Insig Regulates HMG-CoA Reductase by Controlling Enzyme Phosphorylation in Fission Yeast

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DOI 10.1016/j.cmet.2008.09.004

INTRODUCTION
Cholesterol biosynthesis is a tightly regulated pathway that employs multiple feedback mechanisms to maintain homeostasis (Goldstein and Brown, 1990). The first committed step in sterol synthesis, the NADPH-dependent reduction of HMG-CoA to mevalonate, is catalyzed by HMG-CoA reductase (HMGR) at the endoplasmic reticulum (ER) membrane. HMGR, an eight-span integral membrane protein, is regulated by feedback mechanisms operating at multiple levels: transcription, translation, postranslational modification, and protein degradation (Goldstein and Brown, 1990). Insig, a resident ER membrane protein, regulates both transcription and degradation of HMGR (Goldstein et al., 2006). In fission yeast, Insig regulates sterol synthesis by a different mechanism than in mammalian cells, controlling HMGR phosphorylation in response to nutrient supply.

SUMMARY
Insig functions as a central regulator of cellular cholesterol homeostasis by controlling activity of HMG-CoA reductase (HMGR) in cholesterol synthesis. Insig both accelerates the degradation of HMGR and suppresses HMGR transcription through the SREBP-Scap pathway. The fission yeast Schizosaccharomyces pombe encodes homologs of Insig, HMGR, SREBP, and Scap, called ins1+, hmg1+, sre1+, and scp1+. Here, we characterize fission yeast Insig and demonstrate that Ins1 is dedicated to regulation of Hmg1, but not the Sre1-Scp1 pathway. Using a sterol-sensing domain mutant of Hmg1, we demonstrate that Ins1 binding to Hmg1 inhibits enzyme activity by promoting phosphorylation of the Hmg1 active site, which increases the K_M for NADPH. Ins1-dependent phosphorylation of Hmg1 requires the MAP kinase Sty1/Spc1, and Hmg1 phosphorylation is physiologically regulated by nutrient stress. Thus, in fission yeast, Insig regulates sterol synthesis by a different mechanism than in mammalian cells, controlling HMGR phosphorylation in response to nutrient supply.

HMG is also subject to posttranslational modification that regulates enzyme activity. When the cellular AMP-ATP ratio increases, AMP-activated protein kinase (AMPK) phosphorylates a conserved serine in the enzyme active site, corresponding to human HMGR S872 (Omkumar et al., 1994; Sato et al., 1993). Based on the crystal structure of the HMGR catalytic domain, phosphorylation of S872 was proposed to interfere with NADPH binding (Istvan et al., 2000). This phosphorylation reversibly inhibits HMGR and is thought to help the cell limit ATP expenditure in response to metabolic stress (Hardie et al., 2006).

Our recent studies in fission yeast revealed an orthologous SREBP pathway that controls adaptation to hypoxia (Hughes et al., 2005; Todd et al., 2006). S. pombe has single homologs of Scap, SREBP, Insig, and HMGR called scp1+, sre1+, ins1+, and hmg1+, respectively. Scp1 is required for proteolytic activation of Scp1 under low oxygen, which inhibits sterol synthesis. Under low oxygen, Sre1 upregulates genes required for adaptation to low-oxygen growth, promoting cell survival. Interestingly, Ins1 is not required for Scp1-dependent regulation of Sre1 in fission yeast, and the function of fission yeast Ins1 is unknown (Hughes et al., 2005). In budding yeast, the Insig homolog called Nsg1p regulates the proteasomal degradation of HMGR in response to elevated levels of the downstream isoprenoid product farnesol (Flury et al., 2005). However, in contrast to mammalian Insig, Nsg1p is a positive regulator of Hmg2p. Nsg1p is proposed to act as a chaperone, increasing Hmg2p folding and decreasing ubiquitination and degradation.

In the current study, we examined the function of Ins1 and its role in regulation of fission yeast HMGR and the SREBP pathway. In contrast to mammalian cells, we find that Ins1 is dedicated to control of the HMGR pathway. Ins1 controls Hmg1 activity by binding to Hmg1 and promoting phosphorylation of a conserved serine and a nonconserved threonine in the Hmg1 catalytic domain. Kinetic studies demonstrate that Ins1-dependent
phosphorylation inhibits Hmg1 activity and increases the KM for NADPH. Furthermore, we have shown that nutrient availability and osmotic stress regulate Ins1-dependent Hmg1 phosphorylation through the mitogen-activated protein kinase (MAPK) Sty1/Spc1. Our study in fission yeast outlines a mechanism for Insig-dependent regulation of HMGR and establishes a unique model for future Insig-HMGR structure-function studies.

RESULTS

Ins1 Binds Hmg1 but Not Scp1

Sequence homology searches identified a fission yeast homolog of mammalian Insig, which we named Ins1 (Hughes et al., 2005; Loewen and Levine, 2002). Ins1 contains overall low sequence identity with human Insig, but is predicted to contain six transmembrane segments and share membrane topology with its mammalian homolog (Figure 1A) (Feramisco et al., 2004). To investigate the function of fission yeast Insig, we purified Ins1-binding proteins. For this experiment, we constructed ins1-TAP, a yeast strain expressing Ins1 with a COOH-terminal tandem affinity purification (TAP) tag expressed from the endogenous promoter. Using this strain, we conducted a two-step purification of Ins1-TAP and identified copurifying proteins by mass spectrometry (Table S1). With the exception of Ins1 itself, the most abundant protein in the purification was Hmg1, the S. pombe homolog of HMGR (Lum et al., 1996). Ins1-Hmg1 binding was confirmed in a coimmunoprecipitation experiment using a strain expressing Scp1-Myc and Ins1-TAP from endogenous promoters (Figure 1B). These data indicate that Ins1 forms a specific complex with Hmg1, but not with Scp1.
Ins1 Is Required for Lipid Homeostasis and Does Not Control Hmg1 Stability

To investigate whether Ins1 binding to Hmg1 affects enzyme activity, we analyzed sterol pathway intermediates from wild-type or ins1Δ cells by gas chromatography (Figure 1C). We observed increased amounts of the intermediates squalene and lanosterol in ins1Δ cells as compared to wild-type cells. To determine whether the observed changes were consistent with increased HMGR activity, we analyzed a strain overexpressing hmg1Δ from the nmt1Δ promoter (nmt-hmg1Δ). The nmt-hmg1Δ strain had elevated amounts of squalene and lanosterol, suggesting that ins1Δ cells may have increased HMGR activity. These data suggest that Ins1 may negatively regulate Hmg1 activity.

In mammalian cells, Insig binding to HMGR leads to polyubiquitination and degradation of HMGR (Sever et al., 2003). To test whether Ins1 regulates the turnover of Hmg1 in fission yeast, we examined the stability of Hmg1 in wild-type or ins1Δ cells in a cycloheximide chase experiment (Figure 1D). Consistent with a previous study (Lum et al., 1996), we found Hmg1 to be a long-lived protein. No degradation of Hmg1 was apparent after 4 hr of cycloheximide treatment in wild-type cells (Figure 1D, lanes 1–5), and deletion of ins1Δ had no effect on the stability of Hmg1 (Figure 1D, lanes 6–10). In addition, untreated samples from ins1Δ and ins1Δ cells contained equal amounts of Hmg1 (Figure 1D, lanes 1 and 6), indicating that Ins1 does not affect steady-state levels of Hmg1. As a control, the same extracts were immunoblotted for the precursor form of Sre1, which was rapidly turned over in both strains with a half-life of less than 1 hr (Figure 1D, lower panel). These data demonstrate that fission yeast Hmg1 is a stable protein and suggest that Ins1 may regulate Hmg1 by a degradation-independent mechanism.

Ins1 Regulates Hmg1 Activity

Next, we asked whether Ins1 affects the enzyme activity of Hmg1 as measured in vitro. To test this, we prepared microsomes from wild-type or ins1Δ cells. HMGR activity was measured in vitro for each strain and plotted in Michaelis-Menten formats. At near-saturating NADPH concentration (2 mM), microsomes from ins1Δ cells showed a 2-fold increase in HMGR activity compared to Hmg1 microsomes (Figure 2G, lanes 1 and 3), and deletion of ins1Δ had no effect on HMGR activity (Figure 2G, lanes 1 and 3). Microsomes assayed from each strain contained equal amounts of Hmg1 (Figure 2G, lower panel). Thus, Ins1 inhibition of Hmg1 activity was absent in cells expressing Hmg1-FF (Figure 2H). Taken together, these data suggest that Ins1 inhibition of Hmg1 activity requires a physical interaction between the two proteins.

Ins1 Inhibition of Hmg1 Activity Requires Ins1-Hmg1 Binding

Mammalian Insig binds to the membrane-embedded, sterol-sensing domain of HMGR (Sever et al., 2003). To investigate whether Ins1-dependent inhibition of Hmg1 activity required Ins1 binding to Hmg1, we used site-directed mutagenesis of conserved regions in the Hmg1 sterol-sensing domain to generate Hmg1 mutants defective for binding to Ins1. Mutants were integrated onto the chromosome at the hmg1Δ locus, replacing the endogenous gene, and binding to Ins1 was assayed by coimmunoprecipitation with anti-Ins1 antibodies. One Hmg1 mutant (F358A,F359A; called Hmg1-FF) showed reduced Ins1 binding compared to wild-type Hmg1 (Figure 2F, lanes 1 and 2). Disruption of Ins1-Hmg1 binding required mutating both phenylalanine residues (Figure 2F, lanes 3 and 4). Importantly, the Hmg1 mutants were expressed at wild-type levels (Figure 2F, lanes 5–8).

Next, we tested whether Ins1 inhibition of Hmg1 enzyme activity required Ins1-Hmg1 binding. We assayed HMGR activity in both ins1Δ and ins1Δ yeast expressing either hmg1Δ or hmg1-FF. Deletion of ins1Δ caused a 2-fold increase in HMGR activity in cells expressing wild-type Hmg1 (Figure 2G, lanes 1 and 2). Interestingly, Hmg1-FF microsomes showed a 2-fold increase in HMGR activity compared to Hmg1 microsomes (Figure 2G, lanes 1 and 3), and deletion of ins1Δ had no effect on HMGR activity (Figure 2G, lanes 3 and 4). Microsomes assayed from each strain contained equal amounts of Hmg1 (Figure 2G, lower panel). Thus, Ins1 inhibition of Hmg1 activity was absent in cells expressing Hmg1-FF (Figure 2H). Taken together, these data suggest that Ins1 inhibition of Hmg1 activity requires a physical interaction between the two proteins.

Ins1 Promotes Phosphorylation of Two Residues in the Hmg1 Catalytic Domain

Phosphorylation of the mammalian HMGR catalytic domain by AMPK inhibits enzyme activity under conditions of metabolic stress (Hardie et al., 2006). Thus, we investigated whether Ins1 could inhibit Hmg1 activity by controlling enzyme phosphorylation. To determine whether Hmg1 was phosphorylated, we cultured wild-type, ins1Δ, and hmg1Δ cells in the presence of 32P-labeled orthophosphate and immunoprecipitated Hmg1 from detergent-solubilized cell extracts. HMGR activity was supplied to the hmg1Δ strain from a plasmid expressing the catalytic domain of S. cerevisiae Hmg2p. Hmg1 was phosphorylated in wild-type cells (Figure 3A, lane 1). However, in ins1Δ cells, 32P-labeling of Hmg1 was markedly reduced (Figure 3A, lane 2). Cells lacking hmg1Δ showed no signal, confirming the specificity of the assay (Figure 3A, lane 3).

To determine the specific sites of phosphorylation, we constructed a strain that expressed Hmg1 with a COOH-terminal TAP tag, hmg1-TAP. Hmg1-TAP was purified from log phase cells, and mass spectrometry analysis identified peptides for 63% of Hmg1, including 76% coverage of the catalytic domain (amino acids 551–1053) (Figure S1). Two phosphorylated residues were detected: serine 1024 and threonine 1028 (Figure 3B). Of these, only S1024 and T1028 were found in Ins1-containing complexes (Figure 3B). Disruption of the endogenous hmg1Δ locus, replacing the endogenous gene, and binding to Ins1 was assayed by coimmunoprecipitation with anti-Ins1 antibodies. One Hmg1 mutant (F358A,F359A; called Hmg1-FF) showed reduced Ins1 binding compared to wild-type Hmg1 (Figure 2F, lanes 1 and 2). Disruption of Ins1-Hmg1 binding required mutating both phenylalanine residues (Figure 2F, lanes 3 and 4). Importantly, the Hmg1 mutants were expressed at wild-type levels (Figure 2F, lanes 5–8).

Next, we tested whether Ins1 inhibition of Hmg1 enzyme activity required Ins1-Hmg1 binding. We assayed HMGR activity in both ins1Δ and ins1Δ yeast expressing either hmg1Δ or hmg1-FF. Deletion of ins1Δ caused a 2-fold increase in HMGR activity in cells expressing wild-type Hmg1 (Figure 2G, lanes 1 and 2). Interestingly, Hmg1-FF microsomes showed a 2-fold increase in HMGR activity compared to Hmg1 microsomes (Figure 2G, lanes 1 and 3), and deletion of ins1Δ had no effect on HMGR activity (Figure 2G, lanes 3 and 4). Microsomes assayed from each strain contained equal amounts of Hmg1 (Figure 2G, lower panel). Thus, Ins1 inhibition of Hmg1 activity was absent in cells expressing Hmg1-FF (Figure 2H). Taken together, these data suggest that Ins1 inhibition of Hmg1 activity requires a physical interaction between the two proteins.

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To confirm that S1024 and T1028 were the principal sites of Hmg1 phosphorylation, strains were constructed with mutant hmg1Δ alleles in which alanine was substituted for S1024,
T1028, or both S1024 and T1028 (called hmg1-ST). Mutation of S1024 to alanine reduced Hmg1 phosphorylation (Figure 3C, lanes 1 and 2). Mutation of T1028 or both S1024 and T1028 to alanine reduced Hmg1 phosphorylation to the same level seen in hmg1+ cells lacking ins1+ (Figure 3C, lanes 3–5). Equal amounts of Hmg1 protein were immunoprecipitated from each strain (Figure 3C, bottom panel). Importantly, these mutant Hmg1 proteins retained the ability to bind Ins1 (Figure S2). These results confirm that S1024 and T1028 are the primary sites of phosphorylation in Hmg1.

Next, we examined Hmg1 phosphorylation using phosphospecific antibodies. For S1024, we developed a polyclonal antibody that specifically recognized phosphorylated S1024 (see Experimental Procedures). For T1028, we used a commercially available antibody that recognized phosphothreonine followed by proline. T1028 is the only threonine in Hmg1 followed by a proline. To characterize the Ins1-dependent phosphorylation of Hmg1, detergent-solubilized extracts from ins1+ strains expressing wild-type Hmg1, Hmg1-S1024A, Hmg1-T1028A, or Hmg1-ST, or an ins1Δ strain expressing wild-type Hmg1 were subjected to Hmg1 immunoprecipitation followed by immunoblotting with phosphospecific S1024 and T1028 antibodies. In cells expressing wild-type Hmg1, both S1024 and T1028 were phosphorylated (Figure 3D, lane 1). Mutating S1024 to alanine reduced phosphorylation at T1028 (Figure 3D, lane 2). Interestingly, mutating T1028 to alanine completely blocked phosphorylation of S1024 (Figure 3D, lane 3). When either S1024 or T1028 was mutated to alanine, no signal was detected for that residue, confirming antibody specificity (Figure 3D, lanes 2 and 3). Remarkably, deletion of ins1+ resulted in loss of phosphorylation at both S1024 and T1028 (Figure 3D, lane 5). In addition, Hmg1 phosphorylation required binding to Ins1 as Hmg1 was not phosphorylated at either S1024 or T1028 in hmg1-FF cells (Figure 3D). Collectively, these results demonstrate that Hmg1 phosphorylation at S1024 and T1028 requires binding to Ins1.
Figure 3. Phosphorylation of Hmg1 S1024 and T1028 Requires Binding to Ins1 and Is Required for Ins1-Dependent Inhibition of Hmg1 Activity

(A) Hmg1 was immunoprecipitated from the indicated strains labeled with 32P H3PO4 for 3 hr. Samples were analyzed by SDS-PAGE, followed by autoradiography (top panel) or immunoblotting with anti-Hmg1 IgG (bottom panel).

(B) BLAST alignment of fission yeast Hmg1 and human HMGR. Phosphorylated residues are boxed.

(C) Hmg1 was immunoprecipitated from the indicated strains labeled with 32P H3PO4 as in (A).

(D) Hmg1 activity (nmol min^-1 mg^-1)

(E) Hmg1 activity (ins1Δ/ins1+).

(F) Hmg1 activity (nmol min^-1 mg^-1).

(G) Hmg1 activity (ins1Δ/ins1+).

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Ins1-Dependent Inhibition of Hmg1 Activity Requires Hmg1 S1024 and T1028

To investigate whether Ins1-dependent inhibition of Hmg1 activity required the ability to phosphorylate Hmg1 S1024 and/or T1028, we assayed HMGR activity in microsomal extracts from ins1Δ or ins1Δ cells expressing wild-type Hmg1, Hmg1-S1024A, Hmg1-T1028A, or Hmg1-ST. As expected, deletion of ins1Δ had no effect on HMGR activity, demonstrating that these residues are required for Ins1-dependent inhibition of Hmg1 activity (Figure 3F, lanes 3–8; Figure 3G, lanes 2–4). Hmg1-S1024A, Hmg1-T1028A, and Hmg1-ST all showed wild-type binding to Ins1, and Ins1 levels were comparable to wild-type in these strains (Figure S2). Microsomes from hmg1-S1024A or hmg1-ST cells had reduced HMGR activity in the absence of Ins1 (Figure 3F, lanes 4 and 8), suggesting that mutating S1024 altered the catalytic properties of the enzyme. These data show that Ins1-dependent inhibition of Hmg1 activity requires the ability to phosphorylate Hmg1 S1024 and T1028.

Hmg1 Phosphorylation Is Induced by Nutrient Stress

To understand the physiological role of Hmg1 phosphorylation, we sought to identify conditions under which Hmg1 phosphorylation was regulated. We observed that Hmg1 phosphorylation was reduced when cells were grown in rich medium containing yeast extract. To test whether nutrient supply regulates Hmg1 phosphorylation, we grew cells in nutrient-rich medium and then shifted cells to nutrient-poor minimal medium. Collectively, these data demonstrate that the upstream Sty1-activating kinases Wis4 and Wis1.

Sty1 Activation Is Sufficient to Induce Hmg1 Phosphorylation

Because Hmg1 phosphorylation requires Sty1 (Figure 4B), and Hmg1 phosphorylation is rapidly induced by the transition from rich to minimal medium (Figure 4A), we next asked whether Sty1 was required for Hmg1 phosphorylation. To test whether Sty1 activation by high salt was sufficient to induce Hmg1 phosphorylation, we used gas chromatography to analyze a collection of 86 S. pombe kinase deletion strains (Bimbo et al., 2005). We anticipated that strains defective in Hmg1 phosphorylation would have elevated levels of squalene due to increased Hmg1 activity (Figure 1C). Using this method, we identified a strain lacking sty1Δ/spot1Δ, strain regulation of HMG-CoA reducense in fission yeast.

DISCUSSION

In this study, we investigated Insig function and regulation of HMGR in fission yeast. As in mammals, fission yeast Insig is a negative regulator of HMGR. However, in contrast to mammalian cells, Ins1 regulates Hmg1 by a nondegradative mechanism, and Ins1 does not function in the SREBP pathway. Several lines of evidence support a mechanism for Ins1-dependent inhibition of Hmg1, in which Ins1 binds to Hmg1 and promotes phosphorylation of the catalytic domain, thereby increasing the Km for NADPH and reducing Hmg1 activity. First, Ins1 binds to Hmg1.
but does not control Hmg1 stability (Figure 1). Second, binding of Ins1 to Hmg1 inhibits Hmg1 activity and increases the $K_M$ of the enzyme for NADPH (Figure 2). Third, Ins1 binding to Hmg1 promotes phosphorylation of Hmg1 at S1024 and T1028, residues in the enzyme active site (Figure 3) (Istvan et al., 2000). Lastly, S1024 and T1028 are required for Ins1-dependent inhibition of Hmg1 activity (Figure 3).

As in mammals, Ins1 likely binds to the NH$_2$-terminal sterol-sensing domain of Hmg1 insomuch as mutation of two conserved sterol-sensing domain phenylalanines in Hmg1 disrupted both binding to Ins1 and Hmg1 phosphorylation (Figure 2). We were unable to directly assay binding of Ins1 to the Hmg1 NH$_2$-terminal membrane domain because these truncated Hmg1 constructs were not stably expressed in S. pombe (data not shown). These corresponding phenylalanine residues in human HMGR (F211 and F212) may also function in Insig binding as they reside in transmembrane segment 6, which has been shown to be important for sterol-regulated degradation of HMGR (Xu and Simoni, 2003).

Hmg1 phosphorylation at S1024 and T1028 requires binding to Ins1 (Figure 3E). But how does Ins1 promote Hmg1 phosphorylation? Ins1 could recruit a kinase or binding to Ins1 could alter the conformation of Hmg1, making it a kinase substrate. Alternatively, Hmg1 phosphorylation may be regulated at the level of kinase activation or localization, while Ins1 and Hmg1 bind constitutively. In addition, phosphorylation of Hmg1 may

**Figure 4. Sty1 Is Required for Hmg1 Phosphorylation in Response to Nutrient Supply**

(A) Wild-type cells were grown in YES medium overnight. Cells were collected by centrifugation and resuspended in EMM. After 60 min, yeast extract (5 g/L) was added to the culture. Samples were taken at the indicated times, and Hmg1 immunoprecipitates were blotted using anti-Hmg1 IgG or phosphospecific antibodies.

(B) Hmg1 was immunoprecipitated from the indicated strains cultured in EMM and samples were blotted using anti-Hmg1 IgG or phosphospecific antibodies.

(C) sty1-12myc cells were grown in YES medium overnight. Cells were collected by filtration and resuspended in EMM, or were exposed to 0.6 M KCl for 30 min in YES. Protein samples taken at the indicated times were blotted with anti-phospho-p38 MAPK or anti-myc antibodies.

(D) sty1-12myc, sty1Δ, and sty1-12myc ins1Δ cells were grown in YES in the absence or presence of 0.6 M KCl for 45 min. Hmg1 immunoprecipitates (top three panels) or whole-cell extracts (bottom panels) were immunoblotted using indicated antibodies.
be controlled by regulating binding between Hmg1 and Ins1, analogous to sterol-regulated binding of HMGR and Insig in mammalian cells (Sever et al., 2003). Regulation of Hmg1 phosphorylation by both Ins1 binding and kinase activity is an attractive model insasmuch as this would permit integration of feedback signals from the sterol biosynthetic pathway and kinase signaling pathways. Thus far, we have been unable to detect changes in Ins1-Hmg1 binding in response to hypoxia, growth medium, or a variety of sterol synthesis inhibitors (data not shown). This could indicate that while Ins1-Hmg1 binding is required for Hmg1 phosphorylation, the regulated step is kinase activation rather than Ins1-Hmg1 binding. It is also possible that our Ins1-Hmg1 binding assay does not accurately reflect in vivo binding dynamics. Experiments investigating the regulation of Ins1-Hmg1 binding are ongoing.

In our model, Ins1-dependent regulation of Hmg1 activity functions through the phosphorylation of S1024 and T1028. Interestingly, Hmg1 S1024 corresponds to S872 of human HMGR, which is the target of AMPK-dependent phosphorylation. Based on the crystal structure of the HMGR catalytic domain, phosphorylation of HMGR S872 was predicted to interfere with NADPH binding to the active site of the enzyme (Istvan et al., 2000). Our data support this hypothesis as Ins1-dependent phosphorylation increases the $K_M$ of Hmg1 for NADPH (Figure 2).

Despite the conservation of HMGR phosphorylation in fission yeast, AMPK does not appear to regulate yeast Hmg1: (1) The amino acid sequence surrounding Hmg1 S1024 and T1028 does not match the consensus recognition motif for mammalian AMPK or the S. cerevisiae AMPK homolog Snf1p (Dale et al., 1995); and (2) cells lacking the catalytic subunit of S. pombe AMPK, Ssp2, showed no defect in Hmg1 phosphorylation (data not shown) (Townley and Shapiro, 2007). Whether Insig regulates HMGR phosphorylation by AMPK in mammalian cells has not been directly investigated. However, HMGR was subject to phosphorylation in liver extracts from Insig-1-/-/Insig-2-/- mice, indicating that HMGR is regulated by phosphorylation in the absence of Insig (Engelking et al., 2005). Because of this, it is unlikely that the regulatory mechanism described here also functions in mammalian cells.

Hmg1 phosphorylation is regulated by a stress-activated MAP kinase pathway comprised of Wis4-Wis1-Sty1 in response to nutrient supply (Figure 4). Hmg1 phosphorylation is induced in nutrient-poor medium and requires Sty1, which is activated by this nutrient stress (Shiozaki and Russell, 1995). Sty1 is also activated by a variety of other environmental stimuli including osmotic, oxidative, and heat stress (Shiozaki et al., 1998). Importantly, activation of Sty1 by osmotic stress induces Hmg1 phosphorylation, and thus Sty1 may regulate phosphorylation of Hmg1 under a wide range of environmental conditions (Figure 4D). Interestingly, a recent study showed that Sty1 is also activated in response to a poor nitrogen source through the TOR nutrient-sensing pathway (Petersen and Nurse, 2007). Additional experiments are required to determine whether the Sty1-dependent phosphorylation of Hmg1 is direct, and whether TOR signaling also regulates Hmg1.

Sty1 is required for Hmg1 phosphorylation at both S1024 and T1028 in minimal medium, but the phosphorylation defect in cells lacking Sty1 is more pronounced at T1028 than S1024 (Figure 4B). T1028 is adjacent to P1029, forming a typical threonine-proline MAPK phosphorylation site that may serve as a priming phosphorylation to enhance phosphorylation of S1024. In this case, additional Hmg1 kinases may exist. We were unable to determine the relative contribution of the individual residues to inhibition of Hmg1 activity, because phosphorylation of S1024 requires the presence of T1028 (Figure 3D), and mutation of S1024 to alanine reduced Hmg1 activity independent of Ins1 (Figure 3F).

In this study, we describe an Insig-dependent mechanism for phosphoregulation of HMGR in fission yeast. Collectively, our data support a model in which cells sense extracellular nutrient supply and signal through a MAP kinase cascade to regulate Hmg1 and sterol biosynthesis in response to nutrient availability. Future studies will investigate the nutrient signals that regulate the Hmg1 kinase(s). Furthermore, the conservation of Insig-HMGR binding in fission yeast will facilitate structure-function studies of this interaction.

EXPERIMENTAL PROCEDURES

Yeast cell culture, immunoblot analysis, sterol analysis, and gas chromatography were performed as described previously (Hughes et al., 2005, 2007). A complete description of materials, strain construction, TAP, 32P-labeling, and other methods is available in Supplemental Experimental Procedures.

Strains and Media

Wild-type haploid S. pombe KG4125 (h+, his3-D1, leu1-32, ura4-D18, ade6-m210) and derived strains were grown to log phase at 30°C in EMM (Edinburgh minimal medium containing 225 µg/ml each of uracil, adenine, leucine, histidine, and lysine) unless otherwise indicated. YES medium contained 0.5% (w/v) yeast extract plus 3% (w/v) glucose and supplements, 225 µg/ml each of uracil, adenine, leucine, histidine, and lysine. Mutations in hmg1 were made at the endogenous hmg1 locus. hmg1Δ was constructed by transforming RWP492 (h+, hmg1::ura4Δ, -leu1-32, ade6-m216) with the plasmid pLP235 (both kind gifts of Robin Wright), which encodes the catalytic domain of the Saccharomyces cerevisiae HMG2 gene under control of the S. pombe adh1 promoter (Lum et al., 1996). The S. pombe strain GD1942 expressing Sty1-12myc from the endogenous sty1Δ promoter (h+, sty1-12myc::ura4Δ, -leu1-32, ura4-D18) was a gift of Paul Russell and Janni Petersen (Gaits et al., 1998). The nonessential kinase deletion strain collection, including sty1Δ (h+, sty1Δ::ura4Δ, -leu1-32, ura4-D18) and wis4Δ (h+, wis4::ura4Δ, -leu1-32, ura4-D18), was a gift of Jianhua Liu (Bimbo et al., 2005). wis1Δ (h+, wis1::kanR, his3-D1, leu1-32, ura4-D18, ade6-m210) was generated by homologous recombination (Bahler et al., 1998).

Antibodies

Polyclonal antisera recognizing Hmg1 (aa 35–204) and Ins1 (aa 1–80) were generated by immunizing rabbits with bacterially expressed antigen using a standard protocol (Covance). Anti-ins1 IgG was conjugated to horseradish peroxidase using the EZ-Link Activated Peroxidase Kit (Pierce). Rabbit polyclonal antibodies recognizing phosphorylated Hmg1 S1024 were prepared by immunizing rabbits with the peptide SHIGLNR[pS]ALN (21st Century Biochemicals). Anti-phospho-p38 MAPK antibody recognizing phospho- Thr180/Tyr182 was obtained from Cell Signaling Technology (D21C). Anti-phospho-p38 MAPK antibody recognizing phospho-Thr180/Tyr182 was obtained from Cell Signaling Technology (D21C). Monoclonal anti-c-Myc antibody 9E10 was obtained from Santa Cruz Biotechnology (sc-40).

Immunoprecipitation

For Ins1 immunoprecipitation, cells (5 x 10^8) grown as indicated in figure legends were lysed using glass beads (0.5 mm, Sigma) in 50 µl Digitonin Lysis Buffer (6 mM NaHPO4, 4 mM NaH2PO4, pH 7.2, 1% (w/v) digitonin, 150 mM NaCl, 4 mM EDTA, 50 mM NaF, 0.3 mM Na3VO4 plus protease inhibitors. Insoluble material was removed by centrifugation at 1 x 10^6 g for 10 min, and the resulting supernatant was subjected to immunoprecipitation in 1 ml of lysis buffer with 1 µg of the indicated antibody.
Plates were developed in 1:1 acetone/benzene, and the region containing 14C-labeled mevalonolactone was applied to Silica Gel 60 F254 glass-backed thin-layer plates (Merck). Reactions were carried out as previously described with modifications (Philipp and Shapiro, 1979). Microsomes (3.75 µg) were incubated with NADPH and 14C HMG-CoA in a final volume of 50 µl. Unless otherwise indicated, HMGR Activity Assay and NADPH were used at final concentrations of 0.24 mM and 2.0 mM, respectively. Reactions were stopped by addition of 12.5 µl of 5 N HCl, followed by incubation at 37°C for 2 hr. Insoluble material was removed by centrifugation at 16,000 g for 2 min, and 10 µl of the resulting supernatant was applied to Silica Gel 60 F254 glass-backed thin-layer plates (Merck). Plates were developed in 1:1 acetone/benzene, and the region containing 14C-labeled mevalonolactone was identified by direct comparison to an unlabeled mevalonolactone standard visualized by iodine vapor. 14C-labeled mevalonolactone was scraped from the plate and quantified by liquid scintillation counting. Kd and Vmax values were calculated using the program R (R Development Core Team, 2007) and plotted using GnuPlot (Williams and Kelley, 1999).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, two figures, and two tables and can be found online at http://www.cell.com/cellmetabolism/supplemental/S1550-4131(08)00288-X.

ACKNOWLEDGMENTS

We thank Jon Lorsch and Martin Bard (Indiana University-Purdue University Indianapolis) for experimental advice; members of the Espenshade lab for reviewing this manuscript; Shan Zhao and Sung-tse Wu (IUPUI) for excellent technical assistance; Robin Wright (University of Minnesota), Paul Russell (Scripps Research Institute), Janni Petersen (University of Manchester), and Jiangua Liu (Genome Institute of Singapore) for yeast strains and reagents; and Ross Tomai and Dr. Steven Gygi (Harvard Taplin Biological Mass Spectrometry Facility) for performing LC-MS/MS. This work was supported by grants from NIH (HL077588, P.E.; T32-GM007445, J.B.; GM64779, HL68744, ES11993, and CA098131, A.L.; and DK176743 and T32-HL09765, D.P.) A.H. is a recipient of the American Heart Association Predoctoral Fellowship 0515394U. P.E. is a recipient of a Burroughs Wellcome Fund Career Award in the Biomedical Sciences.

Received: March 10, 2008
Revised: July 21, 2008
Accepted: September 10, 2008
Published: December 2, 2008

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