Phosphorylation Regulates myo-Inositol-3-phosphate Synthase

A NOVEL REGULATORY MECHANISM OF INOSITOL BIOSYNTHESIS

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Background: myo-Inositol-3-phosphate synthase (MIPS) catalyzes the first step in de novo biosynthesis of inositol in eukaryotes.

Results: MIPS is a phosphoprotein. Phosphorylation regulates the activity of yeast and human MIPS.

Conclusion: Phosphorylation of MIPS is a novel regulatory mechanism of inositol biosynthesis.

Significance: This may explain the causes and consequences of perturbation of inositol metabolism implicated in human disorders.

myo-Inositol-3-phosphate synthase (MIPS) plays a crucial role in inositol homeostasis. Transcription of the coding gene INO1 is highly regulated. However, regulation of the enzyme is not well defined. We previously showed that MIPS is indirectly inhibited by valproate, suggesting that the enzyme is post-translationally regulated. Using 32P labeling and phosphoamino acid analysis, we show that yeast MIPS is a phosphoprotein. Mass spectrometry analysis identified five phosphosites, three of which are conserved in the human MIPS. Analysis of phosphorylation-deficient and phosphomimetic site mutants indicated that the three conserved sites in yeast (Ser-184, Ser-296, and Ser-374) and humans (Ser-177, Ser-279, and Ser-357) affect MIPS activity. Both S296A and S296D yeast mutants and S177A and S177D human mutants exhibited decreased enzymatic activity, suggesting that a serine residue is critical at that location. The phosphomimetic mutations S184D (human S279D) and S374D (human S357D) but not the phosphodeficient mutations decreased activity, suggesting that phosphorylation of these two sites is inhibitory. The double mutation S184A/S374A caused an increase in MIPS activity, conferred a growth advantage, and partially rescued sensitivity to valproate. Our findings identify a novel mechanism of regulation of inositol synthesis by phosphorylation of MIPS.

Inositol is a six-carbon cyclitol that is ubiquitous in biological systems. It is a precursor for inositol lipids and inositol phosphates that are required for various structural and functional processes, including membrane formation, signaling, and membrane trafficking (1, 2). Inositol plays a major role in the transcriptional regulation of hundreds of genes, including lipid biosynthesis genes that harbor the inositol-sensitive upstream activating sequence (UASINO) in their promoters (3). In addition, inositol plays a key role in osmoregulation. For example, kidney cells rely on myo-inositol along with sodium to offset the change in osmolarity generated by the loss of water molecules (4).

The enzyme responsible for the rate-limiting step of de novo synthesis of inositol is myo-inositol-3-phosphate synthase (MIPS), which catalyzes the conversion of glucose 6-phosphate (G-6-P) to inositol 3-phosphate (I-3-P) in the cytosol in a four-step catalytic reaction using NAD+ as a cofactor (Fig. 1A). MIPS has been isolated from bacteria (5), archaea (6), protozoa (7), plants (8), and animals (9, 10). In yeast, MIPS is encoded by the well characterized INO1 gene (11); in mammals, it is encoded by ISYNA1 and exists as multiple isoforms (12–14). The crystal structure of yeast MIPS shows that it is a homotrimer (Fig. 1B) (15, 16), whereas mammalian MIPS exists as a trimer (10). Each monomer has three major domains: a catalytic domain that binds the substrate glucose 6-phosphate, an NAD+-binding domain, and a central domain, consisting of the N and C termini, which stabilizes the two other domains (17). Sequence analysis showed that the enzyme is highly conserved, with remarkable conservation of the amino acid residues that are important for catalytic activity (18).

Regulation of yeast MIPS at the level of INO1 transcription has been extensively studied and well characterized (19–21). The INO1 gene is tightly regulated by inositol. When inositol is limiting, the transcription activators Ino2p and Ino4p form a dimer that binds to the UASINO leading to the transcription of INO1. However, when inositol is available, the negative regulator Opi1p prevents activation by Ino2p and Ino4p and represses transcription of INO1 (22, 23).

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1 This article contains supplemental Table S1 and Figs. S1 and S2.

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3 The abbreviations used are: UASINO, inositol-sensitive upstream activating sequence; MIPS, myo-inositol-3-phosphate synthase; hMIPS, human myo-inositol-3-phosphate synthase; VPA, valproate; SM, synthetic medium.
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The significance of maintaining inositol homeostasis for cell physiology is exemplified by the inositol-less death phenomenon, in which cells starved for inositol die within a few hours of starvation (24). Inositol depletion leads to various consequences (25). Growth in the absence of inositol elicits profound changes in lipid metabolism and activates numerous stress responses (26). Altered inositol levels in the brain have been associated with a variety of mental disorders, including bipolar disorder (27), Alzheimer disease (28, 29), and manic depressive psychosis (30). Thus, the ability of cells to maintain the right balance of inositol at all times undoubtedly necessitates a high level of regulation.

Although the regulation of inositol biosynthesis at the level of INO1 transcription has been extensively characterized, as discussed above, the regulation of MIPS activity has not been studied. The anticonvulsant drug valproate (VPA) induces depletion of inositol, and MIPS has been suggested as a possible target of the drug (31–33). Our initial finding that VPA leads to a decrease in inositol-3-phosphate in yeast (33) suggested that VPA inhibits MIPS activity. The subsequent observation that VPA-mediated inhibition was indirect (31) suggested that MIPS is regulated post-translationally. Phosphorylation controls the regulation and localization of numerous enzymes, many of which, like MIPS, are transcriptionally regulated by UAS\textsubscript{INO1} elements (34–36). To our knowledge, nothing is known about the post-translationally regulation of MIPS.

Here we report for the first time that MIPS, from yeast and humans, is regulated by phosphorylation of at least three residues, one in the catalytic domain and two in the NAD\textsuperscript{+}-binding domain, thus identifying a novel mechanism of regulation of inositol biosynthesis. These sites are conserved in yeast and human MIPS, suggesting that regulation by phosphorylation is a conserved regulatory mechanism. We also show that mutation of the two inhibitory phosphosites, Ser-184 and Ser-374, leads to decreased sensitivity to VPA, suggesting that VPA may inhibit MIPS activity as a consequence of phosphorylation of these two residues.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were reagent grade or better. Media components were from Difco, EMD Biochemicals, Sigma, or Fisher. Inositol, valproate, glucose 6-phosphate, NaI\textsubscript{O}, Na\textsubscript{2}SO\textsubscript{4}, ascorbic acid, and ammonium molybdate were from Sigma or Fisher. Restriction enzymes, Pfu DNA polymerase, Phusion Hotstart DNA polymerase, and T4 DNA ligase were from Promega and New England Biolabs. The Wizard Plus plasmid DNA purification and DNA gel extraction kits were from Promega. Pureproteome magnetic beads and Centricon filters were from Millipore. Alkaline phosphatase was from New England Biolabs. The protease inhibitor mixture Complete Mini and the phosphatase inhibitor PhoSTOP were from Roche.

Strains used in this study are listed in Table 1. Yeast was grown in synthetic minimal medium containing 2% glucose and lacking inositol unless otherwise stated. Media were supplemented with the amino acids histidine, leucine, methionine, uracil, and lysine using Difco standard concentrations. For selection of cells carrying specific plasmids, appropriate amino acids were omitted from the media. For overexpression purposes, induction medium contained 2% galactose and 1% raffinose with no glucose added. Where indicated, VPA was used at a concentration of 1 mM, and inositol was used at 75 \textmu M. Yeast strains were grown at 30 °C. E. coli strain DH5\textalpha was used for plasmid maintenance and amplifications. Bacteria were grown at 37 °C in LB medium (0.5% yeast extract, 1% tryptone, 1% NaCl), supplemented with ampicillin (100 \mu g/ml) for selection purposes. For growth on plates, media were supplemented with 1.5 and 2% agar for E. coli and yeast, respectively. Growth in liquid cultures was monitored spectrophotometrically by measuring absorbance at 550 nm.

DNA Manipulations, PCR, and DNA Sequencing—Standard methods were followed for isolation of genomic DNA, plasmid purification, digestion with restriction endonucleases, and ligation. Transformation of bacterial and yeast cells was carried out using electroporation. All PCRs were optimized. DNA sequencing was carried out using the ACGT Inc. sequencing facility (Wheeling, IL).

Construction of Plasmids and Expression of Yeast and Human MIPS in S. cerevisiae—All plasmids and primers used in this work are listed in Tables 2 and 3, respectively. The INO1 gene

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**TABLE 1**

| Strains used in this study |
|---------------------------|
| **E. coli**                |
| DH5α                      |
| F′ d88×lacZΔm15A (lacZYA-argF)Il169 deoR recA1 endA1 hsdR17 (rK+ mK+ lfrA15 xspE441 thi-lE96relA1) |
| **S. cerevisiae**          |
| BY4741                    |
| MATa his3Δ1, leu2Δ0, met15Δ0, ura3Δ0 |
| BY4741 ino1Δ             |
| MATa his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, INO1::KanMX |
was amplified from \textit{S. cerevisiae} genomic DNA using the primer pair 5'-GTTGTCGGGTCTCTATATGT-3' and 5'-CAACAAATCTCTTCGTAATC-3' and tagged with His6 and an Xpress epitope on the N terminus using pRSETA as the source of the tag. The tagged INO1 was then subcloned into pYES6 containing the blasticidin marker. HindIII and XhoI
were used to transfer the tagged \( \text{INO1} \) into pRD015 to make the pRD\( \text{INO1} \) construct, a high copy \( \text{GAL1} \)-driven plasmid containing a \( \text{URA3} \) marker. All subsequent manipulations and mutagenesis experiments for the yeast MIPS were carried out using the pRD\( \text{INO1} \) vector. All mutations were confirmed by sequencing. The low copy set of all mutants was prepared by transferring the mutated genes to the centromeric low copy vector p415-ADH purchased from ATCC. For human MIPS, the human \( \text{INO1} \) gene (\( \text{hINO1} \)) was transferred from pRSETA-\( \text{hINO1} \) (31) and cloned into pRD015. All subsequent experiments for the human MIPS, including mutagenesis, growth, and enzyme purification were carried out using pRDh\( \text{INO1} \) and its derivatives.

Site-directed Mutagenesis—Site mutations were constructed using a two-step PCR protocol developed for this study. For each mutation, two overlapping, non-phosphorylated primers were designed. Two PCRs were run simultaneously, using one primer in each reaction. pRD\( \text{INO1} \) and pRDh\( \text{INO1} \) were used as templates for yeast MIPS and human MIPS, respectively. The first PCR was run for 10 cycles. The two samples from the first PCR were then pooled together, and the PCR was resumed for another 20 cycles. After cooling, ligase was added to the reaction mix and incubated for 1 h at room temperature. Gel analysis was used to detect the presence of the amplified plasmid. The parent strands were digested using DpnI treatment for 2 h at 37°C. Transformation of competent \( \text{E. coli} \) cells was carried out using electroporation. Selection was carried out on LB plates supplemented with ampicillin. Plasmids were purified, and all mutations were verified by sequencing. The constructs harboring the mutations were then used to transform yeast \( \text{ino1} \Delta \) cells.

Purification of Recombinant MIPS—\( \text{S. cerevisiae} \) BY4741 \( \text{ino1} \Delta \) mutant bearing the pRD\( \text{INO1} \) construct (or its derivatives) was used to express the recombinant His\(_8\)-tagged MIPS. Cells were grown at 30°C in synthetic minimal medium lacking uracil. Galactose (2%) was used to induce overexpression of the recombinant protein. Cell extracts were prepared by disrupting cells with glass beads (0.5 mm diameter), vortexing for 30 min intermittently, keeping cells on ice. The disruption buffer contained 50 mM Tris-Cl (pH 7.4) and 300 mM NaCl. A mixture of protease and phosphatase inhibitors was added to the disruption buffer before breaking the cells. MIPS was purified using ProBond nickel-chelating resin to bind the protein, gently mixing at 4°C for 1 h. The resin was washed twice with cold 20 mM and then 60 mM imidazole in Tris buffer (50 mM Tris-Cl (pH 7.4) and 300 mM NaCl). The protein was eluted with 300 mM imidazole in Tris buffer, dialyzed, concentrated, and resuspended in 50 mM Tris-Cl, 50 mM NaCl, 10 mM dithiothreitol (DTT). Protein concentration was determined to be 2 mg/ml, which orthovanadate was added to the reaction mix to a final concentration of 10 mM to inactivate the alkaline phosphatase. The beads were then collected and washed three times with Tris purification buffer in preparation for the MIPS assay.

In Vivo Phosphorylation of MIPS—Cells with recombinant MIPS were precultured in synthetic minimal medium, inoculated to \( A_{550} \) 0.03 in 20 ml of phosphate-free medium to which 2 mM of \( \text{[32}^P\) was added, and grown to the early stationary phase with or without VPA. Cells were harvested by centrifugation, washed with water, and disrupted with glass beads in 100 μl of buffer containing 150 mM NaCl, 50 mM Tris-Cl (pH 7.5), 0.5% deoxycholate, 0.1% SDS, 1% Nonidet P-40, 10 mM NaF, 5 mM B-glycerophosphate, 1 mM sodium vanadate, and protease inhibitor mixture. The cell extracts were precleaned with 10 μl of protein G plus protein A-agarose for 1 h at 4°C and incubated with 2 μg of anti-Xpress antibody, 50 μl of protein G plus protein A-agarose overnight at 4°C. After washing with the same buffer, the MIPS anti-Xpress antibody complex was dissociated by boiling for 5 min in protein sample buffer containing 2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris-Cl (pH 6.8), and 0.001% bromphenol blue. After a brief low speed centrifugation, proteins were analyzed by SDS-PAGE. Bands were visualized by Coomassie Blue staining, and \( \text{32}^P \)-labeled proteins were identified by autoradiography.

Phosphoamino acid Analysis—MIPS labeled with \( \text{32}^P \) was digested with 6 N HCl. The acid was evaporated, and the sample was dissolved in 10 μl of TLE buffer (pH 1.9) and mixed with 1 μg of cold phosphoserine, phosphothreonine, and phosphotyrosine standards. The phosphoamino acids were separated in two dimensions, by electrophoresis followed by thin layer chromatography. Standard phosphoamino acids were visualized by ninhydrin staining of the thin layer chromatography plate. Labeled phosphoamino acids were visualized by autoradiography.

Mass Spectrometry Analysis of MIPS Phosphorylation Sites—Purified protein samples were reduced with dithiothreitol, alkylated with iodoacetamide, and digested overnight with sequencing grade trypsin (Promega) or chymotrypsin (Roche).
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Applied Science) at 37 °C. Peptides were desalted and separated by reverse phase chromatography before introduction into a linear ion trap mass spectrometer (LTQ-XL, Thermo Scientific). The top five peaks in the MS1 scan (400–1700 m/z) were sequentially selected for fragmentation by collision-induced dissociation (normalized collision energy = 30, activation Q = 0.25, activation time = 30 ms). Dynamic exclusion was turned on (if 2 hits in 5 s then excluded for 20 s, list size = 200). MS2 spectra were scored against a yeast FASTA protein database (NCBI; 6298 entries) using the SEQUEST algorithm (version 27, revision 13). Search parameters included 1.6 Da/1.0 Da parent/fragment ion tolerances; +57 on Cys fixed modification; +16 on Met and +80 on Ser/Thr/Tyr variable modifications; and up to 2 missed cleavages. Results were imported into Scaffold (version 3.5; Proteome Software), and MS2 spectra were reanalyzed against a subset database using X!Tandem (version 2007.01.01.1). Peptide probabilities were scored using the Peptide Prophet algorithm, and a ≥90% threshold was utilized. Localization probabilities of post-translational modifications were scored using Scaffold PTM (version 2.1.1; Proteome Software).

RESULTS

VPA Causes a Decrease in MIPS Activity—The INO1 coding sequence (1578 bp) was amplified from yeast genomic DNA, tagged with His, and an Xpress epitope, and cloned under the GAL1 promoter in the expression vector pRD015 with URA3 as the selection marker (the construct referred to herein as pRDINO1) (Fig. 2A). To determine if the tagged MIPS is functional, pRDINO1 was transformed into ino1Δ cells that lack endogenous MIPS and therefore cannot grow independently in the absence of inositol. Transformants (ino1Δ-INO1) were selected on Ura− plates and then tested for growth in the absence of inositol (I−) in comparison with cells carrying the empty vector. The tagged MIPS rescued growth on I− medium (Fig. 2B).

We have previously shown that cells grown in the presence of VPA exhibit a decrease in levels of inositol and inositol-3-phosphate (33) and that VPA does not directly inhibit MIPS (31). To test the possibility that VPA indirectly inhibits MIPS activity by bringing about a post-translational change in the enzyme, we expressed and purified MIPS from ino1Δ-INO1 cells grown to the mid-logarithmic phase and treated with 1 mM VPA for 3 h. MIPS purified from the VPA-treated cells showed a decrease in activity of about 40% compared with enzyme purified from untreated cells, suggesting that MIPS is regulated post-translationally (Fig. 3A).

MIPS Is a Phosphoprotein—To determine if MIPS is post-translationally modified by phosphorylation, ino1Δ cells harboring the tagged INO1 were grown in the presence of 32P, with or without VPA, and MIPS was precipitated with anti-Xpress antibody using protein A plus protein G-conjugated Sepharose. The 32P-labeled MIPS protein was resolved using SDS-PAGE and visualized with Coomassie Blue and autoradiography. MIPS purified from VPA-treated cells showed a higher degree of phosphorylation compared with MIPS purified from untreated cells (Fig. 3B). Phosphoamino acid analysis of the 32P-labeled protein indicated that MIPS was phosphorylated mostly at serine residues, although a faint label of threonine residues was also detected (Fig. 3C).

To identify the putative phosphorylated residues, MIPS was overexpressed and purified from ino1Δ-INO1 cells grown to the mid-logarithmic phase. Duplicate samples of control (no treatment) and VPA (1 mM)-treated cells were utilized. The protein was purified using ProBond resin, dialyzed, concentrated, and digested with either trypsin or chymotrypsin. The resulting peptides were separated by reverse phase chromatography before introduction into a linear ion trap mass spectrometer (LTQ-XL, Thermo Scientific). The top five peaks in the MS1 scan (400–1700 m/z) were sequentially selected for fragmentation by collision-induced dissociation (normalized collision energy = 30, activation Q = 0.25, activation time = 30 ms). Dynamic exclusion was turned on (if 2 hits in 5 s then excluded for 20 s, list size = 200). MS2 spectra were scored against a yeast FASTA protein database (NCBI; 6298 entries) using the SEQUEST algorithm (version 27, revision 13). Search parameters included 1.6 Da/1.0 Da parent/fragment ion tolerances; +57 on Cys fixed modification; +16 on Met and +80 on Ser/Thr/Tyr variable modifications; and up to 2 missed cleavages. Results were imported into Scaffold (version 3.5; Proteome Software), and MS2 spectra were reanalyzed against a subset database using X!Tandem (version 2007.01.01.1). Peptide probabilities were scored using the Peptide Prophet algorithm, and a ≥90% threshold was utilized. Localization probabilities of post-translational modifications were scored using Scaffold PTM (version 2.1.1; Proteome Software).
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To determine which of the identified phosphorylation residues are functionally important for the activity of MIPS, two site mutants were constructed for each of the residues: 1) a phosphorylation-deficient mutant in which serine was changed to the unphosphorylatable alanine, or threonine was changed to valine and 2) a phosphomimetic mutant, in which serine was changed to the phosphorylation-mimicking aspartate (Fig. 6A). All of the mutations were confirmed by sequencing. Each mutant was constructed in two vectors: 1) a centromeric low expression vector, p415-ADH, driven by the ADH promoter, used for growth experiments and 2) a high expression vector, pRD015, driven by the GAL1 promoter, used for overexpression and purification of the enzyme. All constructs were transformed into ino1Δ cells. To assess the physiological effect of the mutations on growth, cells transformed with the empty vector, wild type INO1 gene, or mutated genes were grown on synthetic medium with or without inositol. When MIPS was expressed from the high copy vector, all of the site mutants supported growth of ino1Δ cells (data not shown). However, clear differences in growth were observed in ino1Δ cells transformed with the MIPS mutants on the low copy vector (Fig. 6B). Cells carrying the plasmid with wild type INO1 grew normally on inositol-deficient medium. Mutations in residues in the NAD-binding domain, Ser-184 and Ser-296, affected growth. Cells carrying S184A grew well, but S184D did not support growth, suggesting that phosphorylation of this residue inhibits the activity of MIPS and, hence, the synthesis of inositol. Both mutants S296A and S296D did not support growth on inositol-deficient medium, suggesting that a serine residue is essential at that position. Mutants of residue Ser-374 in the catalytic domain were also assessed. Cells carrying S374A grew well, but S374D did not support growth, suggesting that phosphorylation of this residue inhibits MIPS activity. Cells carrying mutations of Thr-48 and Ser-177 showed the same growth pattern as that of WT (data not shown), indicating that these sites are not critical or regulated by phosphorylation.

To determine if the altered growth patterns seen in the mutants were due to altered MIPS activity, wild type and mutant MIPS were overexpressed and purified from ino1Δ cells grown to the late log phase in selective medium supplemented with inositol. Using equivalent amounts of purified protein from all mutants, MIPS activity was assayed using the method of Barnett et al. (37). The activity of MIPS from phosphorylation-deficient mutants S184A and S374A was similar to or slightly greater than that of the wild type MIPS (Fig. 6C). In contrast, MIPS from the phosphomimetic mutants S184D and S374D showed a decrease in activity to about 30 and 60% of wild type levels, respectively. This suggests that phosphorylation of residues Ser-184 and Ser-374 has an inhibitory effect on the activity of MIPS. In contrast to Ser-184 and Ser-374, both mutations of residue Ser-296 (S296A and S296D), led to a decrease in MIPS activity (Fig. 6C), suggesting that a serine residue at this site is important for maintaining catalytic activity.

The effect of phosphorylation on MIPS activity was also addressed by dephosphorylating the protein using alkaline

**FIGURE 3.** MIPS is a phosphoprotein. A, MIPS purified from VPA-treated cells showed decreased activity. inoΔ cells harboring the pRD/INO1 vector were grown to the mid-logarithmic phase in inositol-free SM supplemented with galactose and raffinose. 1 ml VPA was added to one culture, and cells were incubated for 3 h and then harvested. MIPS was extracted and purified, and activity was assayed using the method of Barnett et al. (37). B, inoΔ cells harboring the tagged INO1 were resuspended in phosphate-free induction medium in the presence of 32P, with or without VPA for 1 h and then harvested. Cell extracts were precleared with protein G plus protein A-agarose and incubated overnight at 4 °C with anti-Xpress antibody and 50 μl of protein G plus protein A-agarose. After washing with buffer, the MIPS-anti-Xpress antibody-agarose complex was dissociated by boiling for 5 min and analyzed by SDS-PAGE. Bands were visualized by Coomassie Blue staining (a), and 32P-labeled MIPS was identified by phosphorimaging (b). Data represent three independent experiments. C, phosphoamino acid analysis of MIPS protein. 32P-labeled MIPS was digested with 6 N HCl. The acid was evaporated, and the sample was dissolved in 10 μl of TLE buffer (pH 1.9) and mixed with 1 μg of cold phosphoserine, phosphothreonine, and phosphotyrosine standards. The phosphoamino acids were separated in two dimensions by electrophoresis followed by thin layer chromatography. Ninhydrin staining of the TLC plate (right) shows the migration of the standards. An autoradiogram (left) shows the 32P-labeled phosphoamino acids present in MIPS. The data are representative of three independent experiments. Error bars, S.E.

**TABLE S1.** Complete MS/MS annotations for all phosphopeptide spectral identifications can be found in supplemental Fig. S1 and Table S1.

1. **A**. The termini of peptides do not yield fragmentation information as robustly as the central region; therefore, phosphosite determination for the 274SVDDIIASNDILYNDK91 phosphopeptide was not as direct. The b1–b9 ion series indicated that the phosphosite was either at Ser-374 or Ser-375 and not Ser-383 or Thr-388 (Fig. 5B). A doubly charged Y17 ion was consistently seen in MS/MS spectra that matched this phosphopeptide. Although it has relatively low abundance, this fragment ion indicated that Ser-375 was not phosphorylated (Fig. 5B). Finally, the serine residue at position 374 of yeast MIPS is conserved across disparate species (Fig. 5C). This correlative evidence suggests that Ser-374 has a critical role for protein function.

Three Phosphosites Modulate Activity of Yeast MIPS—To determine which of the identified phosphorylation residues are functionally important for the activity of MIPS, two site mutants were constructed for each of the residues: 1) a phosphorylation-deficient mutant in which serine was changed to the unphosphorylatable alanine, or threonine was changed to valine and 2) a phosphomimetic mutant, in which serine was changed to the phosphorylation-mimicking aspartate (Fig. 6A). All of the mutations were confirmed by sequencing. Each mutant was constructed in two vectors: 1) a centromeric low expression vector, p415-ADH, driven by the ADH promoter, used for growth experiments and 2) a high expression vector, pRD015, driven by the GAL1 promoter, used for overexpression and purification of the enzyme. All constructs were transformed into ino1Δ cells. To assess the physiological effect of the mutations on growth, cells transformed with the empty vector, wild type INO1 gene, or mutated genes were grown on synthetic medium with or without inositol. When MIPS was expressed from the high copy vector, all of the site mutants supported growth of ino1Δ cells (data not shown). However, clear differences in growth were observed in ino1Δ cells transformed with the MIPS mutants on the low copy vector (Fig. 6B). Cells carrying the plasmid with wild type INO1 grew normally on inositol-deficient medium. Mutations in residues in the NAD-binding domain, Ser-184 and Ser-296, affected growth. Cells carrying S184A grew well, but S184D did not support growth, suggesting that phosphorylation of this residue inhibits the activity of MIPS and, hence, the synthesis of inositol. Both mutants S296A and S296D did not support growth on inositol-deficient medium, suggesting that a serine residue is essential at that position. Mutants of residue Ser-374 in the catalytic domain were also assessed. Cells carrying S374A grew well, but S374D did not support growth, suggesting that phosphorylation of this residue inhibits MIPS activity. Cells carrying mutations of Thr-48 and Ser-177 showed the same growth pattern as that of WT (data not shown), indicating that these sites are not critical or regulated by phosphorylation.

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The effect of phosphorylation on MIPS activity was also addressed by dephosphorylating the protein using alkaline
Phosphatase. Dephosphorylation increased the activity of wild type MIPS by about 130% (Fig. 7). Dephosphorylation of the S184A and S374A mutants showed a smaller increase in activity (50 and 83% for S184A and S374A, respectively), suggesting that each of these putative inhibitory sites partially contributes to the overall inhibitory effect. Dephosphorylation of S296A caused more of an increase in activity than either S184A or S374A, suggesting that the two phosphosites Ser-184 and Ser-374 have a greater inhibitory effect than either site alone.

To examine the effect of a phosphorylated Ser-296, a double mutant S184A/S374A was constructed. Dephosphorylation of the double mutant did not significantly alter MIPS activity (Fig. 7), suggesting that under the physiological conditions in which the protein was purified, phosphorylation of Ser-296 was not significant.

Taken together, these results indicate that yeast MIPS is regulated by phosphorylation of at least three residues, two of which are inhibitory when phosphorylated (Ser-184 and Ser-374). The third residue, Ser-296, is strictly dependent on the presence of a serine at that position.

The Phosphorylation Sites are Conserved in Human MIPS—We previously cloned and expressed a human cDNA encoding MIPS and showed that human MIPS (hMIPS) is functional in yeast (31). Amino acid sequence alignment of yeast MIPS and hMIPS showed that the three residues we have found to modulate yeast MIPS are conserved in the human enzyme (Fig. 4B and supplemental Fig. S2). These hMIPS residues are Ser-177 and Ser-279, both of which are in the NAD-binding domain, and Ser-357, which lies in the catalytic domain (Fig. 4B). To determine if hMIPS is regulated by phosphorylation, the hINO1 gene was transferred from pRSETA-hINO1 (31) and cloned into the overexpression vector pRD015. Two independent mutations were created for each residue: Ser to Ala and Ser to Asp (Fig. 8A). All of the mutations were confirmed by sequencing.
To determine if any of the three conserved residues are important for the function of hMIPS, all constructs along with the controls were transformed into \textit{ino1}/H9004 cells. Transformants were grown on selective media with or without inositol. The wild type h\textit{INO1} gene rescued growth on I\textsuperscript{−} medium, indicating that hMIPS expressed from the pRD015 vector is functional in yeast (Fig. 8B). Mutations S177A and S177D did not support growth in the absence of inositol (Fig. 8B). Consistent with this observation, hMIPS purified from these mutants showed decreased activity (Fig. 8C), suggesting that a serine residue is required at this site for the activity of the enzyme. For residue Ser-279, the S279A mutation supported growth on I\textsuperscript{−} medium, although enzyme activity was lower than wild type. The S279D mutation did not support growth on I\textsuperscript{−} medium and caused a big decrease in activity, suggesting that phosphorylation of this residue inhibits hMIPS activity and, hence, the synthesis of inositol. The S357A mutation of the catalytic domain supported growth, but S357D did not. Consistent with this, S357A caused a slight decrease in activity, whereas S357D caused a decrease in activity, suggesting that phosphorylation of Ser-357 inhibits hMIPS activity.

Similar to the yeast enzyme, dephosphorylation of hMIPS led to increased activity (data not shown). Taken together, hMIPS, similar to its yeast counterpart, is regulated by phosphorylation of at least three residues, all of which are inhibitory when phosphorylated.

The S184A/S374A Double Mutation Confers Growth Advantage and Resistance to VPA—To determine the effect of loss of both Ser-184 and Ser-374 phosphosites, \textit{ino1}/H9004 cells were transformed with a vector containing the yeast INO1 gene with both S184A and S374A mutations. As shown in Fig. 9A, cells carrying the S184A/S374A mutated INO1 (DM) exhibited a greatly increased growth rate and reduced lag phase compared with cells carrying the wild type \textit{INO1} gene (WT). Importantly, the double mutant exhibited significantly better growth in the presence of VPA (Fig. 9A and B).

Consistent with increased growth conferred by the double mutation, the activity of the double mutant MIPS was almost twice that of wild type MIPS (Fig. 9C). Importantly, the decrease in activity in response to VPA was twice as much in wild type MIPS (50%) as in the double mutated MIPS (25%). Together, the data show that loss of the two phosphosites Ser-184 and Ser-374 causes a decrease in activity that is twice as much as the decrease caused by loss of either phosphorylation site separately.
DISCUSSION

The current study shows for the first time that MIPS is regulated at the post-translational level by phosphorylation. We report the following novel findings. 1) Yeast MIPS activity is regulated by phosphorylation of at least three residues. 2) Phosphorylation of the corresponding residues affects activity of human MIPS. 3) Eliminating the two inhibitory phosphosites confers resistance to VPA. These findings identify phosphorylation as a novel mechanism of regulation of inositol synthesis and suggest that VPA-mediated inositol depletion may result from phosphorylation of MIPS.

MIPS was identified as a phosphoprotein by phospholabeling and phosphoamino acid analysis (Fig. 3). Consistent with this finding, mass spectrometry identified five phosphosites in MIPS isolated from VPA-treated cells (Thr-48, Ser-177, Ser-184, Ser-296, and Ser-374) (Figs. 4 and 5 and supplemental Fig. S1). Mutation of three of the five sites modulated MIPS activity, including Ser-184, Ser-296, and Ser-374. Phosphomimetic mutations of these three sites decreased enzymatic activity. Among the three residues, only one phosphorylation-deficient mutation, S296A, decreased the activity of MIPS, indicating that this site is crucial for function.

The decreased activity of MIPS carrying either the S184D or S374D mutation suggests that phosphorylation of these two residues promotes conformational changes that alter the activity of the enzyme or block access of the substrate to its catalytic domain. These residues lie in two functionally critical domains. Residue Ser-184 is in the middle of the NAD —/H1001-binding domain, which encompasses residues 66–326, and is one of 14 residues that directly interact with NAD — (17). The adenine portion of NAD — specifically forms tight hydrogen bonds between N1 and Ser-184 (16). Structural studies suggest that the binding of NAD — to the apoenzyme is a prerequisite for the ordered binding of the substrate glucose 6-phosphate to the active site (16, 38). Therefore, it is likely that NAD — binding is disrupted when phosphorylation of the corresponding residues affects activity of human MIPS. 3) Eliminating the two inhibitory phosphosites confers resistance to VPA. These findings identify phosphorylation as a novel mechanism of regulation of inositol synthesis and suggest that VPA-mediated inositol depletion may result from phosphorylation of MIPS.

184 and Ser-374 leads to increased MIPS activity and decreased sensitivity to VPA.
Phosphorylation Regulates myo-Inositol-3-phosphate Synthase

Ser-184 is phosphorylated, resulting in decreased activity of MIPS, which is dependent on NAD+ as a cofactor.

Residue Ser-374 lies within the catalytic domain of MIPS. For MIPS to complete its catalytic cycle, the active site folds and completely encapsulates the substrate in an extreme example of induced fit (16, 17, 38). We have previously shown that multi-substrate adducts that carry a phosphate group on their glucitol side are more potent inhibitors, suggesting that the presence of a phosphate group in the catalytic domain inhibits activity (39). Phosphorylation of Ser-374 may perturb access of the substrate to the catalytic domain or may destabilize the induced fit by creating steric hindrance, thus decreasing catalytic activity. Additionally, the catalytic domain is populated with hydrophobic residues (15, 17). The negative charge of the phosphate group may alter the electrostatic balance of these residues and thus cause further perturbation of enzyme activity.

Whereas the S184A and S374A mutations did not significantly affect activity, both S296A and S296D mutations decreased activity of the enzyme, suggesting that the presence of a serine residue at this location is critical. This residue is also in the NAD+-binding domain. Interestingly, Ser-296 is within a stretch of seven residues, all of which are completely conserved. It is possible that any distortion in this region destabilizes the protein structure or disrupts the interaction between monomers that is needed to form the tetrameric structure of the enzyme, leading to loss of activity.

Alignment of the amino acid sequences of hMIPS and yeast MIPS revealed a consensus of 49.3% (supplemental Fig. S2). Of the 533 amino acids of yeast MIPS, 263 residues are conserved. The highest level of conservation is in the catalytic domain (68%). The observation that the three regulatory sites identified in yeast MIPS are conserved in the human homolog was intriguing. The inability of hMIPS carrying the S357D mutation to rescue inositol auxotrophy of yeast ino1Δ cells (Fig. 8B) and the decreased activity of the enzyme (Fig. 8C) suggest that phosphorylation perturbs the active site or obstructs binding of the substrate. Similarly, phosphorylation of the human Ser-279 may perturb the activity of the enzyme as a consequence of altering its structure or obstructing the binding of NAD+. The decreased activity of both S177A and S177D of hMIPS suggests that a serine residue is critical at that position. The crystal structure of MIPS has been solved for S. cerevisiae (17, 40), Mycobacterium tuberculosis (41), and Archaeoglobus fulgidus (14). To our knowledge, the crystal structure of hMIPS has not been reported. The structural knowledge pertaining to the catalytic mechanism of MIPS is based largely on the S. cerevisiae and A. fulgidus models (14, 17). The catalytic and NAD+-binding domains of hMIPS are recognized based on sequence similarity with the yeast homolog. The current study is the first to shed light on the mechanism of regulation of hMIPS by characterizing the human protein.

Based on the prediction of NetPhos Yeast, the three identified residues Ser-184, Ser-296, and Ser-374 are potential phosphorylation sites for PKA, GSK3, and PKC, respectively. With regard to the human MIPS, human Ser-177 is predicted to be a phosphorylation site for PKA, GSK3, and PKC, respectively. With regard to the human MIPS, human Ser-177 is predicted to be a phosphorylation site for PKA, GSK3, and PKC, respectively. With regard to the human MIPS, human Ser-177 is predicted to be a phosphorylation site for PKA, GSK3, and PKC, respectively.
Phosphorylation Regulates myo-Inositol-3-phosphate Synthase

We have shown in a previous study that VPA causes a decrease in inositol 3-phosphate and myo-inositol (33), suggesting that VPA inhibits MIPS. Inhibition of MIPS by VPA was not observed in vitro (31), suggesting that VPA inhibition is indirect. Our current findings suggest that at least one mechanism whereby VPA causes inositol depletion may involve the phosphorylation of Ser-184 and Ser-374. First, phosphorylation of yeast MIPS is increased in cells grown in VPA (Fig. 3B). Second, the double mutation S184A/S374A partially rescues senitivity of yeast cells to VPA (Fig. 9, A and B) and increases activity of the enzyme (Fig. 9C). We speculate that VPA triggers a signal(s) that culminates in the phosphorylation of MIPS.

It is possible that MIPS is regulated by phosphorylation of additional sites, which may not have been detected in the current study. The use of the GAL1-driven vector was essential for obtaining sufficient amounts of the enzyme for characterizing MIPS activity and for the MS analysis. However, overexpression may have perturbed the stoichiometric balance of phosphorylation, thus underestimating the importance of phosphorylation of some residues. It is also possible that overexpression may have mistargeted the protein and exposed it to non-physiological phosphorylation. Phosphorylation of some amino acids may be transient (rapidly reversible). It is also possible that phosphorylation may occur in a sequential manner, in which a phosphate at one residue may be required to activate a kinase that phosphorylates another residue(s). For example, phosphorylation of Ser-374 may facilitate the phosphorylation of Ser-184 because the increase in activity following dephosphorylation of a single mutant is almost half of that observed for the WT. Alternatively, phosphorylation may occur at different growth stages in response to different cues or signals.

Although the replacement of serine with alanine or aspartate creates phosphodeficient or phosphomimetic mutations, respectively, these mutations may not recapitulate the phosphorylated residues’ functions. The mutations may alter the structural conformation of the enzyme, triggering changes in activity that are not necessarily reflective of a phosphosite. However, our findings are strongly supported by MS data, which unambiguously show that the residues identified in MIPS are phosphosites. The correlation between the growth experiments and enzyme activity demonstrates that MIPS activity is regulated by phosphorylation.

The work reported here identifies for the first time a novel mechanism of regulation of inositol biosynthesis. The knowledge that MIPS is regulated by phosphorylation will facilitate studies to identify signals that play a role in regulating this enzyme, which is crucial for maintaining inositol homeostasis.

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