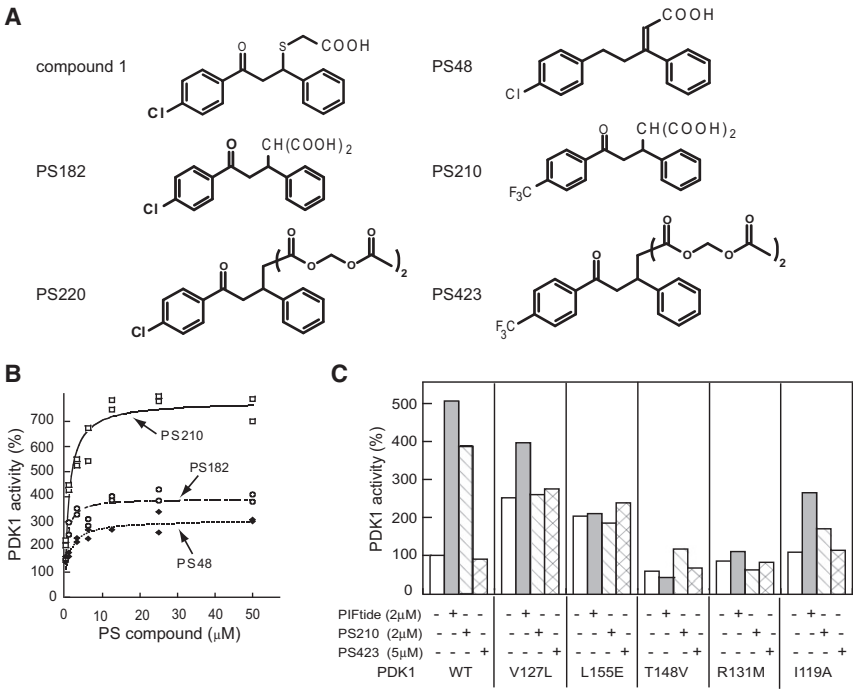

Figure 2. Dicarboxylate Compounds, but Not Their Di-Ester Prodrugs, Are In Vitro Activators of PDK1

The activity of purified full-length PDK1 and its mutants was measured in vitro using the polypeptide T308tide as a substrate in the presence of PIFtide or small compounds.

(A) The structures of the low molecular weight compounds described in this study.

(B) The activation of PDK1 by the dicarboxylates PS210 (AC_{50} 2 μM) and PS182 (AC_{50} 2 μM), relative to that of PS48.

(C) Results of activity assays with PDK1 wt and mutants identify the PIF-binding pocket as the target site for PS210. This compound can activate PDK1 wt but not proteins with mutations in the PIF-binding pocket. The di-ester prodrug, PS423, does not activate PDK1 wt or the mutant proteins. See also Figure S1.

small compounds that activate PDK1 have also been reported by others (Stockman et al., 2009; Wei et al., 2010). However, most of the compounds described to date contain groups that do not allow for the passive diffusion of the compounds into cells. In the present work, we describe both the molecular details by which allosteric PIF-binding pocket compounds induce a close-active conformation of PDK1 in vitro and the effects of prodrug cell-permeable derivative compounds that are cleaved intracellularly to the corresponding active compounds. Treatment of cells with the prodrug PS423 inhibited S6K signaling without affecting PKB/Akt downstream effects in HEK293 or in C2C12 myoblast cells. Notably, PS423 allowed us to elucidate the existence of at least three different PDK1-dependent pathways participating in the insulin-stimulated glucose uptake in C2C12 myoblasts and myotubes. These results open the field for the development of future substrate-selective protein kinase inhibitor drugs that may have use in the treatment of human diseases, including cancer and diabetes.

RESULTS

The Dicarboxylates PS182 and PS210, but Not Their Di-Ester Derivatives, Are Potent Activators of PDK1 In Vitro

We previously developed low molecular weight compound activators of PDK1 consisting of two ring systems joined by a linker and an additional side chain ending in a carboxyl group (e.g., compound 1 and PS48, Figure 2A) (Hindie et al., 2009; Stroba et al., 2009). This carboxyl moiety interacts with three residues that form part of the HM-phosphate-binding site on PDK1, which is participating in the physiologic activation of PDK1. We speculated that a compound with two carboxyl groups might more efficiently mimic a phosphate and be a more potent activator of PDK1. Therefore, we synthesized a series of low molecular weight dicarboxylate compounds (Figure 2A). We observed that PS182 (containing the two ring systems as in compound 1 and PS48) and PS210 (with a trifluoromethyl substitution of the chlorine) were very potent activators of PDK1 (Figure 2B). Notably, PS210 stimulated 650% of basal PDK1 activity, which is 2-fold higher than the maximal activation obtained for our previously described compound, PS48.

The study of compound 1 and the family of PS48 compounds in cells has so far been severely hampered by these compounds' lack of cell permeability that is due to the negative charge associated with the carboxyl group. Therefore, to investigate the pharmacologic effects of blocking the PIF-binding pocket of PDK1 with more potent compounds, we synthesized di-ester (bis-acetoxymethyl ester) derivatives of PS210 (PS423) and PS182 (PS220). We reasoned that these di-ester derivatives would act as cell-permeable prodrugs and could be cleaved by cellular esterases to form the corresponding dicarboxylate variants. PS423 did not affect the activity of PDK1 in vitro, indicating that the carboxyl groups of the compounds are required for activation of PDK1 (Figure 2C). PS210 loses the ability to activate PDK1 upon mutation of Leu155 to Glu, Val127 to Leu or Arg131 to Met, suggesting that the interaction between this compound and PDK1 requires the PIF-binding pocket and that an interaction with the positive charge from Arg131 is important for the activation of PDK1. Mutation of other residues within the PIF-binding pocket resulted in intermediate effects, suggesting that these residues are not essential for the binding and activation by the dicarboxylate compounds.

We originally described the PIF-binding pocket as the site on PDK1 that interacted with PIFtide (REPRILSEEEQEMFRDFDYIA DWC), a polypeptide derived from the HM of PRK2. PIFtide interacts with PDK1 with high affinity (50 nM), and surface plasmon resonance studies revealed that 50% of the dissociation occurred within 40–60 s (Biondi et al., 2000), indicating a low OFF-rate. Here, we demonstrate that the interaction between His-PDK1 (50–360) and biotin-PIFtide can also be detected in an AlphaScreen assay (Figures S1A and S1B available online).

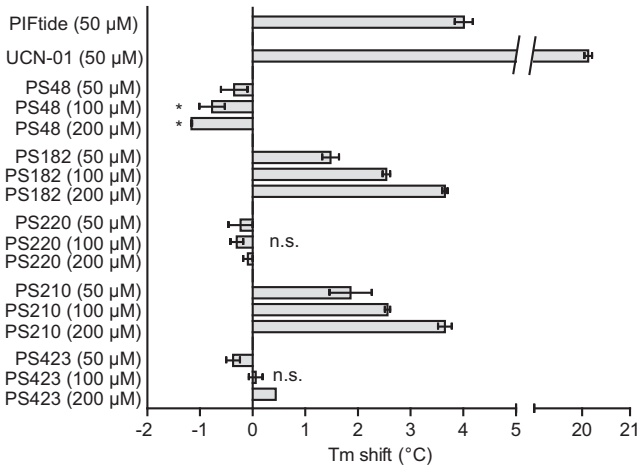

Figure 3. Increased Thermal Stability of PDK1 in the Presence of PS182 and PS210

The thermal stability of PDK1 in the presence of compounds was assessed by differential scanning fluorimetry (DSF) (see also Figure S2). For each condition, the midpoint temperature of transition, or T_m, of PDK1 was calculated. This value changes in the presence of an interacting compound ($\Delta T_m = T_m - T_0$). The data points reflect the averages of a duplicate experiment ± standard deviations. PS182 and PS210 stabilize PDK1 in a concentration-dependent fashion, whereas the di-ester prodrugs PS423 and PS220 show variations only around the standard deviation for T_0, which is 0.3°C (indicated by n.s., not significant). PS48, on the other hand, destabilizes PDK1, which can be concluded because the shifts at the higher concentrations were greater than two times the standard deviation for T_0 (indicated by the asterisk).

We show that PS182 and PS210, but not PS48, were able to fully displace the high-affinity interaction between PDK1 and PIFtide (Figure S1C). Together, mutagenesis analysis and the displacement of the interaction are consistent with the idea that PS182 and PS210 bind with high affinity to the PIF-binding pocket of PDK1.

PS182 and PS210, but Not PS48, Increased the Thermal Stability of PDK1

ATP-competitive compounds have been shown to stabilize different protein kinases up to 20°C in a temperature stability assay (Fedorov et al., 2007; Niesen et al., 2007). Similarly, the melting temperature of PDK1 shifts by 20°C in the presence of UCN-01, a derivative of staurosporine (Figure 3). In comparison to UCN-01, we observed a rather modest stabilization of PDK1 of up to 4°C for PS210 and PS182, which is in agreement with the stabilization induced by PIFtide and with previous findings for other allosteric ligands of PDK1 (Bobkova et al., 2010). The di-ester derivatives PS423 and PS220 did not affect the melting temperature of PDK1, which is in agreement with their lack of effect on PDK1 activity. Interestingly, PS48 did not stabilize full length PDK1 in this assay, but it prompted a small concentration-dependent loss of stability to the temperature gradient. Similarly, PS210 increased the stability of the isolated catalytic domain of PDK1 (residues 50–359) by 4°C, whereas PS48 had virtually no effect (Figure S2). The data indicate that the dicarboxylic compounds PS182 and PS210 stabilize PDK1 but that this stabilization is not required for activation because PS48 has almost no effect on the stability or destabilizes PDK1 to the temperature treatment.

The Crystal Structures of PS182 and PS210 Confirm Their Binding to the PIF-Binding Pocket of PDK1 and Provide Molecular Details on the Mechanism of Allosteric Activation

We previously crystallized and solved the structure of a truncated version of PDK1 that consists of the catalytic domain (residues 50–359) comprising two additional mutations (Y288G and Q292A) in complex with ATP and in complex with both ATP and PS48 (both at 1.9 Å resolution) (Hindie et al., 2009). We now present crystal structures of PDK1 in complex with ATP/PS182 (1.43 Å) and ATP/PS210 (1.68 Å) (Figure 4; Table S1). In agreement with the biochemical data and similarly to PS48, both compounds bind to the PIF-binding pocket in the small lobe of PDK1, with both rings occupying the two subpockets and one carboxyl group interacting with and stabilizing Lys76, Arg131, and Thr148 (Figures 4A–4C). The second carboxyl group from PS182 and PS210 forms an additional salt bridge with Arg131. Because this additional interaction is not present in PS48, this interaction is expected to be responsible for the higher affinity of PS182 and PS210 as compared with that of PS48.

Both dicarboxylate compounds induce a significant 1.0 Å downward motion of helix α-B toward the active site (Figures 4D–4F). However, only compound PS210 closes the structure of the catalytic domain of PDK1. PS210 causes a further shift of helix α-B perpendicular to the first movement and along the axis of the helix by 1.1 Å. This combined conformational shift is accompanied by an upward movement of helix α-C by 1 Å and, more importantly, by a rotational motion of the whole antiparallel β sheet that forms the core of the small lobe. Most notably, the conformational change that originates from compound PS210 allosterically binding to the PIF-binding pocket is relayed to the active site where the Gly-rich loop, which constitutes the upper part of the ATP-binding pocket, is displaced by approximately 1.4 Å (Figure 4G). This conformational change induces a very important relocation of the ATP phosphate groups (Figures 4H and 4I). In particular, the previously uncoordinated β-phosphate is rotated by approximately 120° and now interacts with residues Asp223, Lys111, and Ser94 of the Gly-rich loop. Thus, the PS210-bound structure of PDK1 is very similar to the PKA structure (PDB code 1ATP) in complex with ATP and the peptide inhibitor PKI, which is believed to represent the fully close-active conformation of PKA. Interestingly, in the ATP-binding pocket we now observe strong electron density for small molecules, two of which are not compatible with water molecules as a result of high coordination numbers and short binding distances (up to 2.0 Å), that may correspond to divalent cations. Because the X-ray absorption edge for Mg^{2+} is not within the range of synchrotron beamlines, we soaked a crystal of PDK1 with PS210 and substituted $MgCl_2$ by $MnCl_2$. After transfer of the phases from a high resolution data set, the resulting anomalous density map showed a very strong peak for one of the suspected metal ions and confirms this to be Mn^{2+} or Mg^{2+}. This ion tethers the β- and γ-phosphates of ATP to the Asp223 of the DFG motif. For the other metal ion, no anomalous density was observed, thus it is expected to be a metal with a lower order

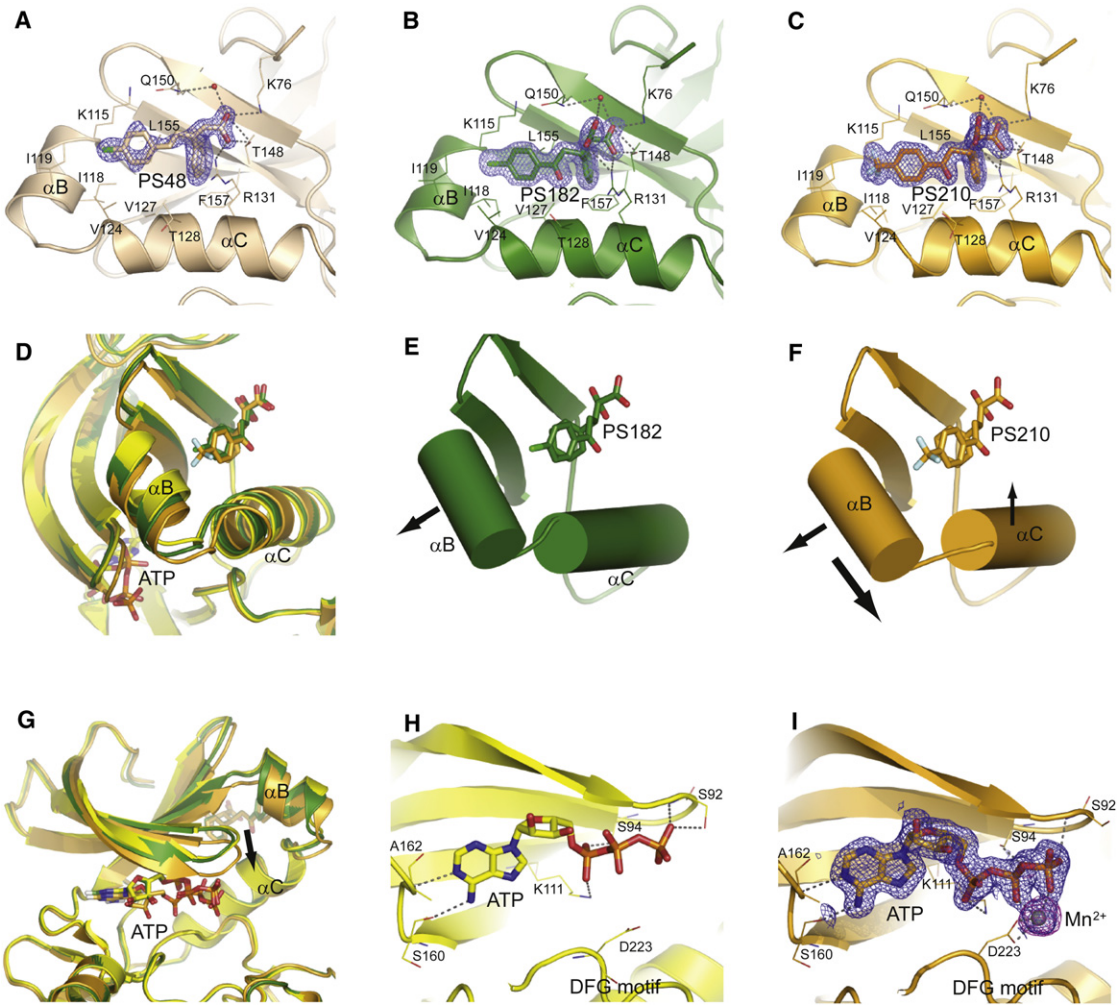

Figure 4. Crystal Structures of PDK1 Bound to PS182 and PS210

(A–C) Crystal structures of PDK1 in complex with allosteric compounds PS48 (PDB code 3HRF) (A), PS182 (B), and PS210 (C). PDK1 is shown in cartoon representation. Only residues contributing to the PIF-binding pocket are shown in stick representation and hydrogen bond interactions are indicated by gray dashed lines. $|2F_o\text{-}F_c|$ electron density of the compounds is indicated in blue and contoured at 1σ.

(D) Comparison of unbound PDK1 (yellow; PDB code 3HRC) with the structures containing PS182 (green) and PS210 (orange). For simplicity, ATP is only shown for the PS210 co-crystal structure.

(E and F) Both dicarboxylate compounds caused a downward motion of helix αB. But only PS210 caused an additional upward shift of helix αC and a rotational movement of large parts of the small lobe.

(G–I) This additional movement converted PDK1 to a close-active conformation by pushing down the glycine-rich loop of the ATP-binding pocket (G). In comparison to the unbound structure (PDB code 3HRC) (H), the ATP of the PS210 structure (I) changed its conformation. In particular, the β-phosphate rotated by approximately 120°. β- and γ-phosphate are now bound by a Mg^{2+} ion to Asp223 of the DFG motif. Mg^{2+} was replaced by Mn^{2+}. Anomalous electron density of the Mn^{2+} ion is shown in purple and contoured at 3σ.

See also Table S1.

number, most likely a Na^+ ion, which is present in high concentrations in the crystallization and soaking solutions.

PS210 Specifically Targets PDK1 In Vitro

To assess the selectivity of PS210 against kinases other than PDK1, a kinase profiling study was carried out. PS210 did not alter the activity of any of the 121 kinases tested in this panel, including PDK1 downstream signaling components such as S6K, PKB/Akt or GSK3 (Table S2).

Remarkably, PDK1 activity remained unchanged in the presence of PS210 in the outsourced profiling study, which employs PDKtide as a substrate (instead of T308tide used by us in our characterizations). PDKtide was designed as an improved substrate by joining PIFtide with T308tide into one polypeptide. The docking of the PIFtide sequence to the PIF-binding pocket of PDK1 resulted in PDKtide being a vastly improved substrate in comparison to T308tide (Biondi et al., 2000). However, PIFtide alone already has a high affinity and low OFF-rate for PDK1. Therefore, it may be difficult to displace the interaction of the 39-amino acid polypeptide PDKtide from PDK1 with small compounds. Indeed, in parallel experiments, we could verify that PS210 potently activates PDK1 using T308tide as a substrate,

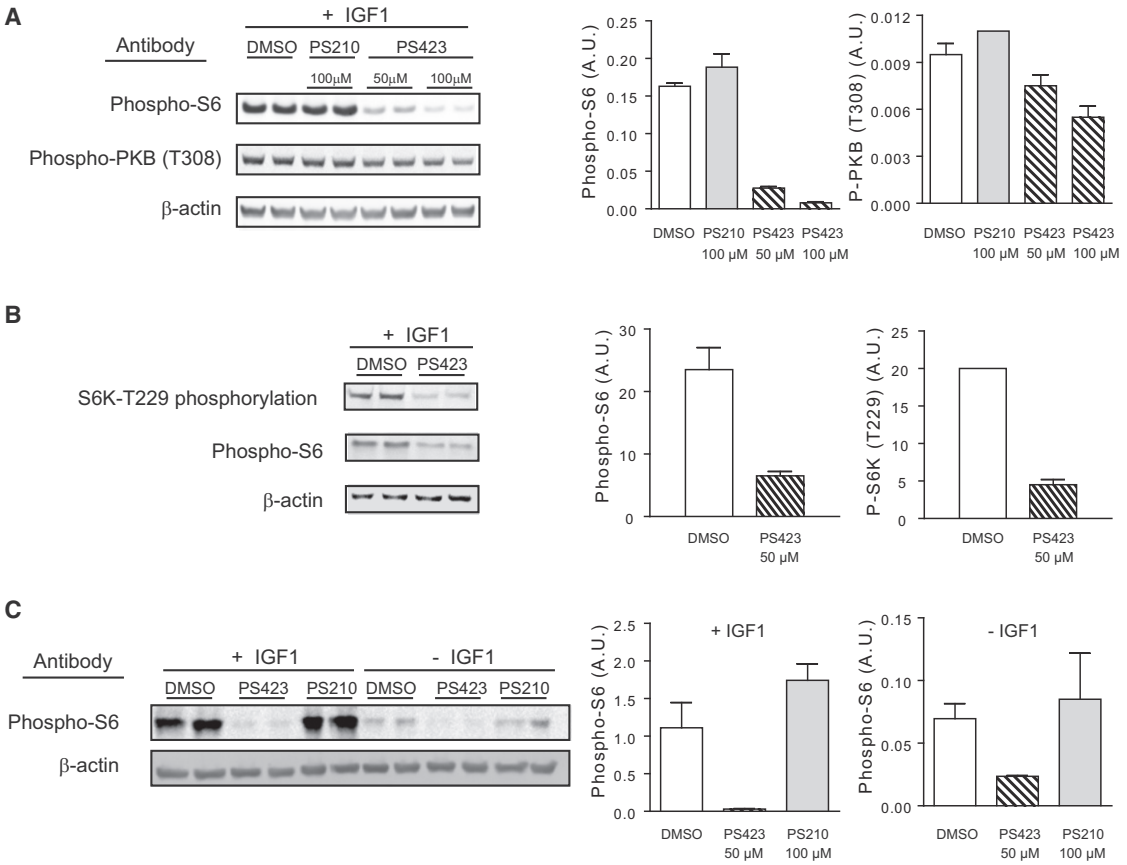

Figure 5. PS423 Inhibits S6K but Not PKB/Akt Downstream Signaling in HEK293 Cells

The immunoblots showing the effects of PS210 and its di-ester prodrug PS423 on the PDK1 signaling pathway in HEK293 cells (left panels) and the quantification of the signals by normalizing to the β-actin signal (right panels, averages of the duplicate samples ± standard deviations are shown).

(A) HEK293 cells were preincubated with 100 μM PS210 and 50 μM or 100 μM PS423 for 2 hr and stimulated with IGF1 20 min prior to lysis, and western blotting using antibodies against phosphorylated S6 and against the activation loop phosphorylation of PKB/Akt.

(B) GST-S6K[T412E] (containing a negatively charged residue in place of the HM phosphorylation site) was overexpressed in HEK293 cells to investigate the phosphorylation of its activation loop and of the substrate S6 after incubation with 50 μM PS423 and stimulation by IGF1.

(C) 50 μM PS423 inhibits the phosphorylation of S6 also without prior stimulation with IGF1, reflecting the effect of the compound on the basal activity.

See also Figure S3 and Table S2.

but does not affect the activity of PDK1 using PDKtide as a substrate (Figure S3). Therefore, we conclude that PS210 is a highly selective compound activator of PDK1 that does not affect the activity of the 120 other protein kinases tested.

The PS210 Prodrug Compound PS423 Inhibits S6K but Not PKB/Akt Downstream Signaling in HEK293 Cells and C2C12 Myoblast Cells

The above data indicated that PS182 and PS210 were potent activators that target the PIF-binding pocket of PDK1. To test the effect of blocking the PIF-binding pocket pharmacologically in cells, we employed PS220 and PS423, the di-ester derivatives of PS182 and PS210, respectively. PS220 was relatively toxic, resulting in the detachment of the HEK293 cells in culture. PS423 was cleaved intracellularly to render PS210 (A.S., L.A.L-G., K.B., Wolfgang Fröhner, Frauke Maurer, Stefan Boettcher, J.O.S., Hua Zhang, R.M.B., and M.E., unpublished data). Therefore, we used PS423 for subsequent experiments. In the first set of experiments, HEK293 cells were serum-starved overnight, incubated for 2 hr with PS423 (50 μM) or PS210 and stimulated with IGF1 for 20 min. The prodrug PS423 potently inhibited the phosphorylation of S6, a substrate of S6K (Figure 5A). At the same concentrations that resulted in 83% and 95% inhibition of S6 phosphorylation, we observed only a minor decrease in PKB/Akt activation loop phosphorylation. This result is in agreement with the previous findings using mutant PDK1 proteins that indicated that docking to the PIF-binding pocket is not necessary for the phosphorylation and activation of PKB/Akt by PDK1.

To correlate the decrease in the phosphorylation of S6 with a diminished phosphorylation of the S6K activation loop [T229] by PDK1, GST-S6K[T412E] (containing a negatively charged residue in place of the HM phosphorylation site) was overexpressed in HEK293 cells. After overnight serum starvation these cells were incubated with 50 μM PS423 for 4 hr, followed by stimulation with IGF1 20 min prior to cell lysis. PS423 produced a strong decrease in the activation loop phosphorylation of S6K, in agreement with the reduced S6 phosphorylation (Figure 5B). A similar reduction in S6 phosphorylation was obtained for cells

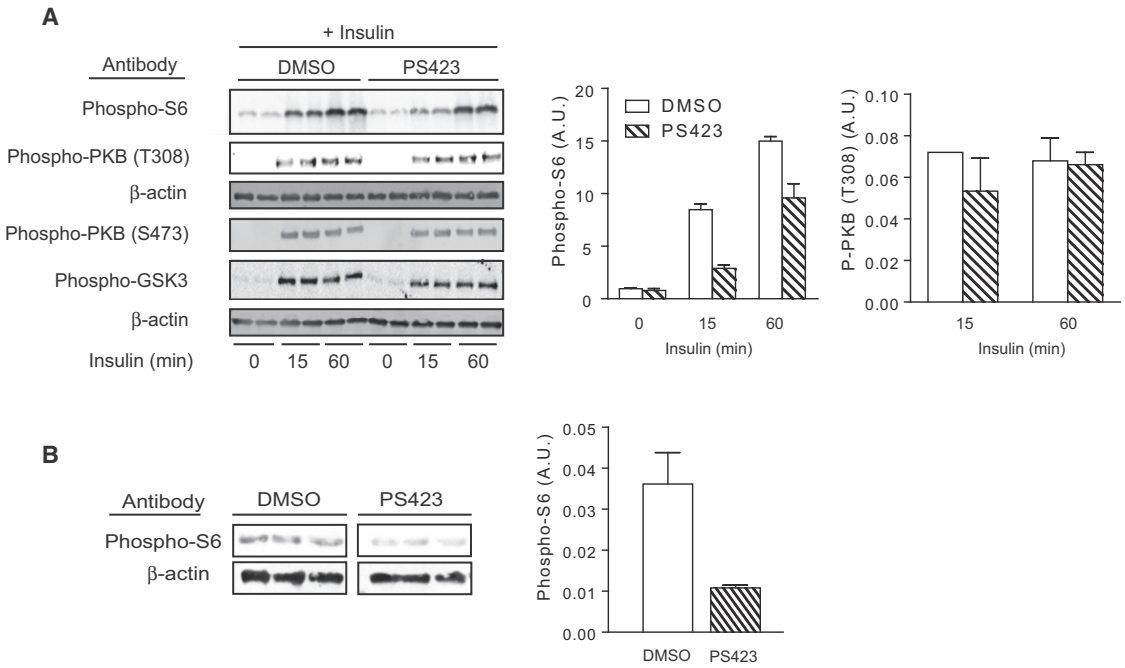

Figure 6. PS423 Inhibits S6K but Not PKB/Akt Downstream Signaling in Muscle Cells

Immunoblots showing the effects of the di-ester prodrug PS423 on the PDK1 signaling pathway in C2C12 myoblasts (left panels) and the quantification of the signals by normalizing to the β-actin signal (right panels, averages ± standard deviations are shown).

(A) C2C12 cells were preincubated with 25 μM PS423 for 2 hr, and then activation of the signaling pathway was achieved by the addition of insulin (incubation for 15 or 60 min). The phosphorylation statuses of S6, the activation loop of PKB/Akt, the hydrophobic motif of PKB/Akt and GSK3 were verified using the indicated antibodies. The quantification of the phospho-PKB(S473) and phospho-GSK3 signals is shown in Figure S4.

(B) To verify the effect of PS423 on the basal kinase activity, C2C12 cells were incubated for 4 hr with 25 μM PS423, followed by immunoblot analysis using the antibody against the phosphorylated S6.

that were not stimulated with IGF1, reflecting the basal turnover of S6 phosphorylation (Figure 5C). Altogether, these results suggest that PS423, the prodrug compound of PS210, inhibits S6K activity without affecting the PKB/Akt signaling pathway in HEK293 cells.

We then addressed the pharmacologic effects of PS423 in a physiologically more relevant system. To this end, we employed the C2C12 myoblast cell line and preincubated the cells with 25 μM PS423 for 2 hr. Activation of the PDK1 signaling pathway was achieved by incubating the cells for 15 or 60 min with insulin (Figure 6A). The phosphorylation of S6 was inhibited by PS423, whereas no significant reduction in PKB/Akt activation loop phosphorylation was detected. Furthermore, we observed no decrease in the phosphorylation of either the PKB/Akt HM S473 residue or, more downstream, GSK3, a substrate of PKB/Akt (Figures 6A and S4). Similar to the findings in HEK293 cells, a reduction (70%) was observed in the basal S6 phosphorylation after a longer preincubation (4 h) of the myoblast cells with the compound (Figure 6B), indicating that the S6 protein is actively phosphorylated and dephosphorylated in serum-starved cells. Thus, as previously observed in HEK293 cells, PS423 inhibited S6K activity but not PKB/Akt downstream signaling in C2C12 myoblast cells, confirming that PS423 can selectively inhibit the phosphorylation of PDK1 substrates. The results confirm the substrate-selective requirement of the PIF-binding pocket of PDK1 by using pharmacologic tools.

PS423 Differentially Inhibits Glucose Uptake in C2C12 Myoblasts but Not in C2C12 Myotubes

We next tested the effect of 25 μM PS423 on glucose uptake in C2C12 myotubes cells (Figure 7A). Insulin stimulated the glucose uptake by 45%. In agreement with the fact that PDK1 mediates PKB/Akt phosphorylation and activation, the non-selective PDK1 inhibitor UCN-01 completely blocked the insulin-stimulated glucose uptake. In addition, UCN-01 also decreased the basal levels of glucose uptake. In parallel, the insulin-mediated increase of glucose uptake was blocked to basal levels in the presence of rapamycin, a TORC1 inhibitor that blocks S6K activation, or in the presence of PS423. Thus, the results are consistent with the idea that, in C2C12 myotubes, both substrates of PDK1, S6K, and PKB/Akt, may be involved in the basal and insulin-stimulated glucose uptake.

C2C12 myoblast cells (C2C12 cells that were not differentiated to myotubes) also responded to insulin by increasing their glucose uptake (Figure 7B). However, in contrast to the results obtained using C2C12 myotubes, we unexpectedly found that PS423 completely inhibited the basal and insulin-stimulated glucose uptake in C2C12 myoblasts cells. As a control, the cell viability (MTT assay) eliminated any potential toxic effect by PS423 (Figure S5). A western blot analysis demonstrated that under these experimental conditions the phosphorylation of S6 was inhibited to 50% by PS423, whereas PKB/Akt phosphorylation was not affected by the treatment. Therefore, the results

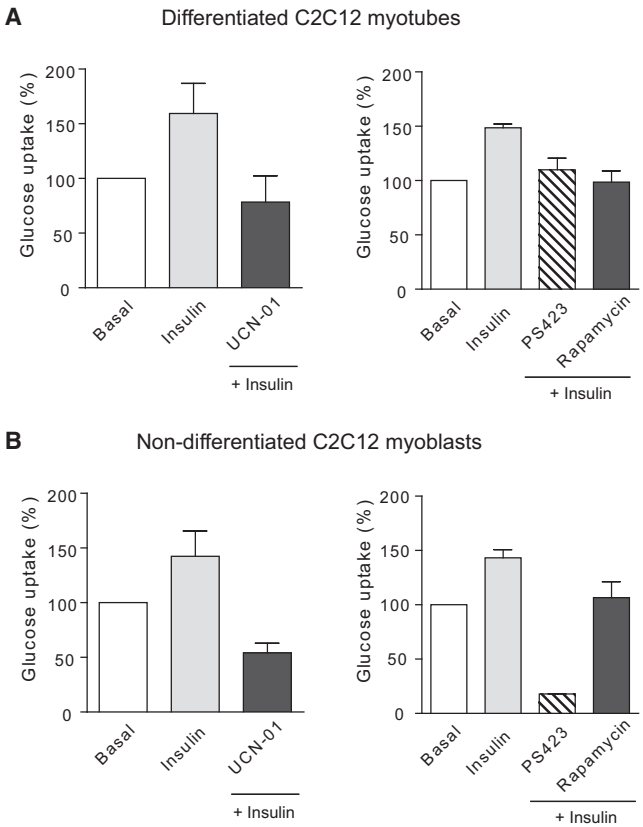

Figure 7. PS423 Inhibits Glucose Uptake in C2C12 Myoblasts but Not in C2C12 Differentiated Muscle Cells

Glucose uptake was measured following insulin stimulation in C2C12 cells that were differentiated to myotubes (A) or in undifferentiated myoblasts (B) and incubated with 25 μM UCN-01 (left panels) or 25 μM PS423 and 25 nM rapamycin (right panels). The basal activity before insulin stimulation was set to 100%. The data points represent the averages ± the standard deviations of duplicate (left panels) or triplicate (right panels) experiments. Related western blot analysis and MTT results are shown in Figure S5.

indicated that PKB/Akt is not involved in the basal or insulin-stimulated glucose uptake in C2C12 myoblasts. Interestingly, rapamycin completely inhibited S6 phosphorylation, but only partially blocked glucose uptake. Therefore, the results suggested that S6K and an additional protein kinase dependent on the PIF-binding pocket of PDK1 were responsible for the basal and insulin-stimulated glucose uptake in C2C12 myoblasts.

In conclusion, the use of PS423 revealed that different PDK1-dependent signaling kinases are involved in glucose uptake in C2C12 myoblasts and myotubes, one independent of the PIF-binding pocket of PDK1 (PKB/Akt), and at least two different kinases that are dependent for their activation on the docking to the PIF-binding pocket of PDK1.

DISCUSSION

Despite its potential importance, the rational design of small compounds to mimic the conformational changes physiologically induced by phosphorylation is hampered by the lack of structural data and by the challenge of rationalizing the development of compounds into pockets that dynamically change shape and transduce conformational changes in ways that are only vaguely understood. We described dicarboxylate allosteric activators of PDK1 that extensively mimic the interactions between the phosphate-binding site of PDK1 and its phophorylated HM and evaluated the properties of the new phosphate-mimic compounds in vitro and in cultured cells. Our results show that the major cellular effect of the binding of the compounds to the PIF-binding pocket of PDK1 is the blockage of the pocket, which is independent of whether the compound is an activator in vitro. The resulting compounds are substrate-selective inhibitors of PDK1 activity in cells and allowed us to distinguish different PDK1-dependent signaling pathways that differed in their requirement for the PIF-binding pocket of PDK1. Together, the development of small compounds that target the regulatory allosteric PIF-pocket of AGC kinases suggests that the site provides a source for the development of allosteric activators, allosteric inhibitors, and substrate-selective inhibitors with potential use in research and for the selective regulation of signaling pathways involved in human diseases. Future drug development programs could benefit from an analogous approach to develop compounds that mimic or antagonize phosphorylation-dependent conformational changes.

Phosphorylation can lead to the activation of enzymes by promoting stability or disorder within the molecule. Our data suggest that the overall neutral or loss of thermal stability in the presence of PS48 is due to a proportionally similar loss of stability of residues from the α-B and α-C helices relative to the stabilization of the phosphate binding site residues. Altogether, neither the overall stabilization of the protein nor the stability of the α-B and α-C helices appeared to be required for compounds to activate PDK1. The finding that activators do not necessarily increase the overall thermal stability of the target may impact on drug discovery approaches to similar allosteric sites, most notably, by careful re-evaluation of thermal stability filtering steps along the process of prioritization of compounds. However, even when they induced a stabilization of PDK1 to the temperature gradient (PS182 and PS210) or its destabilization (PS48), all three activators stabilized the residues Thr148, Arg131, and Lys76, located opposite to the helix α-B at the other extreme of the helix α-C. Thus, the residues forming part of the phosphate-binding site appear to be a fixed point that allows for the relative movement of the helices in the process of PDK1 activation.

Previous hydrogen-deuterium exchange experiments indicated that the helix α-C, within the PIF-binding pocket, is very disordered in solution (Hindie et al., 2009). The initial binding of the small compound activator could take place essentially by conformational sensing of the appropriate conformation of the PIF-binding pocket of PDK1. Alternatively, initial binding could be triggered by interaction of the carboxylates with positively charged residues and thereafter the compound could accommodate into the pocket by induced-fit mechanisms. Independently of the mechanism of initial binding, it is tempting to speculate that the fixing of residues at the phosphate-binding site at one extreme of the helix α-C, together with additional specific interactions of the compound with the helix α-B, would limit the possible movements of the helices α-B and α-C and

allosterically support the stabilization of the active conformation at the ATP-binding site by induced-fit mechanisms.

The downward movement of the helix α-B and its effect on the Gly-rich loop was the key factor that prompted the allosteric conformational change that stabilized the active site of PDK1 in the presence of PS210. The downward movement of the Gly-rich loop excluded water molecules from the ATP-binding site and allowed us to visualize one divalent cation tethering the β- and γ-phosphates of ATP to Asp223 of the DFG motif, which is similar to the close-active conformation of PKA. PDK1 has so far been crystallized in at least five crystal packings (Biondi et al., 2002; Hindie et al., 2009). Interestingly, in all previous cases, the overall relative position of the small and large lobe was ''intermediate,'' suggesting that this intermediate may be the most stable conformation of the catalytic domain of PDK1 in solution as well as in crystal form. The interaction of PDK1 with the phosphorylated HM of substrates could, similarly to PS210, fix residues at the phosphate-binding site and facilitate the downward movement of the Gly-rich loop.

Previous experiments with PDK1 proteins that were mutated at the PIF-binding pocket (e.g., Leu155Glu) showed that the pocket was required for the docking, phosphorylation and activation of S6K but not PKB/Akt in vitro (Biondi et al., 2001) and in vivo (Bayascas et al., 2006; Collins et al., 2003, 2005). Under constitutive activation, S6K mediates an insulin signaling negative feedback loop in muscle, the previous work supported the possibility of sensitizing muscle. We now show that small compounds that bind to the PIF-binding pocket, even if they are potent activators in vitro, selectively inhibit the phosphorylation and activation of S6K. This is in agreement with the previous finding that the PDK1 Leu155Glu mutant, that has higher specific activity than the wild-type protein (Biondi et al., 2000), does not increase the ability of PDK1 to activate PKB/Akt in vitro (Biondi et al., 2001) or in vivo (Collins et al., 2003). It is possible that the kinetics of the PKB/Akt- PIP_3- PDK1 interaction and colocalization are responsible for the absence of an activating effect of PDK1 Leu155Glu and PS210 on PIP_3-dependent phosphorylation of PKB/Akt. The di-ester derivative PS423 was an elegant tool for probing a cellular system pharmacologically because this molecule readily permeated cells and, once inside, was cleaved to produce the active drug PS210. The treatment of HEK293 and myoblast cells with PS423 inhibited S6K signaling without affecting PKB/Akt phosphorylation and downstream effects. The selectivity of the PS210 compound and the observation of its docking to the PIF-binding pocket of PDK1 indicated that the intracellular blockage of the PIF-binding pocket triggered the inhibition of S6K signaling. Thus, to our knowledge, this is the first pharmacological evidence that blocking the PIF-binding pocket with a small compound can lead to the selective inhibition of S6K without affecting PKB/Akt. The selective inhibition of S6K is expected to inhibit the negative feedback loop present in insulin-resistant cells and sensitize them to PKB/Akt-dependent insulin actions. A context-dependent ATP-competitive PDK1 inhibitor that inhibits weakly PKB/Akt was previously described (Najafov et al., 2011). It is possible that such compound binds to the most closed-active form of PDK1, which may be stabilized by the docking interaction with the PIF-pocket by most substrates but not be stabilized by PKB. Interestingly, PS423 also allowed us to identify different PDK1 downstream effectors that transduce glucose uptake in response to insulin in C2C12 myoblasts and in C2C12 myotubes. Thus, PS423 and future compounds blocking the PIF-binding pocket of PDK1 may block insulin-stimulated glucose uptake in nondifferentiated muscle cells. In one knock-in approach where PDK1 Leu155Glu was heterozygously expressed in mice ($PDK1^{fl/155E}MckCre^{+/-}$), an increase in PKB/Akt phosphorylation in response to insulin stimulation was observed (Bayascas et al., 2006), suggesting that blocking the PIF-pocket could sensitize organisms to insulin action and lead to drugs for the treatment of insulin resistance and type 2 diabetes. However, a second PDK1 Leu155Glu knock-in approach did not reveal the same phenotype (Bayascas et al., 2006). The knock-in technique has the drawback of often generating compensatory mechanisms that mask the actual effect of the mutation while conditional knock-in can be incomplete or restricted to a subset of cells in the organism and do not mimic the systemic action of small molecule drugs. Therefore, the validation of the PIF-binding pocket as a drug target will best be assessed by acute pharmacologic treatment with compounds binding to the pocket of PDK1. The PDK1 substrates S6K, RSK and SGK that require docking to the PIF-binding pocket of PDK1, are also involved in cancer progression (Pearce et al., 2010). Therefore, future compounds blocking the pocket could also have potential use for the treatment of diverse cancers.

Targeting allosteric sites in protein kinases with small molecules is an emerging research area because it could overcome drug resistance and lack-of-selectivity issues that are often associated with the more classical ATP-competitive drugs. Moreover, the targeting of truly allosteric sites, i.e., regulatory sites, on protein kinases introduces the possibility both to activate and to inhibit a particular kinase. In support of this possibility, we recently showed that a compound binding to the PIF-pocket could transduce the conformational changes that lead to the allosteric inhibition of an AGC kinase, the atypical PKCζ (Fröhner et al., 2011; Lopez-Garcia et al., 2011). Thus, the PIF-pocket of AGC kinases may be considered a site for the development of agonists and antagonists of a conformational change, acting like a pharmacologic switch that can turn enzymes ON or OFF. Notably, the data presented in the present work confirm a third potential use of allosteric compounds: the development of substrate-selective inhibitors. Since protein kinases can function in different signaling pathways and can also generate negative feedback loops, substrate-selective inhibitors to protein kinases have immense potential for drug discovery. In one example, targeting the differences in the mechanism of substrate recognition of GSK3 was postulated as an approach to obtain the desired effects on neurological disorders or diabetes without affecting the Wnt-signaling substrates and therefore avoiding the risk that the total inhibition of GSK3 would support the development of colon cancers (Frame et al., 2001).

The general model for the mechanism of activation of AGC kinases by HM phosphorylation involves the intramolecular interaction of the HM with the PIF-pocket of its own kinase domain. By binding to the PIF-binding pocket of PDK1, the compounds mimicking the conformational change also block the interaction of PDK1 with its substrate S6K. To our knowledge, the work on the PIF-binding pocket of PDK1 provides the first example

describing the molecular requirements for mimicking phosphorylation-dependent conformational changes in protein kinases with small compounds and has led to the first rationally designed substrate-selective inhibitor. Importantly, this shows that the cellular substrate specificity of protein kinases cannot only be explained by the standard "recognition motifs" located in short segments of the primary sequence around the phosphorylation site of substrates (Biondi and Nebreda, 2003). Therefore, the existence of alternative mechanisms for substrate recognition, such as docking interactions, allows the possibility of developing substrate-selective inhibitors targeting a broader range of protein kinases.

SIGNIFICANCE

Although protein phosphorylation is widely present in human cells to transduce cellular signaling in both normal physiology and disease, the phosphorylation-dependent conformational changes induced in proteins are vastly unknown. Also, the molecular mechanisms by which protein kinases interact with substrates have not been broadly investigated. Consequently, phosphorylation-dependent conformational changes or substrate-selective inhibitors of protein kinases are not considered as possible avenues for the pharmacologic treatment of diseases. The understanding of the mechanisms by which small compounds mimic phosphorylation-dependent conformational changes in target proteins, or the mechanism of protein kinase interaction with substrates, can potentially expand the druggable genome to include new signaling molecules that regulate substrate phosphorylation and phosphorylation-dependent conformational changes. A characteristic of drugs that target regulatory sites is that they can trigger a different set of conformational changes in target proteins, acting as activators or inhibitors of enzymatic activities, or, as here described, as substrate-selective inhibitors. Indeed, we show that the binding of a small molecule to the PIF-pocket regulatory site of PDK1, besides activating the kinase in vitro, affects the mechanism by which PDK1 interacts with its protein substrates and, in cells, acts as a substrate-selective inhibitor of PDK1. The rational design of the substrate-selective inhibitor was based on the deep knowledge of the molecular mechanisms operating on PDK1. Hence, it is possible to envisage that the better understanding of the mechanisms that determine the interaction with substrates could lead to the development of substrate-selective inhibitors of other protein kinases. The present work also completes the picture of the discovery of a phosphorylation-dependent regulatory site with the molecular details showing the activation process mimicked with small molecules. The work could inspire the development of compounds to modulate the activity of other AGC kinases and, more generally, of enzymes regulated by phosphorylation.

EXPERIMENTAL PROCEDURES

The materials and reagents used, the synthesis of small compounds, the expression and purification of proteins, and the crystallization and structure determination are described in the Supplemental Experimental Procedures.

Protein Kinase Activity Assay

The protein kinase activity assays were performed essentially as previously described (Engel et al., 2006). The assays were performed in a 96-well format, and 4 μl aliquots were spotted on P81 phosphocellulose papers (Whatman) using an epMotion 5070 (Eppendorf). The papers were washed in 0.01% phosphoric acid, dried, and then exposed and analyzed using PhosphoImager technology (FLA-9000 Starion, Fujifilm). PDK1 activity assays were performed in a total volume of 20 μl containing 50 mM Tris-HCl (pH 7.4), 0.05 mg/ml BSA, 0.1% (v/v) β-mercaptoethanol, 10 mM $MgCl_2$, 100 μM [γ^{32}P]ATP (5–50 cpm/pmol), 0.003% Brij, 300-600 ng PDK1 and 200 μM T308tide (KTFCGTPEYLA PEVRR) as the substrate. After a 15 min preincubation, the kinase reaction was initiated by the addition of the ATP-Mg mixture. To compare the T308tide and PDKtide substrates, Brij was omitted from the reaction buffer and 100 μM concentration of PDKtide (KTFCGTPEYLAPEVRREPRILSEEE QEMFRDFDYIADWC) and 7 ng PDK1 were used.

The activity assays were performed in duplicate with less than a 10% difference observed between the duplicate pairs. The activity assays whose results are shown were repeated at least twice with similar results. There was variation in the maximal activation of the compounds between different assays. The outsourced kinase profiling study was performed by the International Centre for Kinase Profiling (MRC Protein Phosphorylation Unit, University of Dundee, Scotland).

Differential Scanning Fluorimetry

Protein unfolding was monitored by the increase in the fluorescence of the fluorophor SYPRO Orange (Invitrogen) using a real-time PCR device (StepOnePlus, Applied Biosystems) following the protocol described by Niesen et al. (Niesen et al., 2007). PDK1 was diluted in 10 mM HEPES (pH 7.5) buffer containing 150 mM NaCl. The reactions were performed in a final volume of 10 μl in 96-well PCR microtiter plates (Greiner) and contained 1 μM PDK1, 10 mM HEPES (pH 7.5), 150 mM NaCl, 1/1000 SYPRO Orange, and 1 mM dithiothreitol. PIFtide or compounds (1% final DMSO concentration) were added to this reaction mixture. The temperature gradient was performed in steps of 0.3°C in the range of 25°C to 70°C. To calculate the T_m values, the data were exported to GraphPad Prism and the curves fitted to a Boltzmann sigmoidal equation with all $R^2 > 0.998$.

Cell-Based Experiments

HEK293 cells were grown in 6-well culture dishes, serum starved overnight and incubated for 2 hr with the compounds. All compound dilutions were added as duplicates or triplicates. Cells treated with 0.2% DMSO served as the controls. The cells were stimulated 20 min prior to lysis with 10 ng/ml IGF1. The lysis was performed on ice in a buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 1% (w/v) Triton X-100, 1 mM sodium orthovanadate, 50 μM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% β-mercaptoethanol, and one tablet of protease inhibitor cocktail (Roche) per 50 ml of buffer.

C2C12 cells were preincubated for 2 or 4 hr with PS423 or DMSO. The activation of the PDK1 signaling pathway was achieved by incubating the cells for 15 or 60 min with 100 nM insulin.

For the western blot analysis, we used antibodies against phospho-S6 (Ser235/236 Cell Signaling), phospho-S6K [T229] (Novus Biologicals), phospho-PKB [T308] (Cell Signaling), phospho-PKB [S437] (Cell Signaling), and phospho-GSK3 (Cell Signaling). The IgG IRDye800CW secondary antibodies (anti-mouse and anti-rabbit) were obtained from Li-Cor, and the Cy5-conjugated IgG antibodies (anti-mouse and anti-rabbit) were obtained from Invitrogen. The fluorescence was detected on a Fuji FLA9000, and quantification was performed using a Multi Gauge software (Fuji Film).

2-Deoxyglucose Uptake Assay

On the first day, 8×10^4 C2C12 cells were seeded on 24-wells plates (Falcon) in growth medium and, on the second day, switched to DMEM with 2% horse serum and 1% penicillin/streptomycin to induce differentiation. The differentiation medium was replaced every 24 hr, and cells were used after 6 days. After serum starvation for 3 hr, C2C12 myoblast and myotubes were washed once with PBS and then incubated with DMSO, 25 μM PS423, 25 nM rapamycin, and 25 μM UCN-01 for 1 hr at 37°C. Subsequently, cells were stimulated with 100 nM insulin for 30 min at 37°C in medium without serum, and glucose

transport was determined by the addition of a glucose mix composed of 0.1 mM 2-deoxy-glucose/1 μCi 2-deoxy-[3H] glucose in Krebs-Ringer phosphate-HEPES buffer (KRPH buffer: 25 mM HEPES [pH 7.4], 1.3 mM $MgSO_4$, 1.3 mM $CaCl_2$, 118 mM NaCl, 4.8 mM KCl, 5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 0.007% BSA). After a 4 min incubation, the reactions were stopped by the addition of ice-cold PBS.

The cells were lysed using scintillation liquid (Insta-Gel Plus from Packard), and the glucose uptake was assessed by scintillation counting using a 1450 Microbeta TRILUX reader (Wallac). Cytochalasin B (20 μM) was used to measure the nonspecific background because this drug blocks glucose uptake. The results of the specific uptake were calculated by subtracting the background from the total uptake.

ACCESSION NUMBERS

The RCSB Protein Data Bank (PDB) accession numbers for the crystal structures of the human PDK1 kinase domain in complex with compounds PS182 and PS210 reported in this paper are 4AW0 and 4AW1, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2012.07.017.

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