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Molecular basis of cyclin-CDK-CKI regulation by reversible binding of an inositol pyrophosphate

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Abstract

When Saccharomyces cerevisiae cells are starved of inorganic phosphate, the Pho80-Pho85 cyclin–cyclin-dependent kinase (CDK) is inactivated by the Pho81 CDK inhibitor (CKI). The regulation of Pho80-Pho85 is distinct from previously characterized mechanisms of CDK regulation: the Pho81 CKI is constitutively associated with Pho80-Pho85, and a small-molecule ligand, inositol heptakisphosphate (IP_7), is required for kinase inactivation. We investigated the molecular basis of the IP_7- and Pho81-dependent Pho80-Pho85 inactivation using electrophoretic mobility shift assays, enzyme kinetics and fluorescence spectroscopy. We found that IP_7 interacts noncovalently with Pho80-Pho85-Pho81 and induces additional interactions between Pho81 and Pho80-Pho85 that prevent substrates from accessing the kinase active site. Using synthetic peptides corresponding to Pho81, we define regions of Pho81 responsible for constitutive Pho80-Pho85 binding and IP_7-regulated interaction and inhibition. These findings expand our understanding of the mechanisms of cyclin-CDK regulation and of the biochemical mechanisms of IP_7 action.

In conditions of nutrient limitation, cells maintain homeostasis by altering their metabolomic, proteomic and genomic profiles[1,2]. Many nutrient-starvation response systems have been intensively studied in *S. cerevisiae*[2], and the cellular response to inorganic phosphate (P_i) starvation (the PHO system) is one of the most well characterized[3].

When yeast cells are starved of P_i, the Pho80-Pho85-Pho81 cyclin-CDK-CKI system is inactivated[4], which leads to the dephosphorylation[5] and nuclear localization of the transcription factor Pho4 (refs. 6,[7]) and subsequent transcription of PHO genes[8]. Previous studies of Pho80-Pho85-Pho81 regulation have indicated that Pho81, the CKI, is constitutively associated with the cyclin-CDK (ref. 4), but CDK activity is only inhibited in P_i starvation conditions[4]. For other CKIs, CDK-CKI protein-protein interactions are sufficient to inactivate the CDK (ref. 9). Because Pho81 is constitutively associated with Pho80-Pho85, we hypothesized that there might be an additional factor or modification that is required for P_i starvation–induced Pho80-Pho85 inactivation.

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AUTHOR CONTRIBUTIONS

Y.-S.L. and E.K.O. designed experiments, Y.-S.L. carried out experiments, Y.-S.L. and E.K.O. interpreted data and wrote the manuscript. K.H. and F.A.Q. designed Pho81 peptides and carried out preliminary experiments to study Pho80-Pho85 binding.

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To identify the additional factor required for the regulation of cyclin-CDK, we biochemically isolated a Pho81-dependent Pho80-Pho85 inhibitor from P_i-starved cell extract and identified it as an isomer of IP_7—either 1, 2 or 3 (Fig. 1a)[10], which are all *myo*-D-inositol (4; Fig. 1a) derivatives bearing one pyrophosphate and five phosphates. IP_7 is found in all eukaryotic cells[11] and is produced through the action of a conserved set of inositol multiphosphate kinases from the precursor *myo*-D-inositol 1,4,5-trisphosphate (5; Fig. 1a)[11,12], which can be sequentially phosphorylated to generate inositol poly-phosphates such as *myo*-inositol 1,2,3,4,5,6-hexakisphosphate (IP_6, 6)[11]. Depending on the location of these pyrophosphates, several different IP_7 isomers are possible[11,12]; at least two distinct isomers of IP_7—5-IP_7 (1) and 4-IP_7 or 6-IP_7 (4/6-IP_7; 2 or 3, respectively)—are found in natural systems (Fig. 1a)[12–14].

Studies of loss-of-function mutants have revealed that IP_7 is involved in diverse cellular processes including recombination[15], vacuole function[16], gene expression, endo- and exocytosis[17,18], osmotic stress[16], telomere length control[19,20], nutrient sensing and kinase regulation[10,14]. However, for many of these processes the identities of relevant IP_7 targets are unknown. In one of the best studied examples, 5-IP_7 regulates *Dictyostelium discoideum* chemotaxis by competing with phosphatidylinositol phosphates (PIPs) for binding to pleckstrin homology domains[21]. Additionally, 5-IP_7 and 4/6-IP_7 can nonenzy-matically pyrophosphorylate certain yeast and mammalian proteins *in vitro*[22,23].

In *S. cerevisiae*, there are two enzymes that are known to synthesize IP_7 from IP_6 and ATP (Fig. 1a). The first of these kinases (which are generally known as IP6Ks) identified in yeast, based on its homology to mammalian IP6Ks, was Kcs1 (ref. 24). Kcs1 is thought to produce 5-IP_7 (Fig. 1a)[13]. More recently, another IP6K in yeast was described[14]—Vip1, which synthesizes a distinct isomer of IP_7 tentatively assigned to have a pyrophosphate moiety at its 4 or 6 position (Fig. 1a) and whose absolute configuration is still undefined[14]. We demonstrated that 4/6-IP_7 directly inactivates recombinant Pho80-Pho85 *in vitro* in a manner dependent on the Pho81 minimum domain[25] (a fragment consisting of amino acids 644–723 of Pho81 that is sufficient for the P_i starvation–dependent Pho80-Pho85 regulation *in vivo*)[10], which demonstrates that the inactivation of Pho80-Pho85-Pho81 by 4/6-IP_7 is direct.

This result establishes Pho80-Pho85-Pho81 as a cyclin-CDK-CKI system regulated directly by 4/6-IP_7. Because there are no known examples of CDK regulation by a natural small-molecule ligand, we sought to further characterize the molecular basis of this inactivation mechanism. This inactivation of cyclin-CDK-CKI by 4/6-IP_7 is also noteworthy because it is a rare example in which the molecular targets of IP_7 are known and can therefore be studied mechanistically.

In this paper, we demonstrate that Pho80-Pho85-Pho81 is reversibly regulated by 4/6-IP_7. This reversible regulation of the cyclin-CDK-CKI is distinct from any of the previously known IP_7 functions—competition with PIPs (ref. 21) and covalent modification of proteins[22,23]. Based on further biochemical analysis, we propose a molecular mechanism for Pho80-Pho85 cyclin-CDK inactivation by the concerted action of 4/6-IP_7 and the CKI Pho81.

RESULTS

Reversible regulation of Pho80-Pho85-Pho81 by IP_7

Because there is precedent that 5-IP_7 and 4/6-IP_7 can pyrophosphorylate certain yeast and mammalian proteins *in vitro*[23], we first investigated whether Pho80-Pho85-Pho81 is covalently regulated by 4/6-IP_7 (Fig. 1a). We incubated recombinant Pho80-Pho85 bound to recombinant Pho81 minimum domain[25] (Pho81-MD) with 4/6-[β-^{32}P]IP_7 and analyzed the resulting samples by SDS-PAGE (Fig. 1b). We found no evidence supporting protein

phosphorylation. We next determined whether prolonged incubation alters the half-maximal inhibitory concentration (IC_{50}) of 4/6-IP_7–mediated Pho80-Pho85-Pho81-MD inactivation (Fig. 1c). In typical irreversible protein inhibition, prolonged incubation with inhibitor decreases the apparent IC_{50} (ref. 26). However, we observed little change in the IC_{50} value with different incubation times (IC_{50} calculated to be 23 ± 6, 14 ± 8, 31 ± 14 and 22 ± 9 μM for 10-, 30-, 60- and 120-min incubation, respectively), which implies that the inhibition is at equilibrium and reversible. Finally, in typical irreversible inhibition, the IC_{50} decreases with increasing enzyme concentration because the bimolecular reaction rate is faster at higher enzyme concentrations[26]. Changing the Pho80-Pho85 concentration over two orders of magnitude did not affect the IC_{50} value (Fig. 1d), which further supports the hypothesis that 4/6-IP_7 regulates Pho80-Pho85-Pho81 by a reversible mechanism.

Characterization of IP_7 binding to Pho80-Pho85-Pho81

To gain more information on the mechanism of inhibition, we characterized the binding of 4/6-IP_7 to the kinase complex. We used an electrophoretic mobility shift assay (EMSA) to test which components of the Pho80-Pho85-Pho81-MD complex are required for binding (Fig. 2a). Although Pho81-MD is required for inactivation of Pho80-Pho85 triggered by 4/6-IP_7 (ref. 10), it is unclear whether Pho81-MD is required for binding of 4/6-IP_7 to Pho80-Pho85, or only for the inactivation step. In the EMSA experiment, a protein-dependent electrophoretic mobility shift of the 4/6-[β-^{32}P]-IP_7 was observed only in the presence of Pho80-Pho85, Pho81-MD and divalent metal cations (Fig. 2a). Among several divalent metal cations tested, Mg^{2+} and Mn^{2+} both supported efficient binding. Zn^{2+} also supported binding but Ca^{2+} did not; this metal preference is distinct from that for the IP_7-mediated protein pyrophosphorylation reaction[22,23] (Fig. 2a). All subsequent binding experiments were carried out in the presence of $MgCl_2$. When higher concentrations of Pho80-Pho85-Pho81-MD (μ10 μM) were used, protein–4/6-IP_7 complexes did not enter the gel. Subsequent binding analysis was therefore carried out with 8 μM Pho80-Pho85-Pho81. In these conditions, 22 ± 2% (average ± s.d., n = 50) of the 4/6-IP_7 was bound, as evidenced by a mobility shift. We also varied the amount of 4/6-IP_7 at a constant Pho80-Pho85-Pho81-MD concentration (Supplementary Fig. 1 online). In this experiment, the apparent dissociation constant (K_d^{app}) was 20.3 μM (Hill coefficient 0.94). These observations suggest that 4/6-IP_7 interacts with Pho80-Pho85-Pho81 in a 1:1 complex in a manner dependent on the presence of selected divalent cations and all three proteins.

We explored the specificity of binding of different isomers of IP_7 (Fig. 2b,c). Previously we reported that 4/6-IP_7 regulates Pho80-Pho85 in a Pho81-dependent manner, but 5-IP_7 (Fig. 1a) does not significantly affect kinase activity[10]. We investigated whether this discrimination of the IP_7 isomers in the inhibition assay is due to a difference in the specificity of binding or an inability of 5-IP_7 to induce conformational changes in Pho80-Pho85-Pho81-MD that are necessary for inhibition. We assayed the binding of 5-[β-^{32}P]-IP_7 to Pho80-Pho85-Pho81-MD using EMSA (Fig. 2b) and observed no detectable mobility shift, which indicates that Pho80-Pho85-Pho81-MD binds selectively to 4/6-IP_7.

The concentration of IP_6 in eukaryotic cells is estimated to be up to several hundred micromolar[11], making it far more abundant than 5-IP_7 or 4/6-IP_7, whose concentration is a few micromolar or less at resting conditions[27]. Therefore, for 4/6-IP_7 binding to Pho80-Pho85-Pho81-MD to be physiologically meaningful, Pho80-Pho85-Pho81-MD should be able to bind to 4/6-IP_7 even in the presence of excess IP_6. Indeed, when the binding of isotope-labeled 4/6-IP_7 (0.2 μM) to Pho80-Pho85-Pho81-MD (8 μM each) was competed with inositol derivatives (400 μM), only the unlabeled 4/6-IP_7 showed clear competition (Fig. 2c). This result indicates that the 4/6-IP_7 is a specific ligand for Pho80-Pho85-Pho81-MD, and the presence of cellular levels of 5-IP_7 or IP_6 do not interfere with binding.

Next, we investigated whether the interaction between 4/6-IP_7 and Pho80-Pho85-Pho81-MD observed in the EMSA results from reversible binding or from a covalent protein modification (Fig. 2d). To test whether binding requires the folded structure of Pho80, Pho85 and Pho81-MD, the Pho80-Pho85-Pho81-MD-4/6-IP_7 quaternary complex was boiled at 90 °C for 1 min (Fig. 2d) and then analyzed by native gel electrophoresis. No detectable electrophoretic shift of 4/6-IP_7 was observed after boiling, which suggests that the interaction of 4/6-IP_7 with the complex is reversible. Further, when the pre-formed Pho80-Pho85-Pho81-MD-4/6-IP_7 complex was treated with EDTA (Fig. 2d), 4/6-IP_7 dissociated from the complex, thereby providing further support for the reversible nature of the binding.

Because we observed a reversible interaction of 4/6-IP_7 with the kinase complex, we sought to determine how Pho80-Pho85-Pho81 can be purified in an inactive form from P_i-starved cells. Our previous studies[4,10] demonstrated that Pho80-Pho85-Pho81 can be immunopurified from P_i-starved cells in a kinase inactive form, which indicates that the inactive form is stable enough to survive the immunopurification steps. However, the enzyme inhibition and 4/6-IP_7 binding data (Figs. 1 and 2) indicate that the inhibition is reversible with an IC_{50} value in the micromolar range, which suggests that the half-life of 4/6-IP_7 bound to the cyclin-CDK-CKI complex would not be long enough to remain bound during the immunopurification steps. One possibility is that 4/6-IP_7 dissociation from Pho80-Pho85-Pho81-MD is slow enough at low temperature (conditions of the purification) for the complex to survive immunopurification. To test this possibility, the rate of dissociation of 4/6-IP_7 was measured by diluting pre-formed 4/6-IP_7–protein complex with excess unlabeled 4/6-IP_7 equilibrated at 4 or 30 °C and analyzing complex formation by EMSA (Fig. 2e). The disassembly of the 4/6-IP_7–protein complex, measured by the loss of the electrophoretic mobility shift, was indeed temperature dependent: at 30 °C, the dilution of the complex with unlabeled 4/6-IP_7 caused rapid dissociation; at 4 °C, we observed little disassembly of the pre-formed 4/6-IP_7–protein complex. We also measured the association rate by incubating ^{32}P-labeled 4/6-IP_7 and proteins at 30 °C followed by quenching the association with excess unlabeled 4/6-IP_7 at 4 °C (Fig. 2f). The association rate was also slower at lower temperature. These results indicate that there is a temperature-sensitive step for the association and dissociation of 4/6-IP_7, which may explain why we could immunopurify inactive Pho80-Pho85-Pho81 complex from P_i-starved cells.

Effects of IP_7 on the kinase-substrate interaction

To obtain a better understanding of the roles that 4/6-IP_7 and Pho81 have in Pho80-Pho85 regulation, we examined the effect of 4/6-IP_7 on the kinetics of phosphorylation of Pho4 by Pho80-Pho85-Pho81 (Fig. 3). Addition of Pho81-MD up to ~ 1 μM in the absence of 4/6-IP_7 did not affect the kinase reaction (Fig. 3a). When kinase assays were carried out in the presence of different amounts of ATP, Pho4 and/or 4/6-IP_7, we found that the apparent K_m for Pho4 linearly increases as the concentration of 4/6-IP_7 increases (Fig. 3a,b; K_i ~ 19 ± 8 μM), which indicates that 4/6-IP_7 is an inhibitor that is competitive with Pho4. However, 4/6-IP_7 did not alter the K_m for ATP (Fig. 3c,d), which indicates that 4/6-IP_7 is not competitive with ATP.

Although many models can account for these observations, the simplest explanation is that 4/6-IP_7 binds to the peptide substrate binding region in the active site and acts as a classical competitive inhibitor. However, we disfavor this possibility because 4/6-IP_7 does not bind to or inhibit Pho80-Pho85 in the absence of Pho81-MD (ref. 10). Alternatively, binding of 4/6-IP_7 may induce a conformational change in Pho80-Pho85-Pho81 that subsequently prevents the enzyme from binding to Pho4.

The interaction of Pho4 with Pho80-Pho85-Pho81 is complex; there is a distal Pho4 interaction site on Pho80 away from the enzyme active site[28,29]. The presence of the distal interaction is supported by the observations that Pho80-Pho85 poorly phos-phorylates peptides derived from Pho4 and that Pho80-Pho85 phosphorylates Pho4 semiprocessively, transferring ~ 2

phosphates in each productive kinase-substrate interaction[28]. Based on yeast two-hybrid analysis[30] and glutathione S-transferase pull-down experiments[29], Pho4 sequences involved in the distal binding with Pho80-Pho85 were mapped to two regions distant from the five phosphorylation sites[29]. Pho81-MD and 4/6-IP_7 could interfere with either the active site or the distal site interaction between Pho4 and Pho80-Pho85, which would yield similar enzyme kinetic data.

To determine whether the 4/6-IP_7– and Pho81-MD–mediated inhibition involves blocking the active site or interfering with the distal Pho4 interaction, we tested the effect of 4/6-IP_7 on the phosphorylation of Pho4 mutants whose distal binding sites are mutated[29] (Supplementary Fig. 2 online). In comparison with wild-type Pho4, these mutants have five- to ten-fold higher K_ms but similar k_{cat}s (ref. 29). We observed that phosphorylation of these Pho4 distal site mutants by Pho80-Pho85-Pho81-MD is inhibited by 4/6-IP_7 with an IC_{50} comparable to that observed for wild-type Pho4 (all between 20 and 50 μM; Supplementary Fig. 2a), which suggests that the interaction between Pho4 and its distal binding site is not disrupted in the 4/6-IP_7–mediated inactivation of Pho80-Pho85-Pho81-MD. We also tested the effect of 4/6-IP_7 on the phosphorylation of peptides corresponding to Pho4 phosphorylation site 6 (ref. 28) (Supplementary Fig. 2b). These peptides cannot interact with the distal binding site on the kinase and are phosphorylated less efficiently. Phosphorylation of these peptides was also inhibited by the presence of 4/6-IP_7, with a comparable IC_{50} (25 μM), which again suggests that the interaction between Pho4 and the distal binding site on Pho80-Pho85-Pho81 is not involved in enzyme inactivation. Based on these results, we conclude that the 4/6-IP_7– and Pho81-MD–dependent inhibition of Pho80-Pho85 is likely to be mediated by a change in the interaction of the substrate with the kinase active site.

Secondary interaction between Pho81-MD and Pho80-Pho85

Next, using a fluorescence binding assay[10] in which we labeled Pho81-MD with lissamine, we tested whether the presence of 4/6-IP_7 alters the interaction between Pho80-Pho85 and Pho81-MD (Fig. 4a). In the presence of 4/6-IP_7, the binding of Pho81-MD to Pho80-Pho85 is enhanced by ~ five-fold (K_d ~ 120 and 25 nM in the absence and the presence of 100 μM IP_7, respectively). In contrast, 5-IP_7 had no significant effect on this interaction (K_d = 182 ± 13, 295 ± 50 and 250 ± 29 nM in the presence of 0, 50 and 100 μM 4/6-IP_7, respectively; Supplementary Fig. 3 online). These observations suggest that 4/6-IP_7 may interact with both Pho81 and Pho80-Pho85 or may induce structural changes that stabilize interactions between Pho81 and Pho80-Pho85.

When Pho80-Pho85 kinase activity was measured in different concentrations of 4/6-IP_7 and Pho81-MD, we observed that a high concentration of Pho81-MD (μ10 μM) inactivates Pho80-Pho85 in a 4/6-IP_7–independent manner (Fig. 4b); this observation is consistent with previous results showing that overexpression of Pho81 inhibits Pho80-Pho85 even in high phosphate conditions (conditions in which 4/6-IP_7 levels are low)[4,25]. Because Pho81-MD binds to Pho80-Pho85 with an apparent K_d of 120 nM (ref. 10) (Fig. 4a), the inactivation of Pho80-Pho85 by micromolar Pho81-MD may indicate that additional molecules of Pho81-MD can bind to Pho80-Pho85 at a high concentration of Pho81-MD, thereby leading to the formation of a higher-order complex. In support of this view, the addition of excess Pho81-MD in the EMSA binding (Fig. 4c) or gel filtration (Supplementary Fig. 4 online) assays resulted in the formation of additional complexes, and also changed the structure of Pho85 (Supplementary Fig. 5 online).

Constitutive binding and IP_7-regulated segments of Pho81

What is the relationship between the additional Pho81-MD binding at high concentration and kinase inactivation in the stoichiometric Pho80-Pho85-Pho81 complex? One possibility is that

the additional Pho81-MD binding results from additional contacts between Pho80-Pho85 and Pho81-MD that are not observed in the equimolar Pho80-Pho85-Pho81-MD complex in the absence of 4/6-IP_7. It is possible that, upon the addition of 4/6-IP_7, an additional site on Pho80-Pho85 may be occupied by Pho81-MD, thereby causing kinase inhibition by preventing Pho4 from accessing the active site.

To test this hypothesis, we prepared three nonoverlapping shorter segments derived from Pho81-MD (Fig. 5a) and measured their binding to Pho80-Pho85 in the presence and absence of 4/6-IP_7. To obtain quantitative information, we synthesized peptides with an N-terminal Cys-Gly-Gly linker, labeled them site specifically with Alexa Fluor 488 and monitored their interaction with Pho80-Pho85 using fluorescence polarization (Fig. 5b,c). In the absence of 4/6-IP_7, only the middle segment (S3; corresponds to amino acids 665–701 of Pho81) demonstrated any evidence of binding (apparent K_d ~ 400 ± 160 nM; Fig. 5b). However, in the presence of 4/6-IP_7, the S1 segment corresponding to the C-terminal 22 amino acids of Pho81-MD (amino acids 702–723 of Pho81) showed evidence of binding (apparent K_d ~ 550 ± 170, 30 ± 22 and 25 ± 11 nM for 5, 40 and 200 μM 4/6-IP_7, respectively; Fig. 5c). This result suggests that the middle segment (S3) of Pho81-MD binds to Pho80-Pho85 in a manner independent of 4/6-IP_7, whereas the S1 segment binds to Pho80-Pho85 only when 4/6-IP_7 is present.

We next assessed the functional relevance of the interaction of the smaller Pho81-MD segments with Pho80-Pho85. We preincubated Pho80-Pho85 with Pho81-MD or the smaller segments and measured 4/6-IP_7–dependent inhibition of kinase activity (Fig. 5d). We found that the S3 segment, which is sufficient for binding in the absence of 4/6-IP_7, does not show any evidence of Pho80-Pho85 inhibition. The S2 segment, which did not bind to Pho80-Pho85, also did not have any effect on enzyme activity. However, the S1 segment, which binds to Pho80-Pho85 only in the presence of 4/6-IP_7 (Fig. 5c), inhibited Pho80-Pho85 in the presence of 4/6-IP_7. This result indicates that Pho81-MD can be further divided into two segments: one that binds tightly to Pho80-Pho85 regardless of the concentration of 4/6-IP_7, and one that acts as a 4/6-IP_7–dependent inhibitor of Pho80-Pho85.

Model of Pho80-Pho85 inhibition by IP_7 and Pho81

Based on these biochemical data, we propose a model for the mechanism of inhibition of Pho80-Pho85 (Fig. 6). In the absence of 4/6-IP_7 (or in P_i-rich cells), Pho80-Pho85 is constitutively bound to Pho81, but this protein-protein interaction alone is not sufficient to inhibit Pho80-Pho85 kinase activity (Fig. 6a). This constitutive interaction with Pho80-Pho85 is mediated at least in part by the Pho81 S3 segment. Upon starvation of cells for P_i and binding of 4/6-IP_7, additional contacts mediated by the Pho81 S1 segment occur, thereby preventing Pho4 from accessing the Pho80-Pho85 active site (Fig. 6a).

This model is supported by several observations. First, 4/6-IP_7 binds to the Pho80-Pho85-Pho81-MD ternary complex but not to its individual components (Fig. 2). Second, the 4/6-IP_7– and Pho81-mediated inhibition competes with Pho4 but not with ATP (Fig. 3). Third, binding of 4/6-IP_7 stabilizes the interaction between Pho80-Pho85 and Pho81-MD (Fig. 4a). Finally, Pho81-MD can be divided into two segments: S3 is constitutively bound to Pho80-Pho85 but does not inhibit the kinase, whereas S1 binds and inhibits Pho80-Pho85 only in the presence of 4/6-IP_7 (Fig. 5). It is possible that the S1 segment in full-length Pho81 or in Pho81-MD is in an auto-inhibited state (for example, bound to other parts of Pho81-MD) without 4/6-IP_7.

Based on this model (Fig. 6), we expect that an effect on kinase activity similar to that seen on addition of 4/6-IP_7 can be achieved by the addition of excess Pho81. In the presence of excess Pho81, there may be sufficient amounts of S1 segment to inhibit Pho80-Pho85. In support of

this view, addition of excess Pho81-MD inhibits Pho80-Pho85 independent of 4/6-IP_7 with a higher IC_{50} (~ 20 μM, Fig. 4b). It was also shown previously that addition of excess Pho81 to immunopurified Pho80-Pho85 can inhibit kinase activity *in vitro*[4], and that overexpression of Pho81 can partially inhibit the kinase *in vivo*[4,31,32]. Our model may help to explain the inhibition of Pho80-Pho85 by overexpressed Pho81 (Fig. 6b). At high concentration, excess Pho81 will weakly bind to Pho80-Pho85-Pho81 and inhibit kinase activity. Consistent with our EMSA assay results (Fig. 4c), 4/6-IP_7 can also bind to this higher order complex (Fig. 6b).

DISCUSSION

Since the elucidation of its chemical structure, it has been suggested that IP_7 may act as a phosphate donor33. Indeed, both 5-IP_7 and 4/6-IP_7 were recently shown to pyrophosphorylate mammalian and yeast proteins *in vitro*[22,23]. However, because of a lack of suitable assay systems, it has been difficult to determine the physiological relevance of this phosphor transfer reaction. Our previous work demonstrated that 4/6-IP_7 inhibits Pho80-Pho85-Pho81 in response to phosphate limitation, thus leading to induction of starvation-response genes[10]. Because Pho80-Pho85-Pho81 can be immunopurified in a stably regulated form from yeast cells grown in different phosphate concentrations[4], we speculated that Pho80-Pho85-Pho81 may be covalently modified by 4/6-IP_7. In this report, we provide evidence that 4/6-IP_7 reversibly regulates Pho80-Pho85-Pho81. Therefore, we conclude that Pho80-Pho85-Pho81 is unlikely to be a target of an IP_7 phosphor transfer reaction; instead, 4/6-IP_7 acts as an allosteric regulator of this kinase complex.

Previous structural studies of the CDK inhibitor $p27^{Kip1}$ bound to cyclin A–CDK2 revealed that the mechanism of inhibition of active cyclin A–CDK2 involves insertion of a 3_{10} helix of $p27^{Kip1}$ into the ATP-binding pocket of CDK2 (refs. 9,34). In this mode of inhibition, the K_m for ATP is expected to increase, possibly without a change in K_m for the protein substrate. In contrast, our results indicate that for Pho80-Pho85-Pho81, the K_m for the protein substrate (but not the K_m for ATP) is affected by 4/6-IP_7. These results demonstrate that there are mechanistic differences between the inhibition of Pho80-Pho85 by 4/6-IP_7 and Pho81 and cyclin-CDK2 inhibition by Cip/Kip-family CKI proteins.

The allosteric regulation of a cyclin-CDK-CKI by a small-molecule metabolite is a strategy suitable for cells to respond to fluctuating, transient conditions such as nutrient starvation. By using constitutively bound CKI, whose action is regulated by a metabolite, Pho80-Pho85 cyclin-CDK activity can be rapidly modulated in different nutrient or environmental conditions. When the stimulus is reversed in a short period of time, cyclin-CDK activity can be restored. We have shown that 4/6-IP_7 dissociates from Pho80-Pho85-Pho81 within minutes at 30 °C *in vitro*. When the stimulus is persistent, cells may activate an additional inhibition mechanism and make the inactive form of Pho80-Pho85-Pho81 more kinetically inert. We speculate that the interaction of Pho80-Pho85-Pho81 with an additional molecule of Pho81 may be a mechanism for cells to make the inactive form of Pho80-Pho85-Pho81 persistent. Consistent with this model, expression of Pho81 is increased in a Pho4-dependent manner upon P_i starvation31,32.

In addition to its role in the PHO system, Pho85 also regulates other biological processes[35]. Depending on the identity of its cyclin partners, Pho85 can influence the cell cycle, morphogenesis, carbon source utilization and glycogen metabolism[35]. In many of these processes Pho85 is believed to be regulated by nutrient or environmental stimuli[35]. Other Pho85-cyclin complexes may be regulated by metabolite changes in a manner similar to the mechanism described in this paper.

METHODS

Materials

Recombinant proteins used in this study were prepared as described[10,28,29]. We used both chemically synthesized Pho81-MD (amino acids 644–723 of Pho81; NH_2-LLQRPLNLPSAPLNEINSQSSTQRLNTIDLTPNDDK FDLDIQDSI PDFALPPPIIPLRKYGHNFLEKKIFIKLKLRPGLE-$CONH_2$; Biosynthesis, Inc.) and recombinant His_6-Pho81-MD (ref. 25), which had similar biochemical properties. Peptide fragments of Pho81-MD were synthesized by Genscript (S1 (amino acids 702–723 of Pho81): NH_2-CGGKYGHNFLEKKIFIKLKLRPGLE-CO_2H; S2 (amino acids 644–664 of Pho81): NH_2-CGGLLQRPLNLPSAPLNEINSQSS-CO_2H; S3 (amino acids 665–701 of Pho81): NH_2-CGGTQRLNTIDLTPNDDKFDLDIQDSIPDFALPPPIIPLR-CO_2H; residues added for fluorophore labeling are underlined). Enzymatic preparations of 5-IP7 and 4/6-IP7 were prepared and characterized as previously reported[10,14]. See Supplementary Methods online for additional description of materials and methods.

Kinase assays

Pho80-Pho85 kinase assays were performed essentially as previously described[10]. For typical experiments, Pho80-Pho85 (final concentration 0.1–10 nM after mixing with the kinase assay solution) and Pho81-MD (typically 450 nM final concentration) were mixed with 4/6-IP_7 for the noted time period and then diluted in kinase assay solution (40 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 0.01% NP-40, 1 mM PMSF, 2 mM benzamidine, 10 mM NaF, 10 mM β-glycerophosphate, 0.1 mg ml^{-1} bovine serum albumin) containing ATP (typically 1 mM with 0.1–0.2 μCi μl^{-1} [γ-^{32}P]ATP and Pho4 (typically 1–2 μM). After incubation at 30 °C (1–10 min depending on the Pho80-Pho85 concentration), the reaction was quenched by the addition of SDS gel loading buffer and samples were analyzed by SDS-PAGE. Dried gels were exposed to storage phosphor screens and analyzed using a Typhoon imaging system (Molecular Dynamics) equipped with ImageQuant software (GE Healthcare). Enzyme kinetic analysis was done by fitting the data using SigmaPlot software (Systat Software, Inc.) to the following Michaelis-Menten equation:

$$k = \frac{V_o}{[\text{Pho80} - \text{Pho85}]_o} = \frac{k_{cat} \cdot [\text{Pho4}]}{K_m + [\text{Pho4}]}$$

where [Pho80-Pho85]o, V_o, k_{cat}, K_m and [Pho4] indicate initial Pho80-Pho85 concentration, phosphorylation rate, apparent catalytic constant, apparent Michaelis-Menten constant and Pho4 concentration, respectively. Michaelis-Menten constants obtained from these fittings were plotted against the concentration of 4/6-IP_7 (Fig. 3b,d) and fitted into the following linear equation[36]:

$$K_{m,app} = K_m \cdot (1 + \frac{[I]}{K_i})$$

where K_m,app, K_i and [I] indicate apparent K_m in the presence of 4/6-IP_7, the dissociation constant for the protein–4/6-IP_7 interaction and the concentration of 4/6-IP_7. For the analysis of peptide phosphorylation, Pho4 site 6 peptide (400 μM; NH_2-SAEGVVVASESPVIAPHGSTHARSY-CO_2H; phosphorylation site underlined) was used instead of Pho4 protein. The reaction was carried out for 5 min with Pho80-Pho85 (100 nM) pre-equilibrated with Pho81-MD (450 nM) and 4/6-IP_7. After quenching and Tris-Tricine SDS-PAGE, phosphorylation of peptides was analyzed as above.

IP_7 binding assays

In typical binding experiments, Pho80-Pho85-Pho81-MD (8 μM each) was mixed with 4/6-[β-32P]IP_7 (5 Ci mmole–1; 0.2 μM) in buffer A (20 mM HEPES, pH 7.4, 6 mM $MgCl_2$, 5 mM NaF), and the mixture was incubated for 30 min at 30 °C. The solution was then placed on ice and mixed with glycerol (final concentration 10 % v/v) and a trace amount of bromophenol blue. Samples were loaded onto 4–20% gradient gels (Bio-Rad) in Tris-glycine, pH 8.3 running buffer. After 1 h of electrophoresis (120 V; initial current was approximately 30 mA per gel) at 4 °C, gels were dried and analyzed as above. For the test of covalent phosphotransfer, Pho80-Pho85-Pho81-MD-4/6-IP_7 complex solution described above was mixed with SDS gel loading buffer and subjected to SDS-PAGE. The gel was stained with Colloidal Coomassie Blue (Invitrogen), photographed and then dried and exposed to a storage phosphor screen.

Fluorescence binding assays

Pho81-MD binding to Pho80-Pho85 was measured as previously described10. Lissamine-labeled Pho81-MD (ref. 10) (5 nM) was placed in a glass-bottom 96-well plate containing buffer A (100 μl per well), Pho80-Pho85 (0–1 μM) and/or 4/6-IP_7. After 1 h incubation at room temperature (22 ± 2 °C), the Lissamine fluorescence was monitored (λ_{ex} 532 nm; λ_{em} 580 nm). Binding of smaller Pho81-MD segments was performed using Alexa Fluor–labeled S1-S3 segments. S1–S3 segments (~ 0.5 mg) in 0.5 ml 20 mM HEPES, pH 7.4, 1 mM tris(2-carboxyethyl)phosphine were mixed with Alexa Fluor 488 C5 maleimide (0.5 mg in 100 μl dimethyl-sulfoxide). After 1 h incubation at room temperature, labeled peptides were purified through a HiTrap Desalt column (5 ml), yielding quantitative labeling. Labeled peptides were analyzed using MALDI-TOF MS (2,5-dihydroxybenzoic acid matrix; 50% acetonitrile and 0.1% trifluoroacetic acid); *m*/*z*: MW calcd. for S1, 2889.5; found, 2889.9; MW calcd. for S2, 2549.9; found, 2551.1; MW calcd. for S3, 4474.1; found, 4474.5; MW calcd. for Alexa 488 S1, 3610.2; found, 3611.0; MW calcd. for Alexa 488 S2, 3270.6; found, 3272.8; MW calcd. for Alexa 488 S3, 5195.7; found, 5195.9. For the polarization experiments, samples (100 μl per well) containing 10 nM Alexa Fluor 488–labeled S1–S3 peptides in buffer A supplemented with 125 mM NaCl, Pho80-Pho85 and/or 4/6-IP_7 (250 μM) were incubated at room temperature for 30 min, and fluorescence polarization measurements were performed (1 s averaging with fluorescein excitation and emission filters) with a Wallac 1420 Victor3V fluorescence microplate reader.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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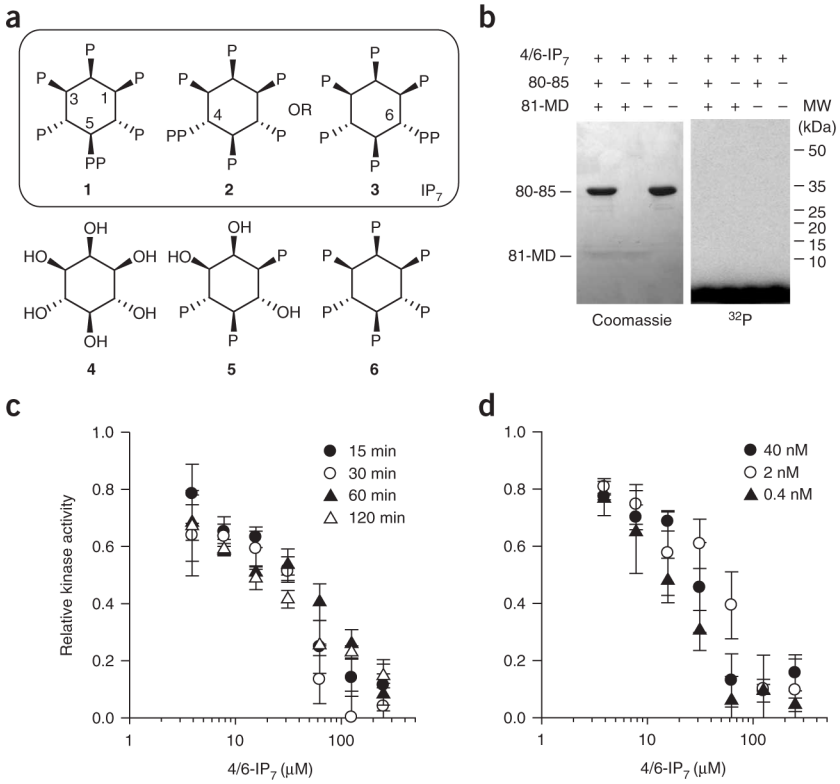

Figure 1.

Reversible regulation of Pho80-Pho85-Pho81-MD by IP_7. (**a**) Structures of *myo*-D-inositol derivatives used in this study (P and PP denote phosphate and pyrophosphate, respectively). The absolute configuration of 4/6-IP_7 is not completely defined (either 4-IP_7 or 6-IP_7, which are mirror images of each other). (**b**) SDS-PAGE of Pho80-Pho85-Pho81 (8 μM each) preincubated with 40 μM 4/6-[β-^{32}P]IP_7 (5 μCi μmol^{-1}). Coomassie staining of the gel (left); autoradiogram of the gel (right). (**c,d**) Time-dependent (**c**) and Pho80-Pho85 concentration-dependent (**d**) IC_{50} changes. For **c**, following 15 (●), 30 (○), 60 (▲) and 120 (△) min of incubation with 4/6-IP_7, the kinase activity of Pho80-Pho85-Pho81-MD (1 nM Pho80-Ph85, 450 nM Pho81-MD) was measured by the addition of buffer containing ATP and Pho4 (1 mM and 1 μM, respectively). Average and s.d. (n = 3) are shown. For **d**, the IC_{50} value for the inhibition by 4/6-IP_7 (30 min incubation) was measured at different Pho80-Pho85 concentrations (40 (●), 2 (○) and 0.4 nM (▲); data represent mean values ± s.d.; n = 3).

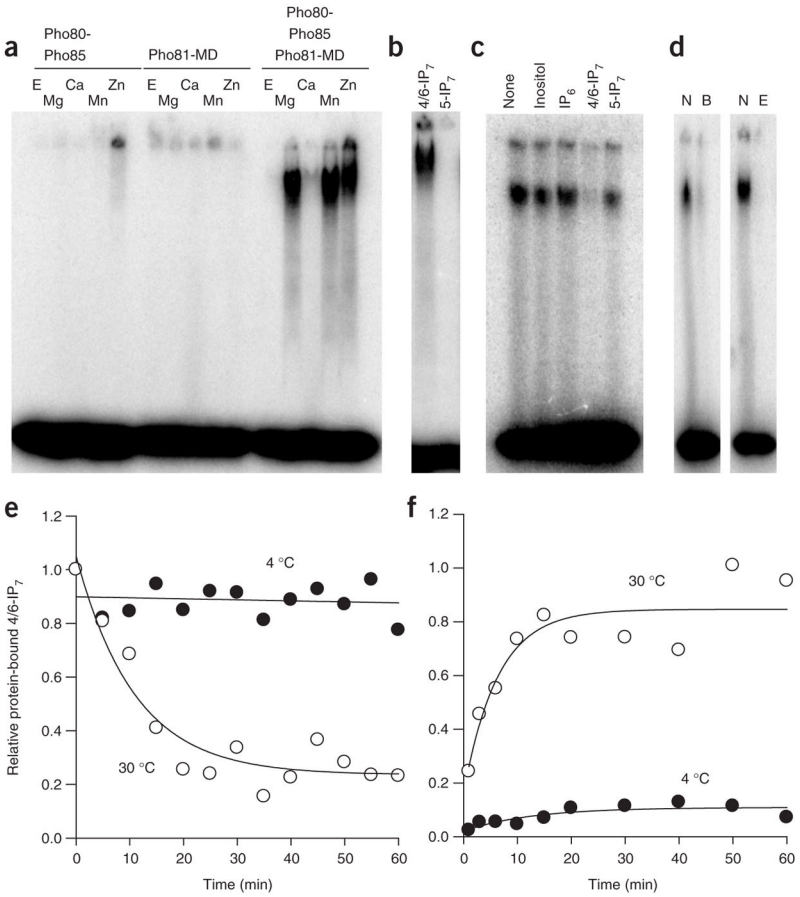

Figure 2.

Characterization of IP_7 binding to Pho80-Pho85-Pho81-MD. (**a**) Native gel EMSA. Pho80-Pho85 (8 μM), Pho81-MD (8 μM) and divalent metal cations (6 mM each; E, EDTA; Mg, $MgCl_2$; Ca, $CaCl_2$; Mn, $MnCl_2$; Zn, $ZnSO_4$) were mixed and incubated with 0.2 μM 4/6-[β-^{32}P]IP_7 (5 μCi mmol^{-1}) for 30 min at 30 °C, followed by electrophoresis at 4 °C. All subsequent binding assays were performed in the presence of 6 mM $MgCl_2$ unless noted otherwise. (**b**) Isomer selectivity. Pho80-Pho85-Pho81-MD (8 μM each) was incubated with 0.2 μM 5-[β-^{32}P]IP_7 or 4/6-[β-^{32}P]IP_7 (30 °C, 30 min) and analyzed as in **a**. (**c**) Specificity of the binding. Binding of 4/6-[β-^{32}P]IP_7 (0.2 μM) to Pho80-Pho85-Pho81-MD (8 μM each) was monitored as in a in the presence of 400 μM competitors. (**d**) Binding reversibility. After formation of the complex between Pho80-Pho85-Pho81-MD and 4/6-IP_7 as in **a**, reversibility of the binding was tested by the addition of 20 mM EDTA (E), or by heating (B; 90 °C, 1 min). N, not treated. (**e**) Dissociation kinetics. After formation of the complex, samples were diluted in 400 μM unlabeled 4/6-IP_7 at 4 or 30 °C, and the dissociation of the complex was monitored by EMSA as a function of the time following the dilution. (**f**) Association kinetics. After adding 4/6-[β-^{32}P]IP_7 to Pho80-Pho85-Pho81-MD and incubating at 4 or 30 °C, a portion of the sample was diluted into ice-cold (4 °C) unlabeled 4/6-IP_7 (400 μM) and analyzed by EMSA. For **d** and **e**, data were fit to single exponential equations.

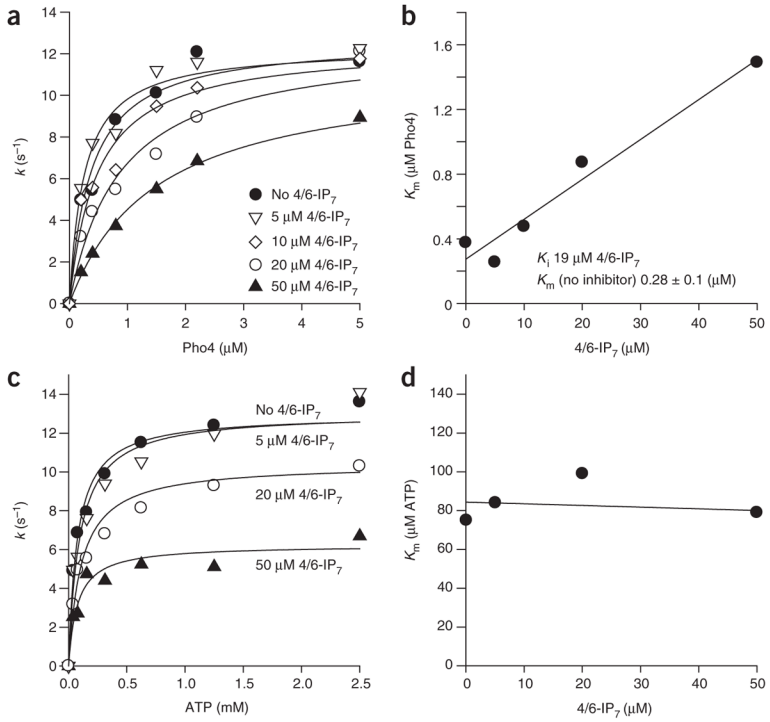

Figure 3.

Enzyme kinetic analysis of IP_7-mediated Pho80-Pho85-Pho81-MD inactivation. Effect of 4/6-IP_7 on Pho80-Pho85-Pho81-MD–catalyzed Pho4 phosphorylation. Pho4 phosphorylation was measured at different Pho4 concentrations (**a,b**) or ATP concentrations (**c,d**) in the absence (**a**) or presence (c) of 4/6-IP_7, and the rate constants were plotted against substrate concentrations. Plots of K_m, obtained by fitting data shown in a and **b** to a Michaelis-Menten equation (see Methods), for Pho4 (**b**) and ATP (**d**) against the concentration of 4/6-IP_7. All reactions were carried out with 0.5 nM Pho80-Pho85 and 450 nM Pho81-MD as described in the Methods.

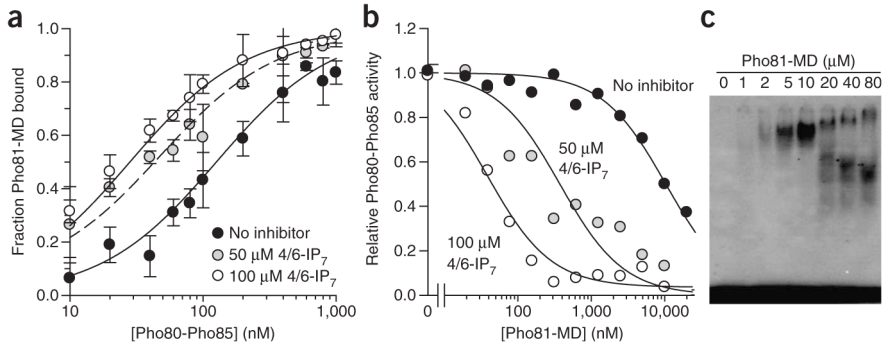

Figure 4.

Additional interaction between Pho81-MD and Pho80-Pho85. (**a**) Binding of Lissamine-labeled Pho81-MD (5 nM) to Pho80-Pho85 in the presence of the indicated concentrations of 4/6-IP_7. Protein-binding-dependent lissamine fluorescence quenching was monitored (λ_{ex} 532 λ_{em} 580 nm; data represent mean values ± s.d.; $n = 3$). (**b**) Inhibition of Pho80-Pho85 (0.5 nM) by excess Pho81-MD at different concentrations of 4/6-IP_7. (**c**) Additional Pho80-Pho85-Pho81-MD-4/6-IP_7 complex formation in the presence of excess Pho81-MD. EMSA experiments were carried out with 8 μM Pho80-Pho85, 0.2 μM 4/6-[β-^{32}P]IP_7 and different concentrations of Pho81-MD.

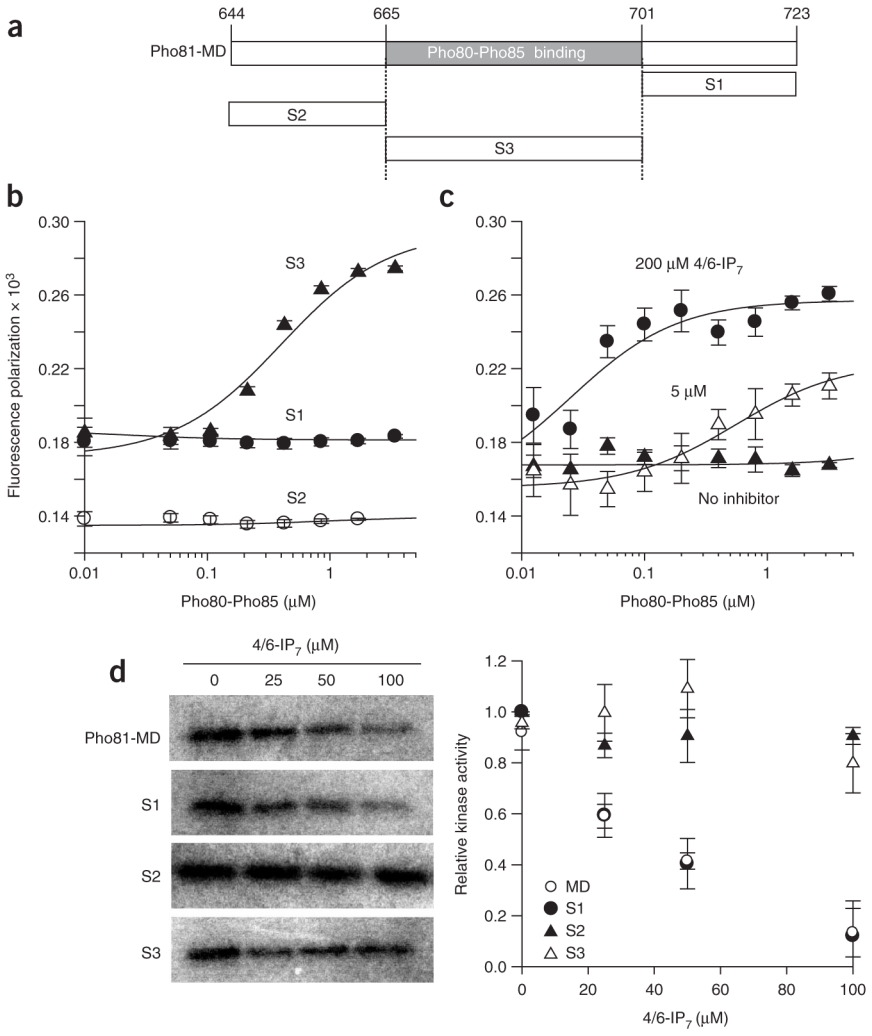

Figure 5.

Dissection of Pho81-MD into binding and inhibitory segments. (**a**) Design of peptide fragments of Pho81-MD. (**b,c**) Fluorescence polarization binding of Alexa Fluor 488–labeled Pho81-MD (10 nM) segments to Pho80-Pho85 (data represent mean values ± s.d.; $n = 3$). (**b**) Binding of S1 (●), S2 (○) and S3 (▲) to Pho80-Pho85 in the absence of 4/6-IP_7. (**c**) Binding of S1 to Pho80-Pho85 in the presence of 0 (▲), 5 (△) and 200 (●) μM 4/6-IP_7. (**d**) Effect of Pho81-MD, S1, S2 and S3 on Pho80-Pho85 inhibition by 4/6-IP_7. Experiments were carried out with 1 nM Pho80-Pho85, 400 nM Pho81-MD or shorter segments, 1 μM Pho4, 500 μM ATP, 0.1 μCi μl^{-1} [γ-^{32}P]ATP for 5 min (data represent mean values ± s.d.; $n = 3$). Symbols note Pho80-Pho85 activity in the presence of IP_7 and Pho81-MD (○), S1 (●), S2 (▲) and S3 (△).

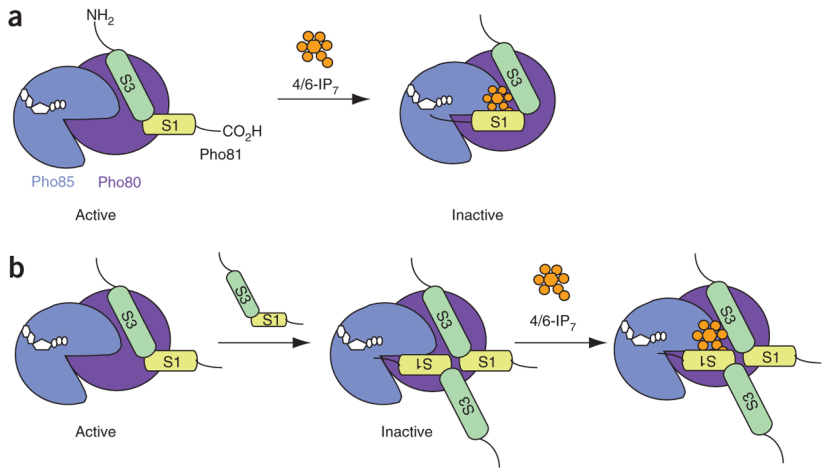

Figure 6.

Model of Pho80-Pho85 regulation by 4/6-IP_7 and Pho81. (**a**) The S3 region of Pho81 binds constitutively to Pho80-Pho85 (left); addition of 4/6-IP_7 triggers a structural change that results in the S1 segment of Pho81 occluding interaction of Pho4 with the kinase active site (right), without affecting ATP binding (white molecule). (**b**) The effect of binding additional molecules of Pho81-MD. When a high concentration Pho81-MD is present, it can bind and block substrate access to the active site. Our data suggest (Fig. 4c) that 4/6-IP_7 can still bind to this higher order complex.