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Regulation of a Cyclin-CDK-CDK Inhibitor Complex by Inositol Pyrophosphates

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Abstract

In budding yeast, phosphate starvation triggers inhibition of the Pho80-Pho85 cyclin–cyclin-dependent kinase (CDK) complex by the CDK inhibitor Pho81, leading to expression of genes involved in nutrient homeostasis. We isolated *myo-D*-inositol heptakisphosphate (IP₇) as a cellular component that stimulates Pho81-dependent inhibition of Pho80-Pho85. IP₇ is necessary for Pho81-dependent inhibition of Pho80-Pho85 in vitro. Moreover, intracellular concentrations of IP₇ increased upon phosphate starvation, and yeast mutants defective in IP₇ production failed to inhibit Pho80-Pho85 in response to phosphate starvation. These observations reveal regulation of a cyclin-CDK complex by a metabolite and suggest that a complex metabolic network mediates signaling of phosphate availability.

In response to nutrient limitation, cells increase transcription of starvation response genes. Many proteins that function in nutrient response systems have been identified, but the molecular details of their regulation are not well understood (1). The *Saccharomyces cerevisiae* Pho80-Pho85 cyclin-CDK complex is a key regulator in the phosphate (P_i)–responsive (PHO) signaling pathway (2,3). When cells are grown in medium that is rich in P_i, Pho80-Pho85 is active and phosphorylates the transcription factor Pho4 (4), resulting in its export from the nucleus (5). Upon P_i starvation, Pho80-Pho85 kinase activity is decreased, leading to nuclear accumulation of Pho4 and transcription of PHO genes. In vivo, the Pho81 CDK inhibitor (CKI) is bound to Pho80-Pho85 regardless of P_i conditions and is required for the inhibition of Pho80-Pho85 activity in response to P_i-limitation (6).

We used a biochemical strategy to identify cellular components that influence Pho80-Pho85-Pho81 activity. The Pho80-Pho85-Pho81 complex immunopurified from cells grown in P_i-rich conditions actively phosphorylated Pho4 in vitro, whereas the complex isolated from P_i-starved cells was less active (6) (Fig. 1A). To identify regulators of Pho80-Pho85-Pho81, we added extracts from cells grown in P_i-rich and P_i-starved conditions to active (from P_i-rich) and inactive (from P_i-starved) immunopurified Pho80-Pho85-Pho81 complexes and performed kinase assays with recombinant Pho4 serving as the substrate. Extracts from P_i-starved cells efficiently inactivated Pho80-Pho85-Pho81, whereas the P_i-rich cell extract had little effect (Fig. 1B). When the extract of P_i-starved cells was fractionated into fractions of large (>3 kD) and small (<3 kD) molecular size, most inhibitory activity was found in the fractions of small molecular size. This activity had little effect on Pho80-Pho85 purified from *pho81Δ* cells (Fig. 1C), indicating that, as is true in vivo (6), the inhibitory activity requires Pho81 for its function.

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To determine the identity of the inhibitory activity, we enriched it through several fractionation steps and characterized its properties (fig. S1) (7). The low-molecular weight inhibitor contained acid-labile P_i (8), and characterization by nuclear magnetic resonance and mass spectrometry suggested that it was inositol heptakisphosphate (9), also known as diphospho-*myo*-D-inositol pentakisphosphate, PP-InsP₅, PP-IP₅, InsP₇, or IP₇ (Fig. 1D).

Inositol polyphosphates are signaling molecules found in all eukaryotic cells. They are produced through the action of a conserved set of inositol polyphosphate kinases from inositol 1,4,5-trisphosphate (IP₃) (9). IP₃, which regulates Ca^{2+} release from the endoplasmic reticulum (10), can be sequentially phosphorylated to generate IP₄, IP₅, inositol hexakisphosphate (IP₆), and inositol pyrophosphates such as IP₇. Loss-of-function studies have implicated IP₇ in recombination (11), vacuole function (12), gene expression, endo- and exocytosis (13,14), osmotic stress (12), and telomere length control (15,16), but it is unclear whether IP₇ directly regulates these processes. One well-characterized role for IP₇ is the regulation of chemotaxis in *Dictyostelium*, in which intracellular concentrations of IP₇ increase upon chemoattractant addition and IP₇ competes with phosphatidylinositol 3,4,5-trisphosphate (PIP₃) to bind to proteins containing a pleckstrin homology domain (17).

To test whether the inhibitory activity was IP₇, we synthesized IP₇ by a nonenzymatic method (8) and tested its effect on the Pho80-Pho85 complex. When the Pho80-Pho85-Pho81 complex that had been immunopurified from P_i -rich cells was treated with synthetic IP₇, kinase activity was inhibited with an apparent median inhibitory concentration (IC₅₀) of ~50 μ M (Fig. 2A). IP₇ had little or no effect on the kinase purified from *pho81* Δ cells, suggesting that IP₇-mediated inactivation of Pho80-Pho85 requires Pho81. Inactivation of Pho80-Pho85-Pho81 by IP₇ appears to be specific, given that inositol and several inositol derivatives did not affect kinase activity (Fig. 2B).

We next tested whether IP₇-mediated inactivation of Pho80-Pho85-Pho81 is direct or whether it requires other factors from yeast. Neither synthetic IP₇ nor the activity from P_i -starved cell extract could inactivate recombinant Pho80-Pho85 purified from *Escherichia coli* (Fig. 2C). Similarly, addition of recombinant Pho81 minimum domain (Pho81-MD) (18), a fragment that functionally substitutes for full-length Pho81 in vivo, did not cause inhibition of recombinant Pho80-Pho85 in vitro, even in two- to threefold molar excess (Fig. 2C). Because Pho81-MD binds to Pho80-Pho85 (apparent dissociation constant $K_d \sim 170$ nM, fig. S2), we concluded that neither Pho81-MD nor IP₇ is sufficient for the inactivation of Pho80-Pho85. However, when both IP₇ and Pho81-MD were added, Pho80-Pho85-Pho81-MD was inactivated (Fig. 2C). Therefore, IP₇ directly inactivates Pho80-Pho85-Pho81 and this inactivation requires Pho81.

To test whether the cellular concentration of IP₇ increases when cells are starved of P_i , we monitored inositol polyphosphate levels in cells labeled with [³H]-inositol and grown in P_i -rich or P_i -limiting conditions (12). In P_i -rich conditions, the IP₇ concentration was approximately 3% or less of the IP₆ concentration (Fig. 3) (12). However, upon P_i limitation, IP₇ levels increased to more than 20% of the levels of IP₆ (Fig. 3). This increase in abundance of IP₇ is not due to Pho4-dependent transcriptional activation (fig. S3) (7), and the time course and dependence of the IP₇ increase on extracellular P_i are comparable to those observed for PHO pathway regulation (fig. S3). Assuming that the IP₆ concentration is ~100 μ M in cells (9) and that the IP₆ concentration does not change in response to P_i limitation, the concentration of IP₇ may reach ~30 μ M during P_i limitation (fig. S3), a value similar to the apparent IC₅₀ measured in vitro (Fig. 2).

Many gene products involved in yeast inositol polyphosphate metabolism are known (Fig. 4A) (9). Kcs1 is a homolog of IP₆ kinases in other organisms (19) and is a major IP₆ kinase in yeast

(12). However, IP₇ is still observable in *kcs1Δ* cells (16,20), suggesting that another IP₆ kinase exists. A second yeast IP₆ kinase, Vip1, generates an isomer of IP₇ distinct from that produced by the mammalian IP₆ kinase and likely distinct from that produced by Kcs1 (21). To determine which IP₆ kinase is involved in the PHO pathway, we assayed the ability of cellular extracts from P_i-starved wild-type, *kcs1Δ*, and *vip1Δ* cells to inactivate Pho80-Pho85-Pho81 in vitro (Fig. 4B). The extract from *kcs1Δ* cells inactivated Pho80-Pho85 in a Pho81-dependent manner, whereas extract from *vip1Δ* cells did not, suggesting that Vip1 mediates synthesis of IP₇ relevant to the PHO system.

To further assess the physiological role of Vip1-generated IP₇ in P_i signaling in vivo, we determined whether yeast strains with mutations in enzymes involved in inositol polyphosphate metabolism (Fig. 4A) (9) had defects in the regulation of Pho80-Pho85-Pho81. We monitored the activity of Pho80-Pho85 by measuring the subcellular localization of its substrate, Pho4 (Fig. 4C). In the *ipk1Δ* and *vip1Δ* strains, Pho4 was localized to the cytoplasm even in conditions of P_i starvation, suggesting that the kinase cannot be inactivated. In the *kcs1Δ* strain, Pho4 became localized to the nucleus upon P_i starvation, consistent with the model that Kcs1 does not generate the IP₇ that inhibits Pho80-Pho85-Pho81. A fraction of the *kcs1Δ* cells grown in high-P_i medium exhibited nuclear localization of Pho4; the cause of this phenotype is not understood (22,23). Pho4 was constitutively nuclear in *ddp1Δ* cells (16), which have high levels of IP₇, but was constitutively cytoplasmic in *kcs1Δvip1Δddp1Δ* and *arg82Δ* cells, which cannot produce IP₇ and IP₅, respectively (fig. S4A). Pho4 was constitutively nuclear in a *vip1Δpho80Δ* strain and was constitutively cytoplasmic in *ddp1Δpho81Δ* cells, suggesting that IP₇ acts upstream of Pho80-Pho85-Pho81 (fig. S4B). Additionally, IP₇ enzymatically synthesized by recombinant Vip1 inhibited Pho80-Pho85-Pho81-MD in a manner dependent on Pho81-MD, but IP₇ enzymatically synthesized by the human homolog of Kcs1, hIP6K1, did not (Fig. 4D; apparent IC₅₀ ~ 50 μM; fig. S4C) (21).

We conclude that IP₇ produced by Vip1 acts as a signaling molecule that controls the yeast PHO signaling pathway: IP₇ concentrations increase in response to P_i limitation, and IP₇ is required for inhibition of Pho80-Pho85-Pho81 in vivo and in vitro. This work illuminates the molecular mechanisms underlying previously reported genetic interactions between inositol polyphosphate metabolism and the phosphate-responsive signaling pathway (22,24). Our work reveals regulation of a cyclin-CDK-CDK inhibitor complex by a small molecule, IP₇. It is unclear whether IP₇ acts by allosterically regulating Pho80-Pho85-Pho81 or by covalently modifying one or more of these components, as it has been demonstrated to do for other yeast and mammalian proteins (25). IP₇ may be a more general signal for nutrient limitation given that P_i starvation of budding yeast and nutrient limitation of *Dictyostelium* (17) leads to elevation of IP₇ levels. In addition, considering that a mammalian IP₆ kinase is known to stimulate P_i uptake (26), a phenotype similar to P_i-starved yeast cells, it is possible that connections between phosphate metabolism and inositol polyphosphate are conserved in other organisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References and Notes

1. Wilson WA, Roach PJ. Cell 2002;111:155. [PubMed: 12408859]
2. Lenburg ME, O'Shea EK. Trends Biochem Sci 1996;21:383. [PubMed: 8918192]
3. Carroll AS, O'Shea EK. Trends Biochem Sci 2002;27:87. [PubMed: 11852246]
4. Kaffman A, Herskowitz I, Tjian R, O'Shea EK. Science 1994;263:1153. [PubMed: 8108735]
5. O'Neill EM, Kaffman A, Jolly ER, O'Shea EK. Science 1996;271:209. [PubMed: 8539622]

6. Schneider KR, Smith RL, O'Shea EK. *Science* 1994;266:122. [PubMed: 7939631]
7. Materials and methods are available as supporting material on *Science* Online.
8. Mayr GW, Radenberg T, Thiel U, Vogel G, Stephens LR. *Carbohydr Res* 1992;234:247.
9. Irvine RF, Schell MJ. *Nat Rev Mol Cell Biol* 2001;2:327. [PubMed: 11331907]
10. Streb H, Irvine RF, Berridge MJ, Schulz I. *Nature* 1983;306:67. [PubMed: 6605482]
11. Luo HR, et al. *Biochemistry* 2002;41:2509. [PubMed: 11851397]
12. Dubois E, et al. *J Biol Chem* 2002;277:23755. [PubMed: 11956213]
13. Ye W, Ali N, Bembenek ME, Shears SB, Lafer EM. *J Biol Chem* 1995;270:1564. [PubMed: 7829485]
14. Fleischer B, et al. *J Biol Chem* 1994;269:17826. [PubMed: 8027036]
15. Saiardi A, Resnick AC, Snowman AM, Wendland B, Snyder SH. *Proc Natl Acad Sci USA* 2005;102:1911. [PubMed: 15665079]
16. York SJ, Armbruster BN, Greenwell P, Petes TD, York JD. *J Biol Chem* 2005;280:4264. [PubMed: 15561716]
17. Luo HR, et al. *Cell* 2003;114:559. [PubMed: 13678580]
18. Huang S, Jeffery DA, Anthony MD, O'Shea EK. *Mol Cell Biol* 2001;21:6695. [PubMed: 11533256]
19. Saiardi A, Erdjument-Bromage H, Snowman AM, Tempst P, Snyder SH. *Curr Biol* 1999;9:1323. [PubMed: 10574768]
20. Seeds AM, Bastidas RJ, York JD. *J Biol Chem* 2005;280:27654. [PubMed: 15944147]
21. Mulugu S, et al. *Science* 2007;316:106. [PubMed: 17412958]
22. Auesukaree C, Tochio H, Shirakawa M, Kaneko Y, Harashima S. *J Biol Chem* 2005;280:25127. [PubMed: 15866881]
23. Huang S, O'Shea EK. *Genetics* 2005;169:1859. [PubMed: 15695358]
24. Flick JS, Thorner J. *Genetics* 1998;148:33. [PubMed: 9475719]
25. Saiardi A, Bhandari R, Resnick AC, Snowman AM, Snyder SH. *Science* 2004;306:2101. [PubMed: 15604408]
26. Schell MJ, et al. *FEBS Lett* 1999;461:169. [PubMed: 10567691]
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Fig. 1.

Purification of a cellular component that inactivates Pho80-Pho85 in a Pho81-dependent manner. **(A)** Phosphorylation of recombinant Pho4 by Pho80-Pho85-Pho81 immunopurified from P_i -rich (High) and P_i -starved (Low) cells expressing Pho80-hemagglutinin tag (3HA). **(B)** Effects of cell fractions on Pho80-Pho85 activity. Extracts from cells (3×10^9 cells per ml extract) grown in high- P_i medium (High P_i extract) or starved for phosphate (Low P_i extract) were added either directly or after filtration (molecular weight cut-off 3 kD; Low and High indicate low- and high-molecular weight fractions, respectively) to Pho80-Pho85-Pho81 immunopurified from P_i -rich cells, and kinase assays were done as in (A). U, untreated Pho80-Pho85-Pho81 from the same gel. **(C)** The low- P_i low-molecular weight fraction in (B) was serially diluted and added to Pho80-Pho85 immunopurified from wild-type (WT) and *pho81Δ* cells that had been grown in P_i -rich medium. Kinase assays were then performed using recombinant Pho4 as a substrate. **(D)** Structures of relevant IP₇ isomers. 4-PP-IP₅ and 6-PP-IP₅ are mirror images of each other. P, phosphate; PP, pyrophosphate.

Fig. 2.

Inhibition of Pho80-Pho85-Pho81 by synthetic IP₇. **(A)** Synthetic IP₇ was serially diluted and added to immunopurified Pho80-Pho85 isolated from wild-type (WT) or *pho81Δ* cells that had been grown in *P*_i-rich medium (Fig. 1C). Kinase activity was then measured. **(B)** Serial dilutions of inositol, *myo*-D-inositol 1,4,5-triphosphate (IP₃), IP₆, or IP₇ were added to Pho80-Pho85-Pho81 immunopurified from wild-type cells grown in *P*_i-rich medium, and kinase activity was measured. **(C)** Synthetic IP₇ was added to recombinant Pho80-Pho85 or Pho80-Pho85-Pho81-MD complex (50 nM Pho80-Pho85 and 250 nM Pho81-MD) and incubated 20 min at room temperature. Kinase activity was then measured.

Fig. 3.

Increase in IP_7 upon P_i starvation. Strong anion exchange chromatograms of extracts derived from wild-type (WT) cells labeled with [^3H]-inositol grown in medium containing high P_i (10 mM) or low P_i (5 μ M). Cells were starved for 2 hours at 30°C before lysis and analysis. CPM, counts per minute.

Fig. 4. Effect of disrupted inositol polyphosphate metabolism on Pho80-Pho85 activity, monitored by Pho4 localization. **(A)** Schematic diagram showing inositol polyphosphate metabolism in *S. cerevisiae*. **(B)** Wild-type (WT), *kcs1Δ*, or *vip1Δ* cell extracts from P_i -starved cells were added to immunopurified Pho80-Pho85 from P_i -rich wild-type (top) or *pho81Δ* (bottom) cells, and kinase activity was measured. U, untreated. **(C)** Fluorescence microscopic (100× magnification) analysis of wild-type, *ipk1Δ*, *kcs1Δ*, *vip1Δ*, or *ddp1Δ* cells expressing Pho4–green fluorescent protein, grown in medium containing high (top) or low (bottom) concentrations of P_i . **(D)** Isomer specificity of the IP₇-mediated Pho80-Pho85 inactivation. Pho80-Pho85 (8 nM) in the presence (top) and the absence (bottom) of His₆-Pho81-MD (250 nM) was incubated with IP₇ isomers synthesized with recombinant Vip1 (top) and human IP6 kinase 1 (bottom), and kinase activity was measured.