Activation of an MAP Kinase Cascade Leads to Sir3p Hyperphosphorylation and Strengthens Transcriptional Silencing

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Abstract. During cell division and growth, the nucleus and chromosomes are remodeled for DNA replication and cell type–specific transcriptional control. The yeast silencing protein Sir3p functions in both chromosome structure and in transcriptional regulation. Specifically, Sir3p is critical for the maintenance of telomere structure and for transcriptional repression at both the silent mating-type loci and telomeres. We demonstrate that Sir3p becomes hyperphosphorylated in response to mating pheromone, heat shock, and starvation. Cells exposed to pheromone arrest in G_1 of the cell cycle, yet G_1 arrest is neither necessary nor sufficient for pheromone-induced Sir3p hyperphosphorylation. Rather, hyperphosphorylation of Sir3p requires the mitogen-activated protein (MAP) kinase pathway genes *STE11*, *STE7*, *FUS3/KSS1*, and *STE12*, indicating that an intact signal transduction pathway is crucial for this Sir3p phosphorylation event. Constitutive activation of the pheromone-response MAP kinase cascade in an *STE11-4* strain leads to hyperphosphorylation of Sir3p and increased Sir3p-dependent transcriptional silencing at telomeres. Regulated phosphorylation of Sir3p may thus be a mechanistically significant means for modulating silencing. Together, these observations suggest a novel role for MAP kinase signal transduction in coordinating chromatin structure and nuclear organization for transcriptional silencing.

SIGNAL transduction through mitogen-activated protein (MAP)[1] kinase cascades communicates extracellular signals to the nucleus and elicits distinct responses. In yeast, six different MAP kinase pathways have been described; they coordinate signals induced by such diverse stimuli as mating pheromone, heat shock, starvation, and changes in osmolarity (for reviews see Levin and Errede, 1995; Herskowitz, 1995). Induction of any of these MAP kinase pathways leads to transcriptional activation of many genes that are responsible for alterations in cellular structure and physiology. For example, reorganization of the nucleus may enable the cell to respond properly to extracellular signals.

Chromosomal structures, like telomeres and centromeres, as well as specialized regions of transcription, such as the nucleolus, specify discrete structural and functional domains in the nucleus. Some of these nuclear domains are distinguished by the nature of their resident chromatin: heterochromatic domains remain condensed during interphase, are often localized to the nuclear periphery, and are generally transcriptionally inactive; euchromatic regions, in contrast, have a more open chromatin conformation, are dispersed throughout the nucleus, and are typically transcriptionally active. The posttranslational modification of key chromatin proteins has been shown to contribute to local differences in chromatin structure (for review see Reeves, 1992). For example, phosphorylation of the linker histone H1 in *Tetrahymena* macronuclei is associated with nuclear domains that contain transcriptionally active chromatin, whereas dephosphorylated H1 is predominantly found within the condensed, transcriptionally inactive, chromatin bodies (Lu et al., 1995). In *Drosophila*, heterochromatin-associated protein HP1 becomes multiply phosphorylated during embryonic assembly of pericentric heterochromatin (Eissenberg et al., 1994). The kinases and phosphatases that regulate phosphorylation of these critical chromatin proteins have not been identified, nor are the signals known that must be activated for temporal and spatial modulation of chromatin structure during the life cycle of these organisms.

Silenced chromatin at the silent mating-type loci and telomeres in *Saccharomyces cerevisiae* defines functionally distinct nuclear domains. This chromatin is transcription-

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1. *Abbreviations used in this paper*: CIP, calf intestinal alkaline phosphatase; 5-FOA, 5-fluoroorotic acid; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAP kinase kinase; MEKK, MEK kinase; YPD, yeast extract/peptone/dextrose medium.

ally inactive, late replicating, and refractory to DNA-modifying enzymes, and it may be restricted to the nuclear periphery (for review see Laurenson and Rine, 1992; see also Nasmyth, 1982; Singh and Klar, 1992; Gottschling, 1992; Palladino et al., 1993; Loo and Rine, 1994). Sir3p, a key component of silenced chromatin in yeast, is essential for transcriptional silencing at both the silent mating-type loci and telomeres. Haploid *sir3* mutants are mating defective, due to the simultaneous expression of genes at the transcriptionally active mating-type locus *MAT* and the normally inactive *HM* silent loci (Rine and Herskowitz, 1987). Silencing of telomere-proximal reporter genes is exquisitely sensitive to Sir3p; such genes are completely transcriptionally active in *sir3* mutants and increasingly inactive upon increased *SIR3* gene dosage or tethering (Gottschling et al., 1990; Aparicio et al., 1991; Renauld et al., 1993; Lustig et al., 1996). Telomeres are shorter in *sir3* mutants, and the normal localization of telomeres at the nuclear periphery is disrupted (Palladino et al., 1993). Sir3p, in fact, localizes to distinct perinuclear foci (Palladino et al., 1993), similar to the patterns identified by immunolocalization of the telomere-binding protein Rap1p (Klein et al., 1992), suggesting that Sir3p is present at telomeres. In addition, Sir3p localization is lost in histone H4 mutants that are known to abolish silencing (Hecht et al., 1995), as well as in certain Rap1p mutants (Cockell et al., 1995). These observations point to a link between silencing functions and the structural integrity of the telomeres.

Data indicating that Sir3p interacts physically with other proteins involved in transcriptional silencing suggest that Sir3p is part of a macromolecular silencing complex. Sir3p coimmunoprecipitates with histones H3 and H4 glutathione-*S*-transferase fusion proteins (Hecht et al., 1995). In addition, interactions between Sir3p and Rap1p or Sir4p hybrid proteins have been observed in vivo (Moretti et al., 1994). Genetic interactions have also been observed between *SIR3* and *SIR1*, *SIR4*, *RAP1*, histone H4, and the telomeres themselves (Ivy et al., 1986; Marshall et al., 1987; Rine and Herskowitz, 1987; Johnson et al., 1990; Stone et al., 1991; Enomoto et al., 1994; Liu et al., 1994; Liu and Lustig, 1996). Together, these observations support a model in which Sir3p, histones H3 and H4, Rap1p, Sir4p, and possibly other silencing proteins form a multiprotein complex that modulates chromatin structure at the silent mating-type loci and at telomeres.

The wealth of evidence that establishes Sir3p as a central component of a silencing complex prompted us to begin evaluating properties of the protein itself. We present evidence that Sir3p is a phosphoprotein and that its phosphorylation is altered as a function of cellular physiology. Specifically, Sir3p is hyperphosphorylated in response to mating pheromone, heat shock, and starvation. Furthermore, we demonstrate that signal transduction through an MAP kinase cascade is required for pheromone-induced Sir3p hyperphosphorylation. Substantially increased transcriptional silencing occurs simultaneously with activation of the pheromone-response MAP kinase pathway and Sir3p hyperphosphorylation. Our data suggest that global signaling pathways may be critical for regulating Sir3p phosphorylation and transcriptional silencing in response to a variety of physiological challenges. Sir3p phosphorylation may thus link crucial physiological responses, via signal transduction, to changes in chromatin structure and nuclear organization.

Materials and Methods

Yeast strains, Media, and Growth Conditions

Yeast strains are listed in Table I. LPY771 was made with the use of plasmid YIpΔpep4 (kindly provided by A. Brake, University of California, San Francisco). LPY1683 is a haploid segregant from UCC3542 (isolated by E. Ray, University of Colorado, Boulder). LPY2495 and LPY2497 were disrupted for *STE7* or *STE12*, respectively, by PCR-directed mutagenesis (Baudin et al., 1993). Yeast extract/peptone/dextrose (YPD)–, yeast extract/peptone/glycerol (YPGlycerol)–, and yeast extract/peptone/galactose (YPGal)–rich media, or supplemented synthetic medium lacking the appropriate nutrient for plasmid selection, were prepared as described (Sherman, 1991), except 3% galactose was used for YPGal (McKinney et al., 1993). 5-fluoroorotic acid (5-FOA) plates were prepared by adding 5-FOA to 0.1% final concentration (Sikorski and Boeke, 1991) to supplemented synthetic medium. Phosphate-free growth medium has been described (Warner, 1991). Strains containing plasmids were grown in selective medium overnight, and then diluted into fresh rich medium for two or three generation times to reach logarithmic growth before making cell extracts. Strains were grown at 30°C unless otherwise indicated. α-factor (P. Chou, Chemical Synthesis Facility, Howard Hughes Medical Institute, University of Washington) was added to *bar1* strains to 1 μg/ml, or to *BAR1* strains to 10 μg/ml; nocodazole (Sigma Chemical Co., St. Louis, MO) was added to 10 μg/ml; and hydroxyurea (Sigma Chemical Co.) was added to a final concentration of 0.15 M. Cultures were then arrested by incubating for 2.5 h. To release cells from α-factor arrest, cells were washed with 5 vol of growth medium and collected by centrifugation or filtration. Cell cycle arrest and release was confirmed by flow cytometric analysis and/or by budding index determination (typical arrest resulted in >95% unbudded cells by α-factor, >90% budded cells by hydroxyurea, and >95% large-budded cells by nocodazole). Cyclin depletion experiments were performed as described (McKinney et al., 1993), except that cells were removed from galactose medium by centrifugation and washed in 2 vol of YPD, followed by arrest in YPD for 2.5 h with shaking at 30°C. Heat shock experiments performed at 37°C as reported here, or at 39°C after Kamada et al. (1995), yielded similar results. The sporulation medium used in this work consisted of 0.3% potassium acetate, 0.02% raffinose, and 25 μg/ml zinc acetate.

Yeast Plasmids and Transformation

pLP27, also known as pJR104 (kindly provided by J. Rine, University of California, Berkeley), is a 2μ-based plasmid containing the *URA3* selectable marker and the *SIR3* gene under its own promoter. pLP304 (kindly provided by C. Nislow, University of Colorado, Boulder) was made by introducing *SIR3* as a 4.5-kb SalI fragment in the 2μ-based *LEU2* plasmid YEp351 (Hill et al., 1986), where *SIR3* expression is also under control of its natural promoter. These constructs result in increased levels of Sir3p compared with endogenous levels of the protein (see Fig. 1 *A*). Experimental results for immunoblots were qualitatively identical, comparing strains expressing endogenous levels of *SIR3* from its chromosomal locus and strains carrying a multicopy *SIR3* plasmid. Strains carrying Sir3p-overproducing plasmids behave normally in all aspects examined, and the plasmids fully complement a *sir3* null mutant strain. To facilitate data presentation, many of the immunoblot experiments presented use strains carrying the multicopy *SIR3* plasmid pLP27, as indicated in the figure legends, since overproduction results in readily visible Sir3p bands.

pLP421 contains the *STE11-4* allele as a 3.6-kb XbaI fragment from pSL1655 (Stevenson et al., 1992) in the centromeric plasmid pRS315 (Sikorski and Heiter, 1989). Plasmid transformations into various yeast strains were performed with lithium acetate as described (Schiestl and Gietz, 1989).

Yeast Protein Extracts

Protein extracts were prepared as follows: cultures were harvested in 5 ml at an OD_{600} of 1.0 (~10^7 cells per ml). Cell pellets were washed in cold H_2O, frozen in liquid N_2, and stored at −70°C. Frozen cell pellets were thawed and resuspended in 100 μl cold PBS with protease inhibitors (final concentration 1 μg/ml leupeptin, 0.067 U/ml aprotinin, 0.2 mM PMSF, and

Table I. Yeast Strains

Strain	Genotype	Source
W303-1a	*MAT* **a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	R. Rothstein
RS862	W303-1a *sir3::TRP1*	R. Sternglanz
LPY771	W303-1a *pep4*	This study
1255-5C	*MAT* **a** *ade1 bar1 his2 leu2 trp1 ura3*	F. Cross
372	*MAT* **a** *ade1 bar1 his6 leu2-3,112 lys2 trp1-289 ura3-52 gal1*	R. Sclafani
1608-21C	1255-5C *cln1 cln2 cln3 leu2::LEU2::GAL-CLN3*	F. Cross
1630-4A-1a	1255-5C *far1Δ*	"
EY957	W303-1a *bar1Δ*	E. Elion
EY940	Ey957 *fus3::LEU2*	"
EY1119	EY957 *kss1::HIS3*	"
EY966	EY940 *kss1::HIS3*	"
EG123	*MAT* **a** *his4-519 leu2 trp1 ura3 can1-101*	S. Fields
DC40	EG123 *ste11-Δ1*	"
L5528	MAT **a** *his3::hisG ura3-52*	G. Fink
L5559	L5528 *ste7::LEU2 leu2::hisG*	"
L6016	L5528 *kss1::hisG fus3::TRP1 trp1::hisG leu2::hisG*	"
L5573	L5528 *ste12::LEU2 leu2::hisG*	"
SY1390	*MATα FUS1::HIS3 leu2 trp1 ura3 his3Δ200::ura3 pep4Δ::ura3 can1*	G. Sprague
SY1866	SY1390 *STE11-4*	"
RS927	W303-1a *HML::TRP1*	R. Sternglanz
UCC3542	*MAT***a***/MATα ade2101/ade2-101 his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/lys2-801 TRP1/trp1-Δ63 ura3-52/ura3-52 PPR1/ppr1::LYS2 adh4::URA3*-TELVIIL*/adh4::URA3*-TELVIIL *DIA5-1(ADE2*-TELVR*)/DIA5-1*	D. Gottschling
LPY1683	*MAT* **a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 adh4::URA3*-TELVIIL *DIA5-1(ADE2*-TELVR)	This study
LPY2495	LPY1683 *ste7::HIS3*	This study
LPY2497	LPY1683 *ste12::HIS3*	This study
UCC1001	*MAT* **a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 adh4::URA3*-TEL	D. Gottschling
UCC1003	*MAT* **a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 adh4::URA3*	D. Gottschling
UCC3249	UCC1001 *sir3::HIS3*	"
UCC3107	*MAT* **a** *his3-11 leu2 trp1Δ ura3-52 can1Δ::hisG ade2::hisG VR::ADE2*-TEL	"

1 μg/ml pepstatin; Sigma Chemical Co.). An equal volume of glass beads was added (0.4–0.52-mm, acid-washed; Thomas Scientific, Swedesboro, NJ), and cells were broken by vortexing four to six times, for 30 s each, with ≥30 s on ice between vortexing. After adding sample loading buffer, samples were boiled for 2 min, followed by a 5-min microfuge spin before gel loading. Cell extract equivalent to an OD_{600} of ~1.0 was loaded in each lane.

Antisera, Immunoprecipitation, and Immunoblotting

The rabbit polyclonal antiserum raised against a bacterially expressed βgal–Sir3p fusion protein has been previously described (Palladino et al., 1993). For probing Western blots, crude serum from rabbit 2936 (bleeds 2, 3, or 4) was used at a 1:5,000 dilution. For immunoprecipitation, this antiserum was purified on a protein A–agarose column (Sigma Chemical Co.), eluted in 100 mM glycine, pH 2.4, neutralized by addition of Tris, pH 8.0, to 100 mM, and used at ~5 mg/ml. Conjugation of 5 μl of anti-Sir3p antiserum and 20 μl of protein A–Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) per sample was performed in IP buffer (Axelrod, 1991) (25 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, pH 8.0, 10% glycerol, 50 mM KCl) + 0.2% NP-40 for 1 h at 4°C. Conjugated beads were washed three times in IP buffer + 0.2% NP-40 before immunoprecipitation.

Protein extracts for immunoprecipitation were made as described above, except cells were broken in IP buffer + 1% NP-40. SDS was added to 1% before boiling, 400 μl cold IP buffer was added to reduce the detergent concentration to 0.2%, and then debris was removed with a 10-min microfuge spin at 4°C. The supernatant was mixed with 20 μl of the anti-Sir3p antiserum–conjugated protein A–Sepharose beads for 1–3 h at 4°C. Immunoprecipitates were washed three times in IP buffer + 0.1% NP-40, followed by one wash in IP without detergent, and then boiled in sample loading buffer for 2 min.

Samples were subjected to SDS-PAGE electrophoresis on 7.5% gels (30:0.8 acrylamide/bis-acrylamide). Prestained standards (Bio Rad Laboratories, Hercules, CA) were used as molecular weight markers. Proteins were transferred from gels to nitrocellulose (0.2 μm; Schleicher & Schuell, Inc., Keene, NH) in transfer buffer (Harlow and Lane, 1988) containing 15% methanol. Immunoblotting was performed as described (Harlow and Lane, 1988) at room temperature, blocking in TBSTM buffer (150 mM NaCl, 10 mM Tris, pH 8.0, 0.05% Tween 20, 2% powdered skim milk), with 2-h primary antibody incubations, 1-h secondary antibody (anti-rabbit, HRP-conjugated; Amersham Corp. [Arlington Heights, IL]; or alkaline phosphatase–conjuated; Promega [Madison, WI]) incubations, and developing with ECL (Amersham Corp.) or alkaline phosphatase (Harlow and Lane, 1988).

In Vivo Labeling and Phosphatase Treatment

For ^{32}P-in vivo labeling, a logarithmically growing culture was inoculated into phosphate-free growth medium and incubated for one generation of growth. [^{32}P]orthophosphate (8,500–9,120 Ci/mmol; Dupont–New England Nuclear, Wilmington, DE) was added to 0.75 mCi per 5 ml culture, and cells were labeled for one doubling time before harvesting and processing for immunoprecipitation. Gels were fixed (7% acetic acid/50% methanol) and dried for autoradiography.

For phosphoaminoacid analysis, Sir3p immunoprecipitates from in vivo-labeled cells were run on SDS-PAGE and transferred to polyvinylidene fluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA). The Sir3p band was excised, eluted, and processed as described (Kamps and Sefton, 1989; Cooper et al., 1983).

For in vitro phosphatase experiments, immunoprecipitates were resuspended in 40 μl of IP buffer + 0.1% 2-mercaptoethanol immediately after the last IP wash. Calf intestinal alkaline phosphatase (CIP) (Boehringer Manheim Biochemicals, Indianapolis, IN) was added to 1 U per reaction. When used, the phosphatase inhibitor sodium orthovanadate was included at 100 μM. Reactions were incubated at 30°C for 1 h, before adding sample loading buffer and boiling.

In initial experiments, a mammalian phosphatase 2A (a gift from N. Ahn, University of Colorado, Boulder) and *Yersinia enterocolitica* (Boehringer Mannheim Biochemicals) tyrosine phosphatase were also examined; CIP was the only phosphatase that appeared active on Sir3p immunoprecipitates. Moreover, treating ^{32}P-labeled immunoprecipitates with CIP (data not shown) led to the conclusion that the Sir3p protein sample was not completely accessible to dephosphorylation by the CIP phosphatase, perhaps explaining why CIP treatment (see Fig. 1 *B*) resulted in a Sir3p band that comigrated with the lowest band in untreated samples.

Flow Cytometric Analysis

Flow cytometry was performed as described (Hutter and Eipel, 1979). Propidium iodide– (Sigma Chemical Co.) stained cells were analyzed on a flow cytometer (FACScan®; Becton Dickinson & Co., Mountain View, CA) using the LYSYS software package (Becton Dickinson & Co., Immunocytometric Systems, San Jose, CA).

Telomere Silencing Assays

Transcriptional repression from the reporter genes *URA3* and *ADE2* at telomeric locations was monitored using qualitative and quantitative methods described (Aparicio et al., 1991). For monitoring *URA3* expression, 14-h cultures were inoculated from selective synthetic medium containing recently plated transformants. Logarithmically growing cells were then plated in a series of fivefold dilutions onto supplemented synthetic medium lacking leucine for plasmid selection and onto the same medium containing 5-FOA. The ability of *STE11-4* to improve telomeric silencing was most readily apparent under these growth conditions where silencing of the parental plasmid control strain was relatively inefficient. For monitoring *ADE2* expression, ~200 cells from each stationary culture were plated onto synthetic medium supplemented with only the minimal required nutrients, including 6 μg/ml adenine, which aids in distinguishing the white colonies and sectors expressing *ADE2* from the red colonies and sectors that are silent for *ADE2*.

Results

Sir3p Is a Phosphoprotein

Sir3p has a central role in yeast transcriptional silencing and nuclear organization as defined by genetic and cell biological assays. To initiate biochemical analysis of Sir3p, we prepared yeast whole cell extracts for immunoprecipitation and immunoblot analysis. As shown in Fig. 1 *A*, we detected multiple species of Sir3p. The Sir3p bands were more intense when *SIR3* was overexpressed from a yeast 2μ plasmid than when expressed from its normal chromosomal locus (compare lanes *1* and *3*). These immunoreactive bands were not detectable in a *sir3* null mutant strain (lane *2*).

Since complex protein patterns often result from post-translational modification, we asked if the multiple electrophoretic species of Sir3p that we detect are due to phosphorylation. Accordingly, anti-Sir3p immunoprecipitates were treated with phosphatase and examined for changes in the pattern of Sir3p bands. Untreated immunoprecipitates showed the characteristic multiple bands for Sir3p (Fig. 1 *B*, lane *1*). Treatment of anti-Sir3p immunoprecipitates with CIP resulted in a shift of the heterogeneous Sir3p bands to a distinct lower band (Fig. 1 *B*, lane *2*), suggesting that Sir3p is indeed modified by phosphorylation. To confirm that the change in electrophoretic mobility was due to phosphatase activity, immunoprecipitates were treated with CIP in the presence of the phosphatase inhibitor sodium orthovanadate, in which case the shift to a lower band was not observed (Fig. 1 *B*, lane *3*).

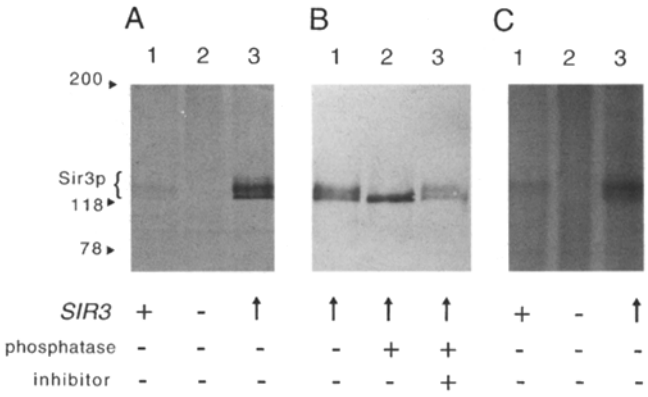

Figure 1. Sir3p is modified by phosphorylation. (*A*) Immunoblot of Sir3p immunoprecipitated from wild-type (lane *1*), *sir3* null mutant (lane *2*), and Sir3p-overproducing strain (lane *3*). (*B*) Sir3p immunoprecipitates were mock phosphatase treated (lane *1*), phosphatase treated (lane *2*), and phosphatase treated in the presence of the phosphatase inhibitor sodium orthovanadate (lane *3*), followed by immunoblot analysis. (*C*) Autoradiograms of Sir3p immunoprecipitates from in vivo ^{32}P-labeled cultures of wild-type (lane *1*), *sir3* null mutant (lane *2*), and Sir3p-overproducing strain (lane *3*). Although as many as five distinct Sir3p bands have been detected, their resolution is variable. Since we always detect at least two prominent forms of Sir3p, alternately modified Sir3p bands are referred to as upper and lower bands. Molecular weight markers of 200, 118, and 78 kD are indicated at the left. Strains used were: (*A*) W303-1a, RS862, and LPY771 with the 2μ-*SIR3* plasmid pLP27, respectively; (*B*) LPY771 with pLP27; and (*C*) W303-1a (lane *1*), RS862 (lane *2*), and LPY771 with pLP27 (lane *3*).

To ask if Sir3p is a phosphoprotein in vivo, yeast cultures were metabolically labeled with [^{32}P]orthophosphate followed by immunoprecipitation of whole cell lysates with anti-Sir3p antiserum. Autoradiograms revealed that at least two species of phosphorylated Sir3p exist in vivo in wild-type cells (Fig. 1 *C*, lane *1*). Again, the phosphorylated species were more prominent in cultures overexpressing *SIR3* (Fig. 1 *C*, lane *3*), and undetectable in a *sir3* null mutant (Fig. 1 *C*, lane *2*). Immunoblotting these same samples showed that the two major ^{32}P-labeled phosphorylated species comigrated with the two major bands seen on the immunoblot (data not shown). Phosphoaminoacid analysis of ^{32}P-labeled Sir3p samples detected phosphorylation only on phosphoserine (data not shown).

Since results from these experiments and all others were qualitatively identical for strains expressing endogenous levels of *SIR3* and for strains carrying a multicopy *SIR3* plasmid, the remaining experiments in this paper are shown for strains carrying the multicopy *SIR3* plasmid to facilitate data presentation, unless otherwise indicated.

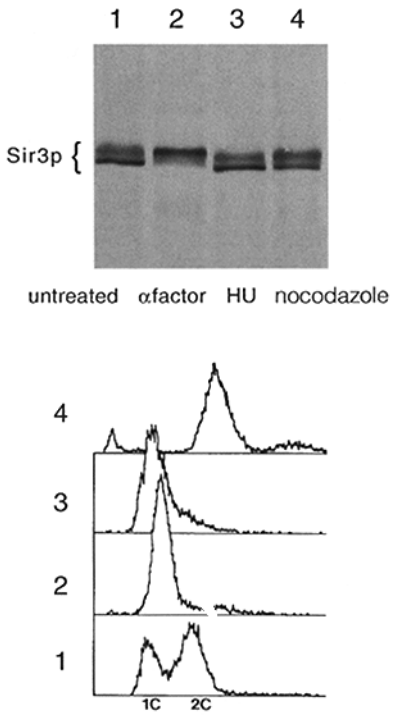

Figure 2. Sir3p patterns change upon G_1 arrest by α-factor exposure. Immunoblot of whole cell lysates from cultures that were mock treated (lane *1*), or cell cycle arrested with α-factor (lane *2*), hydroxyurea (lane *3*), or nocodazole (lane *4*) as described in Materials and Methods. Flow cytometric analysis of the same samples is presented below. The x-axis represents DNA content (1C or 2C) as measured by relative fluorescence, and the y-axis represents relative cell number (sample size, 5,000 cells). Flow cytometry and budding index determination suggested arrest as expected at G_1/START by α-factor, at S by hydroxyurea, and at G_2/M by nocodazole. The strain used was 1255-5C with pLP27.

Sir3p Is Hyperphosphorylated in Response to α-Factor Treatment

Phosphorylation states of many cellular proteins change in response to different growth conditions and during the cell cycle. To determine whether phosphorylation of Sir3p might vary during the cell cycle, we arrested cells at three different stages of the cell cycle: (*a*) in G_1, before the commitment point START, with the mating pheromone α-factor; (*b*) in S phase with the DNA replication inhibitor hydroxyurea; and (*c*) at G_2/metaphase with the microtubule-depolymerizing agent nocodazole. Flow cytometric analysis (Fig. 2), to evaluate DNA content, and budding index determination (not shown) confirmed that these cultures arrested at the expected cell cycle point. Immunoblots of whole cell lysates showed that Sir3p was shifted substantially to a higher, more slowly moving band upon treatment with α-factor (Fig. 2, compare lane *1*, untreated, with lane *2*, α-factor treated). In contrast, no marked change was observed for Sir3p at S phase arrest or at G_2/metaphase arrest (Fig. 2, lanes *3* and *4*), indicating that not all cell cycle arrests result in increased Sir3p phosphorylation.

The decrease in Sir3p mobility observed upon α-factor exposure was consistent with hyperphosphorylation. To confirm that the shift in Sir3p mobility was due to hyperphosphorylation, immunoprecipitates from α-factor–arrested samples were treated with phosphatase. Phosphatase treatment resulted in a similar shift to a lower Sir3p band both in samples that were exposed to α-factor or that were mock treated, demonstrating that the upper band that resulted from α-factor treatment is sensitive to phosphatase (Fig. 3 *A*). Additional evidence that the higher Sir3p band in α-factor–treated cells is a phosphoprotein was obtained from cultures labeled with ^{32}P in vivo, as described before, and arrested with α-factor. Only a higher, more slowly migrating phosphospecies was observed for such samples (Fig. 3 *B*), which comigrated with the upper band detected immunologically for the same samples (data not shown). We observe similar hyperphosphorylation of Sir3p in a strain expressing *SIR3* from its endogenous chromosomal locus (Fig. 3 *C*).

Since α-factor exposure led to G_1 arrest, the observation that Sir3p was hyperphosphorylated upon treatment with α-factor raised the possibility that Sir3p phosphorylation may be modulated in G_1 of each cell cycle. To determine if Sir3p phosphorylation changes occur normally in G_1, a culture was synchronized by first treating with α-factor, and then releasing from α-factor arrest as a synchronous population of dividing cells. We observed that the lower band reappeared as soon as pheromone was removed from the medium and the cells began to re-enter the cell cycle. The Sir3p pattern did not shift to that of α-factor–arrested cells during the second synchronous G_1, or at any subsequent time after release from α-factor. In a separate experiment, the lower Sir3p band reappeared immediately after release from α-factor even in the presence of the protein sythesis inhibitor cycloheximide, suggesting that reappearance of the lower band occurred in the absence of new synthesis of Sir3p (unpublished data). Therefore, it is

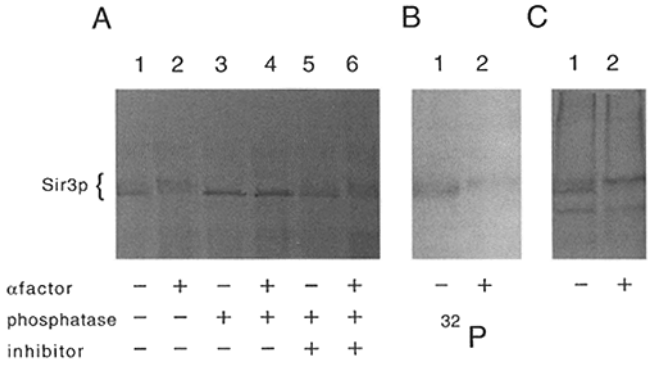

Figure 3. Sir3p is hyperphosphorylated upon exposure to α-factor. (*A*) Immunoblot of Sir3p immunoprecipitates from an asynchronously growing culture (lanes *1*, *3*, and *5*) or an α-factor–exposed culture (lanes *2*, *4*, and *6*). Immunoprecipitates were mock treated (lanes *1* and *2*), phosphatase treated (lanes *3* and *4*), or phosphatase treated in the presence of a phosphatase inhibitor (lanes *5* and *6*). (*B*) Autoradiogram of Sir3p immunoprecipitates from in vivo ^{32}P-labeled cultures that were untreated (lane *1*) or α-factor treated (lane *2*). (*C*) Immunoblot of untreated (lane *1*) or α-factor–treated (lane *2*) cells from a strain expressing *SIR3* at endogenous levels, overdeveloping the blot to facilitate visualization of bands. Strains used were: (*A*) 1255-5c with pLP27; (*B*) 372 with pLP27; and (*C*) 372 with no plasmid.

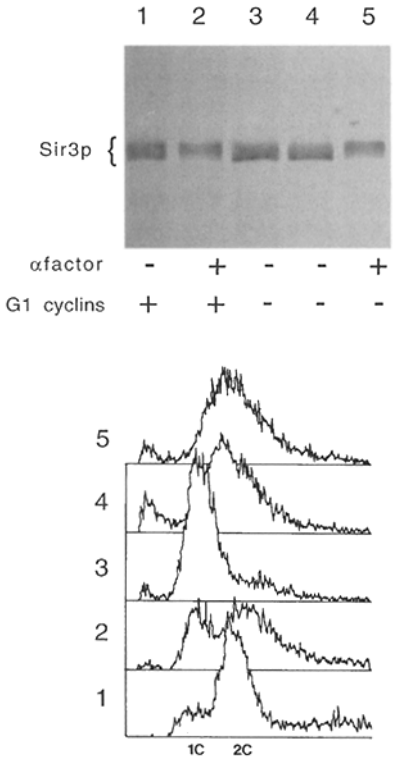

Figure 4. G_1 arrest is not sufficient for Sir3p hyperphosphorylation. Cells were arrested by depletion of G_1 cyclins, by shifting a growing culture in galactose medium to glucose medium. Immunoblot of whole cell lysates from a galactose culture that was asynchronously growing (lane *1*) or exposed to α-factor (lane *2*), arrested in G_1 by shifting to glucose for 2.5 h (lane *3*), and subsequently mock treated (lane *4*) or α-factor treated (lane *5*) for an additional 2 h. Flow cytometric analysis of the same samples is presented in corresponding panels in the graph below. The x-axis represents DNA content (1C or 2C) as measured by relative fluorescence, and the y-axis represents relative cell number (sample size, 5,000 cells). Note that flow cytometric analysis reveals that α-factor treatment of this strain in galactose does not result in uniform arrest in G_1; rather, this strain has been observed to arrest at positions throughout the cell cycle in response to α-factor exposure (Oehlen, B., personal communication). Cells are arrested with 1C DNA content upon depletion of cyclins by 2.5-h growth in glucose (panel *3*); cultures that were subsequently mock or α-factor treated (panels *4* and *5*) continue to maintain 1C DNA content that is seen slightly shifted to the right, probably due to increased cell volume and proliferation of mitochondrial DNA during continued incubation at G_1 arrest. The strain used was 1608-21C (*MAT***a** *cln1 cln2 cln3 GAL-CLN3*) with pLP27.

likely that an endogenous phosphatase activity dephosphorylates Sir3p as cells re-enter the cycle and approach S phase. These data suggest that, like Sir3p hyperphosphorylation, dephosphorylation of Sir3p may also be regulated.

G_1 Arrest Is Neither Necessary Nor Sufficient for Sir3p Hyperphosphorylation

Hyperphosphorylation of Sir3p occurred at G_1 arrest in response to α-factor treatment, yet it did not occur during G_1 of cycling cells. It was therefore important to determine whether a pheromone-independent G_1 arrest would result in Sir3p hyperphosphorylation. To test this possibility, we took advantage of the observation that yeast cells can be arrested in G_1 by simultaneous depletion of the three G_1 cyclins, *CLN1*, *CLN2*, and *CLN3*. Although G_1 cyclin function is essential for cell viability (Richardson et al., 1989), a strain that is triply mutant for *cln1 cln2 cln3* can be kept alive with *CLN3* under control of the carbon source–regulated *GAL1-10* promoter (McKinney et al., 1993). This strain was arrested in G_1 by shifting a culture growing in galactose to glucose-containing medium to repress transcription of the *CLN3* gene. Flow cytometric analysis showed that the culture was arrested in G_1 (Fig. 4, *bottom panel 3*), yet there was no change in Sir3p phosphorylation patterns (Fig. 4, compare lane *1*, galactose-growing cells, to lane 3, 2.5-h shift to glucose). Subsequent treatment of the *CLN*-depleted culture with α-factor resulted in the same hyperphosphorylation of Sir3p previously observed upon α-factor treatment of an asynchronous population of cells, showing that these cells were capable of α-factor–induced Sir3p hyperphosphorylation (Fig. 4, see lane *2*, treatment of asynchrous galactose culture, and lane *5*, 2.5-h shift to glucose followed by 2-h α-factor treatment). A control in which *CLN*-depleted cells were arrested in glucose for as long as 4.5 h showed no changes in Sir3p phosphorylation (Fig. 4, lane *4*). Thus, the *CLN*-depleted strain had the potential to respond to α-factor by hyperphosphorylating Sir3p, even though G_1 arrest due to absence of the G_1 cyclins did not result in hyperphosphorylation. Hence, changes in Sir3p phosphorylation were not a direct consequence of G_1 arrest, but appear to be the result of a pheromone response. We also tested whether **a**-factor exposure led to Sir3p hyperphosphorylation, which would be expected. We observed similar results to those with α-factor treatment (not shown).

The results above demonstrated that G_1 arrest is not sufficient for Sir3p hyperphosphorylation. We next asked if G_1 arrest is necessary for Sir3p hyperphosphorylation by examining Sir3p in a *far1* mutant. The *far1* mutant does not arrest the cell cycle in G_1 upon exposure to pheromone, although other responses to α-factor appear normal (Chang and Herskowitz, 1990). An immunoblot of a wild-type and a *far1* mutant strain treated with α-factor showed the same shift in mobility for Sir3p, demonstrating that Sir3p was indeed hyperphosphorylated in the absence of G_1 arrest (Fig. 5, lane *2*, wild-type; lane *4*, *far1* mutant). Flow cytometric analysis (data not shown) confirmed that the *far1* mutant did not arrest in G_1 with exposure to α-factor. Thus, G_1 arrest is not necessary for pheromone-induced Sir3p hyperphosphorylation.

Sir3p Is Hyperphosphorylated in Response to Heat Shock and Starvation

We reasoned that, in principle, temperature-sensitive *cdc* mutants could be tested for potential Sir3p hyperphosphorylation effects at different cell cycle arrest points. However, raising the temperature in wild-type strains led to changes in Sir3p phosphorylation patterns, confounding such analysis. Sir3p mobility was reproducibly altered when a wild-type strain was shifted from 22°C to 37°C for 2.5 h (Fig. 6, lanes *1* and *2*, respectively). We determined that changes in Sir3p patterns seen upon temperature shift

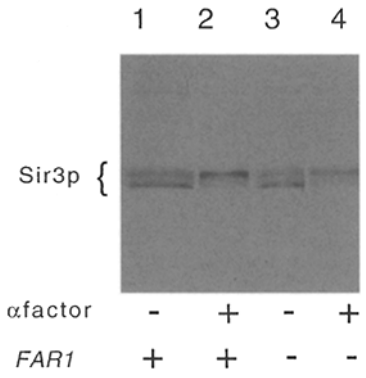

Figure 5. G_1 arrest is not necessary for Sir3p hyperphosphorylation. Immunoblot of whole cell lysates from wild-type or *far1* mutant strain (lanes *1* and *3*, untreated; lanes *2* and *4*, α-factor exposed, respectively). The strains used were 1255-5C and 1630-4A-1a, respectively, both with pLP27.

were due to altered phosphorylation, because phosphatase treatment of Sir3p immunoprecipitates from such cultures resulted in a quantitative shift of the higher, slower moving material to a lower band, similar to the shift seen in Fig. 3 *A*. Hyperphosphorylation of Sir3p in response to a temperature shift, although reproducibly detectable, was not as great as that seen with pheromone treatment. These observations are reminiscent of those described by Kamada et al. (1995) in which mild heat shock activates protein kinase C and downstream MAP kinases.

The observation that mild heat shock resulted in Sir3p hyperphosphorylation prompted us to explore whether Sir3p phosphorylation is a general cellular response to physiological stress. Accordingly, Sir3p patterns were analyzed after both nitrogen and glucose starvation, as well as upon osmotic shock. Cultures harvested after 48-h starvation in sporulation medium showed a shift to a more slowly migrating Sir3p band, which was distinctly higher than that seen for α-factor–exposed cells (Fig. 7 *A*, compare lanes *1* and *2*). This effect was seen for both haploid and diploid cells (data not shown), indicating that the shift occurs in response to starvation and is independent of meiosis. Phosphatase treatment of Sir3p immunoprecipitates from starved cells resulted in reduction of the upper band to a lower band, similar to the mobility shift seen in Fig. 3 *A*, indicating that the changes in Sir3p mobility were due to phosphorylation.

Sporulation medium starves cells by depriving them of both nitrogen and carbon sources (for review see Mitchell, 1994). Another starvation regime that caused a similar upward shift in the Sir3p pattern was growth to saturation in rich medium, during which cells also enter stationary phase as a result of nutrient starvation, although the shift was not as severe as that seen for cultures in sporulation medium (data not shown). To distinguish whether changes in Sir3p phosphorylation were due to nitrogen depletion, carbon depletion, or a combination of the two, cultures were grown to saturation in sporulation medium supplemented with glucose. Hyperphosphorylation of Sir3p was still observed when glucose was added to sporulation medium, although to a lesser extent than in the absence of glucose (data not shown). Both glucose and nitrogen limitation thus contributed to Sir3p phosphorylation.

In recent studies, distinct influences on transcriptional silencing have been reported for cells starved for glucose and for cells growing in a nonfermentable carbon source (Shei and Broach, 1995). To test if growth in a nonfermentable carbon source likewise influences Sir3p phosphorylation, cells were grown in glycerol-containing medium. Cultures harvested after growth in glycerol showed changes in the Sir3p pattern (Fig. 7 *B*, cultures grown in glucose, lane *1*; then shifted to glycerol, growing for 6 h, lane *2*; or growing for 24 h, lane *3*). The Sir3p upward shift was exacerbated upon starvation in glycerol medium by growing cells to saturation (Fig. 7 *B*, lane *4*), illustrating another example of an additive or synergistic effect on Sir3p phosphorylation, in this case for glycerol growth and starvation conditions.

We showed that Sir3p phosphorylation was altered by several different stress conditions, such as heat shock and starvation. In contrast, exposure to osmotic stress in culture media containing 0.4 M NaCl for 10 or 30 min (Brewster et al., 1993) did not appear to influence Sir3p phosphorylation (data not shown). Thus, Sir3p was hyperphosphorylated in response to many but not all stress conditions,

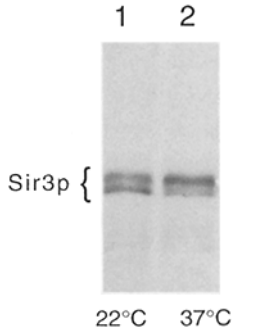

Figure 6. Sir3p phosphorylation changes upon heat shock. Immunoblot of whole cell lysates from cultures grown at 22°C (lane *1*) or heat shocked at 37°C for 2.5 h (lane *2*). The strain used was W303-1a with pLP27.

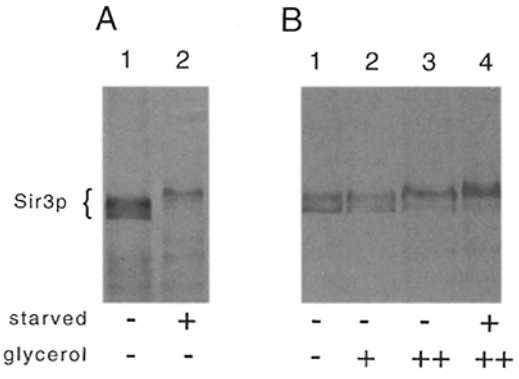

Figure 7. Starvation induces Sir3p hyperphosphorylation. Immunoblots of whole cell lysates from haploid cultures (*A*) growing asynchronously (lane *1*) or starved for 48 h in sporulation medium (lane *2*); and (*B*) growing asynchronously in glucose medium (lane *1*), or shifted to glycerol medium and growing asynchronously for 6 h (lane *2*) or 24 h (lane *3*), or starved in glycerol medium by growth to saturation (lane *4*). Flow cytometric analysis for starved samples indicated that cultures were arrested with 1C DNA content, consistent with G_0 arrest (data not shown). The strains used were (*A*) W303-1a and (*B*) LPY771, both with pLP27.

thereby suggesting specific regulation of the phosphorylation response.

An Intact MAP Kinase Signaling Pathway Is Required for Sir3p Hyperphosphorylation in Response to Pheromone

Diverse physiological responses, including pheromone response and heat shock, are regulated by MAP kinase pathways (for reviews see Levin and Errede, 1995; Herskowitz, 1995). In particular, the MAP kinases (MAPK) Fus3p and Kss1p have critical and overlapping functions in the pheromone-response pathway (Elion et al., 1991). To determine if α-factor–induced Sir3p hyperphosphorylation depends on *FUS3* and/or *KSS1*, Sir3p phosphorylation was examined in *fus3* or *kss1* single mutants, and in a *fus3 kss1* double mutant. The Sir3p pattern was the same in asynchronously growing wild-type and mutant strains (Fig. 8 *A*, lanes *1*, *3*, *5*, and *7*). When wild-type and *fus3* or *kss1* single mutant strains were treated with α-factor, hyperphosphorylation of Sir3p was observed (Fig. 8 *A*, lanes *2*, *4* and *6*). In the *fus3 kss1* double mutant, however, no change in Sir3p pattern was seen upon exposure to α-factor (Fig. 8 *A*, lane *8*). Thus, although *FUS3* and *KSS1* are not required for normal modification of Sir3p in cycling cells, at least one of these MAPK genes is required for pheromone-induced Sir3p hyperphosphorylation. This suggests that α-factor–induced Sir3p hyperphosporylation requires an intact pheromone-response MAP kinase pathway.

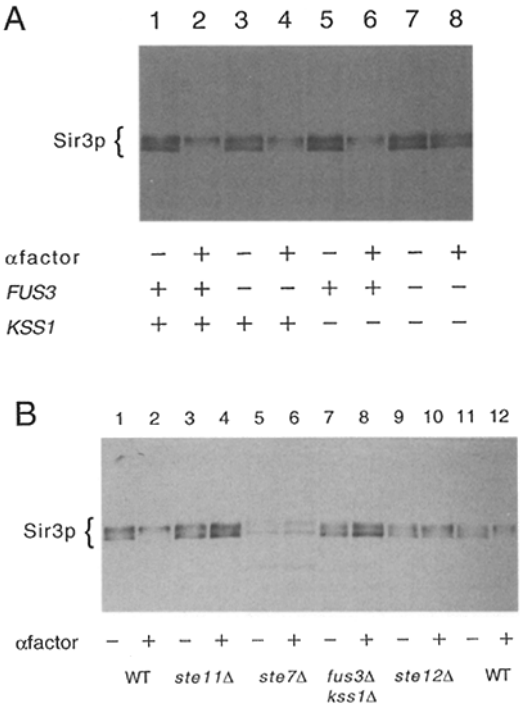

Figure 8. The pheromone-response MAP kinase pathway is required for α-factor–induced hyperphosphorylation of Sir3p. Immunoblot of whole cell lysates from (*A*) wild-type, *fus3* mutant, *kss1* mutant, or *fus3 kss1* double mutant (lanes *1*, *3*, *5*, and *7*, untreated; lanes *2*, *4*, *6*, and *8*, α-factor exposed); and (*B*) wild-type, *ste11* mutant, *ste7* mutant, *fus3 kss1* double mutant, *ste12* mutant, or wild type (lanes *1*, *3*, *5*, *7*, *9*, and *11*, untreated; lanes *2*, *4*, *6*, *8*, *10*, and *12*, α-factor exposed). The first two strains and the final four strains are in the same genetic strain background. *ste7* and *ste12* mutants were also examined in the EG123 background with the same results. Immunoblotting the *ste7* mutant strain consistently resulted in a fainter Sir3p signal. Strains used were: (*A*) EY957, EY940, EY1119, and EY966, respectively; and (*B*) EG123, DC40, L5559, L6016, L5573, and L5528, respectively, all with pLP27.

The MAPK kinase (MEK) gene *STE7* and the MEK kinase (MEKK) gene *STE11*, like *FUS3* or *KSS1*, are also required for signal transduction through the pheromone-response MAP kinase pathway. We therefore examined Sir3p phosphorylation in *ste11* and *ste7* mutants, and found that Sir3p was not hyperphosphorylated in these mutant strains in response to α-factor exposure. Samples of untreated vs α-factor–treated cells for each mutant strain are shown in lanes *1–8* of Fig. 8 *B*, which also demonstrates the *FUS3/KSS1* requirement again. To address the possibility that Sir3p is hyperphosphorylated by one of the kinases in the MAP kinase cascade itself (MEKK/MEK/MAPK) or by a kinase that is activated downstream of the MAP kinase cascade, we analyzed Sir3p phosphorylation patterns in an *ste12* mutant. *STE12* encodes a transcription factor that is activated by the MAP kinase pathway in response to pheromone (for review see Herskowitz, 1995). Ste12p in turn activates a number of downstream genes that encode proteins important for the pheromone response. When an *ste12* mutant strain was treated with α-factor, Sir3p hyperphosphorylation did not occur (Fig. 8 *B*, compare α-factor–exposed *ste12* mutant in lane *10* to α-factor–exposed wild-type strain in lane *12*). Although it remains a formal possibility that a kinase in the MAP kinase cascade itself directly phosphorylates Sir3p in response to pheromone (see Discussion), we interpret this result to indicate that pheromone-induced Sir3p hyperphosphorylation probably requires a downstream kinase that is directly or indirectly activated by Ste12p.

To test if the changes in Sir3p phosphorylation that occur under starvation conditions also require the MAP kinase genes, Sir3p patterns were examined in strains harboring mutations in *FUS3* and *KSS1*. Wild-type, *fus3* single mutants, *kss1* single mutants, and *kss1 fus3* double mutants all respond similarly to incubation in sporulation medium, revealing slower mobility Sir3p bands (Fig. 9 *A*, lanes *2*, *4*, *6*, and *8*) compared with logarithmically growing strains (Fig. 9 *A*, lanes *1*, *3*, *5*, and *7*). Thus, although *FUS3* or *KSS1* is required for Sir3p hyperphosphorylation upon pheromone treatment, they are not required for the changes in Sir3p phosphorylation upon starvation. Moreover, neither *STE11* (Fig. 9 *B*) nor *STE7* or *STE12* (data not shown) is required for starvation-induced Sir3p hyperphosphorylation. These results demonstrate that other kinases in addition to those active in the pheromone-response MAP kinase pathway must play a role in regulating Sir3p phosphorylation. Whether these kinases are parts of other MAP kinase cascades, or other regulated phosphorylation pathways, remains to be determined.

Activation of the Pheromone-Response MAP Kinase Pathway in an STE11-4 Mutant Strengthens Transcriptional Silencing at Telomeres

A key question is whether changes in the state of Sir3p phosphorylation affect its function in transcriptional si-

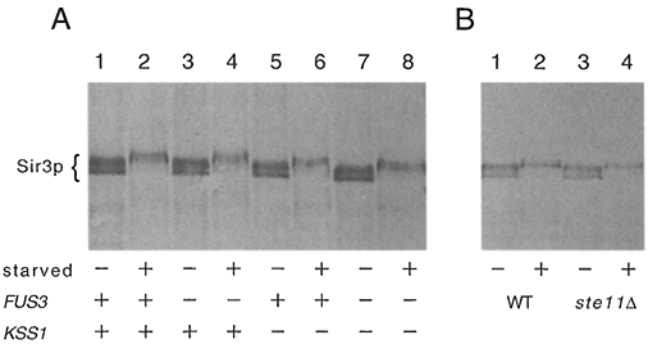

Figure 9. The pheromone-response MAP kinase pathway is not required for starvation-induced hyperphosphorylation of Sir3p. Immunoblot of whole cell lysates from (*A*) wild-type, *fus3* mutant, *kss1* mutant, or *fus3 kss1* double mutant (lanes *1*, *3*, *5*, and *7*, asynchronously growing; lanes *2*, *4*, *6*, and *8*, starved for 24 h in sporulation medium); and (*B*) wild-type or *ste11* mutant (lanes *1* and *3*, asynchronously growing; lanes *2* and *4*, starved for 24 h in sporulation medium). Strains used were: (*A*) EY957, EY940, EY1119, and EY966, respectively; and (*B*) EG123 and DC40, all containing pLP27.

lencing. We reasoned that developing a strain that could continually maintain a state of Sir3p hyperphosphorylation would be invaluable in addressing this question. We thus assessed Sir3p phosphorylation in the dominant *STE11-4* mutant, in which the MAP kinase cascade is constitutively activated in the absence of pheromone (Stevenson et al., 1992). The Sir3p pattern was shifted to a slower mobility in the *STE11-4* mutant (Fig. 10, lane *2*) compared with wild type (Fig. 10, lane *1*), consistent with the Sir3p changes seen upon exposure to α-factor. Therefore, not only must the pheromone-response MAP kinase pathway be intact for Sir3p hyperphosphorylation to occur in the presence of α-factor, but activation of the pathway in the absence of α-factor is capable of constitutively hyperphosphorylating Sir3p.

Since Sir3p was constitutively hyperphosphorylated in the dominant *STE11-4* mutant even in the absence of pheromone, we were able to ask if transcriptional silencing is altered in an *STE11-4* strain in which Sir3p is hyperphosphorylated as a result of MAP kinase pathway activation. Using a strain with the *TRP1* reporter gene at the silent mating-type locus *HML*, no changes in silencing were observed when a plasmid carrying the *STE11-4* allele was introduced (data not shown). Thus, *STE11-4* did not appear to interfere with silencing at *HML::TRP1*. Since this *TRP1* reporter is likely to already be fully silenced, however, no further increases in silencing would be detected if *STE11-4* were to improve transcriptional repression. We therefore chose to evaluate telomeric transcriptional silencing using the well-characterized *URA3* and *ADE2* reporter genes, because at these loci it is possible to detect either increases or decreases in transcriptional silencing (Gottschling et al., 1990).

We examined the efficiency of silencing in the presence of the *STE11-4* plasmid in strains with both the *URA3* and the *ADE2* genes at telomeres. To evaluate *URA3* gene expression, growth of control and *STE11-4* transformants was compared on medium selecting for plasmid maintenance (−leu) and on medium containing the suicide substrate 5-FOA, (continuing to maintain selection for plasmid). Growth on 5-FOA is a sensitive measure of transcriptional silencing of the telomere-positioned *URA3* gene because cells expressing *URA3* cannot grow on 5-FOA, such that only silenced cells form colonies. In this assay we observed that *STE11-4* transformants were more 5-FOA resistant than control transformants (Fig. 11 *A*, rows 1 and 2). In a parallel quantitative evaluation of 5-FOA resistance, we determined that the *STE11-4* plasmid increased resistance from 2.2×10^{-3} to 7.1×10^{-2}%, reflecting a 32-fold increase in transcriptional silencing of *URA3*. We also observed significantly larger colonies on 5-FOA with *STE11-4* compared with controls (see Chien et al., 1993, for similar observations of increased colony size with increased silencing). Since overexpression of *SIR3* increases silencing of *URA3* at the telomere (Renauld et al., 1993), we compared the *STE11-4* effect to that of *SIR3* overexpression. The degree of improved silencing with overexpression of *SIR3* was greater than that of the *STE11-4* plasmid in these strains (Fig. 11 *A*, row 3), increasing to 2.2×10^{1} in a quantitative assay. We further examined the effect of the *STE11-4* plasmid on telomeric silencing using the *ADE2* reporter gene by monitoring colony color, and observed similar improved silencing at the telomeric *ADE2* locus in the form of increased red and sectored colonies with *STE11-4* (data not shown).

To investigate the specificity of the *STE11-4* effect on silencing, we tested whether the *STE11-4* plasmid could also improve silencing in *ste7* and *ste12* mutant strains. *STE7* is directly downstream of *STE11* in the pheromone-response MAP kinase cascade (Levin and Errede, 1995; Herskowitz, 1995), and it is required for pheromone-induced Sir3p hyperphosphorylation (Fig. 8). We observed that *STE11-4* was incapable of improving silencing in the *ste7* mutant (Fig. 11 *A*, rows 4 and 5), although increased silencing as a result of *SIR3* overexpression was comparable in the wild-type and the *ste7* mutant strain (row 6). As the Ste12p transcription factor is essential for the pheromone response but is downstream of the MAP kinase cascade itself, we also tested if the increased silencing seen with *STE11-4* occurred in the *ste12* mutant. As for the *ste7* mutant, we saw that *STE11-4* did not result in increased silencing in the *ste12* mutant strain (Fig. 11 *A*, rows 7–9). In addition, *STE11-4* had no effect on expression of an internal *URA3* locus (data not shown), thus demonstrating specificity for loci subject to silencing. Moreover, in an *sir3* mutant, the *STE11-4* plasmid was unable to improve silencing of *URA3* at the telomere (Fig. 11 *B*). These results demonstrate that *STE11-4* requires both *SIR3* and an intact pheromone-response MAP kinase pathway to im-

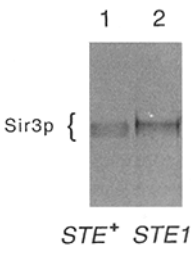

Figure 10. Constitutive activation of the MAP kinase pathway by *STE11-4* results in constitutive Sir3p hyperphosphorylation. Immunoblot of whole cell lysates from asynchronously growing wild-type (lane *1*) or *STE11-4* mutant strain. The strains used were SY1390 and SY1866, both with pLP27.

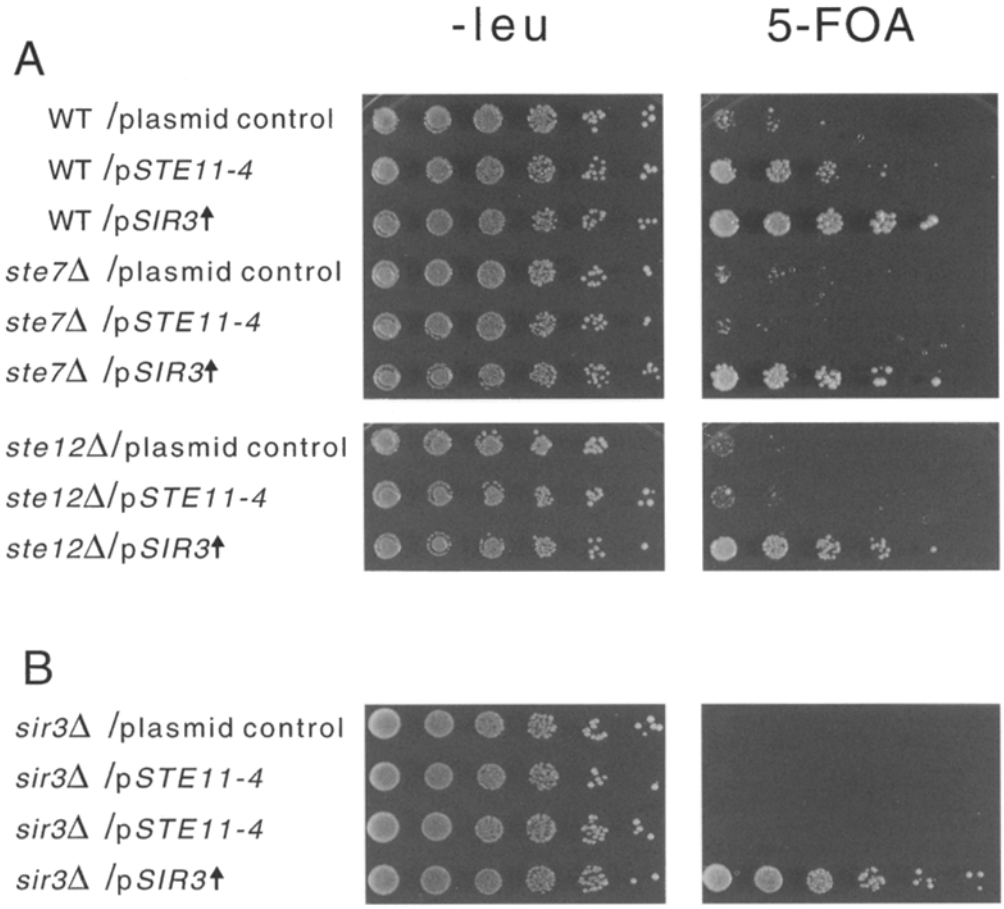

Figure 11. MAP kinase pathway activation by *STE11-4* strengthens transcriptional silencing of a telomere-proximal *URA3* reporter. Telomeric silencing of the *URA3* gene is measured by growth on 5-FOA. A fivefold dilution series of growing cultures were plated on supplemented synthetic medium lacking leucine for plasmid selection (*-leu*, *left*) or the same medium containing 5-FOA (*right*). (*A*) Wild-type, *ste7*, or *ste12* mutant containing plasmid control (pRS315, rows 1, 4, and 7), the *STE11-4* plasmid (pLP421, rows 2, 5, and 8), or a plasmid overexpressing *SIR3* (pLP304, rows 3, 6, and 9), respectively. (*B*) *sir3* mutant strain containing pRS315 (row 1), pLP421 (rows 2 and 3, two independent transformants), or pLP304 (row 4). Strains used were LPY1683, LPY2495, LPY2497, and UCC3249.

prove telomeric silencing. Thus, transcriptional silencing at telomeres is strengthened upon activation of the pheromone-response MAP kinase cascade and Sir3p hyperphosphorylation.

Discussion

Gene silencing, an important form of transcriptional repression, is regulated in part by local differences in chromatin structure. We have shown that Sir3p, a protein essential for yeast silencing, is subject to dynamic phosphorylation, and that signal transduction through an MAP kinase cascade leads to Sir3p hyperphosphorylation and increased transcriptional silencing. Since phosphorylation of other key chromatin components is correlated with their function (Reeves, 1992), similarly, phosphorylation of Sir3p may potentially be important for its function in the assembly and maintenance of transcriptionally silent chromatin. Our results suggest a novel role for signal transduction in regulating changes in chromatin structure via Sir3p phosphorylation.

Signal Transduction through an MAP Kinase Cascade Results in Hyperphosphorylation of Sir3p

We have demonstrated that Sir3p is hyperphosphorylated when exposed to mating pheromone. Although pheromone exposure causes cell cycle arrest in G_1 before START, G_1 arrest is not sufficient for Sir3p hyperphosphorylation because phosphorylation is unchanged when cells are arrested in G_1 by depletion of the G_1 cyclins. Moreover, G_1 arrest is not necessary for Sir3p hyperphosphorylation because it occurs in the G_1 arrest–defective *far1* mutant when exposed to α-factor. Thus, Sir3p phosphorylation changes specifically in response to pheromone. Indeed, this phosphorylation requires an intact pheromone-response signal transduction pathway, as revealed by the requirement for at least one of the functionally redundant MAP kinase pair Fus3p/Kss1p. Hyperphosphorylation of Sir3p does not occur in the MAP kinase–defective *fus3 kss1* double mutant when exposed to α-factor, although both *fus3* and *kss1* single mutants are capable of Sir3p hyperphosphorylation when treated with pheromone (see Fig. 8 *A*). MAP kinases Fus3p and Kss1p are known to act redundantly in phosphorylating Ste12p, a transcription factor that activates a number of downstream genes (Herskowitz, 1995). Although Sir3p contains a single MAP kinase consensus site (LTSP; Nigg, 1993) at residues 293–296, we think it is more likely that Ste12p activates a downstream kinase that is responsible for Sir3p hyperphosphorylation because hyperphosphorylation does not occur in an *ste12* mutant when treated with α-factor. This interpretation is complicated by the apparently contradictory observations that the Ste11p MEKK remains active toward the Ste7p MEK in an *ste12* mutant (Zhou et al., 1993), yet Ste12p modulates expression of several upstream genes in the MAP kinase cascade (Fields et al.,

1988; Elion et al., 1991) such that the activity of MEKK/MEK/MAP kinases may be decreased. The complex feedback loops within the pathway confound simple predictions, but future experiments will help in determining if Sir3p is directly phosphorylated by a MAP kinase and/or if Sir3p is a substrate of a kinase downstream of Ste12p.

Sir3p is hyperphosphorylated upon heat shock or starvation, as well as in response to mating pheromone. Several lines of evidence suggest that different kinase activities target specific Sir3p sites under different conditions. First, the hyperphosphorylated Sir3p bands that result from pheromone treatment, heat shock, and starvation are qualitatively distinct. Second, basal levels of Sir3p phosphorylation appear identical in MAP kinase pathway mutants that are defective in pheromone-induced Sir3p phosphorylation changes. Third, starvation-induced Sir3p hyperphosphorylation seems to occur by a different pathway than pheromone-induced Sir3p hyperphosphorylation. This point is emphasized by our evaluation of starvation-induced Sir3p phosphorylation in the MAP kinase pathway mutants that interfered with pheromone-induced Sir3p hyperphosphorylation. Since many of the same components of the pheromone-response MAP kinase cascade are also required for the invasive growth that occurs in response to nitrogen starvation (Roberts and Fink, 1994), it might have been predicted that starvation-induced Sir3p hyperphosphorylation would not occur in these mutants. However, Sir3p hyperphosphorylation in response to starvation was the same in wild-type and MAP kinase pathway–defective mutants *ste11*, *ste7*, *fus3 kss1*, and *ste12*. Our results therefore indicate that hyperphosphorylation of Sir3p induced by starvation occurs by a pathway distinct from the MAP kinase cascade required for pheromone response or invasive growth. Overall, the complexity observed for Sir3p phosphorylation suggests that Sir3p serves as a substrate for a number of different kinase activities. Sir3p phosphorylation occurs predominantly on serine residues, of which 91 exist in the protein. There is thus great potential for finely tuned regulation of Sir3p phosphorylation in response to altered cellullar physiology.

Functional Implications of Sir3p Phosphorylation: Increased Transcriptional Silencing Is Associated with Pheromone-induced Sir3p Hyperphosphorylation

Sir3p is essential for transcriptional repression and for telomeric positioning and integrity, raising the possibility that transcriptional silencing or telomere structure responds to Sir3p hyperphosphorylation. A critical test of this possibility was to ask if silencing is altered when Sir3p is hyperphosphorylated. We examined silencing in an *STE11-4* strain that is known to cause dominant activation of the pheromone-response MAP kinase cascade (Stevenson et al., 1992), and that we showed resulted in hyperphosphorylation of Sir3p in the absence of pheromone. Under conditions in which either increased or decreased silencing could be detected, we observed improved silencing of the telomeric *URA3* and *ADE2* reporter genes. The increased silencing with *STE11-4* is dependent upon an intact pheromone-response signaling pathway because it was not observed if the pathway was disrupted by mutation of *STE7* or *STE12*. Thus, Sir3p hyperphosphorylation is correlated with strengthened transcriptional silencing at telomeres. It is a possibility that Sir3p hyperphosphorylation is not the direct cause of the increased silencing observed upon *STE11-4* activation. Further investigation should reveal how phosphorylation of other silencing proteins may be affected during the pheromone response. Due to the wealth of evidence that implicates Sir3p as a crucial and limiting component of the silencing complex, we feature Sir3p phosphorylation in our discussion of how MAP kinase regulation of silencing may occur.

Aparicio and Gottschling (1994) proposed that both starvation and heat shock may strengthen transcriptional silencing. Specifically, they observed that telomeric transcription was irreversibly silenced after starvation-induced stationary phase arrest. In their experiments, the *trans*-activator protein Ppr1p was normally able to activate transcription of a reporter gene during stationary arrest, but not if the reporter was subject to telomeric silencing. Heat shock at 37°C also compromised activated expression of the telomeric reporter gene. Our results raise the possibility that the strengthened silencing that they observed was a result of Sir3p hyperphosphorylation. We predict that regulation of Sir3p phosphorylation is a key step in strengthening silencing during heat shock and starvation as well as during the pheromone response.

What might be the significance of Sir3p hyperphosphorylation in response to pheromone, heat shock, and starvation? It may be crucial for the cell to have tight control over silencing to avoid inappropriate expression of silenced genes during nuclear reorganization that occurs during mating and as a protective response to stress. Likewise, since Sir3p is necessary for maintenance of telomere structure, strict positioning of the telomeres at the nuclear periphery may be required during nuclear fusion at mating and during the stress response. Our results suggest that Sir3p phosphorylation may play an important role in regulating changes in chromatin structure and nuclear organization.

Sir3p phosphorylation might also influence its interactions with other cellular components. Sir3p is known to interact physically with other molecules involved in transcriptional silencing and telomere function, including histones H3 and H4, Sir4p, Rap1p, and with the telomeres themselves (see Introduction). Our observations raise the possibility that alternatively phosphorylated forms of Sir3p interact with different members of the silencing apparatus. We examined phosphorylation of Sir3p in *sir1*, *sir2*, and *sir4* mutants in logarithmically growing cells and under pheromone-response and starvation conditions. Our experiments revealed that Sir3p phosphorylation patterns appeared comparable in wild-type strains and *sir* mutants under all conditions tested (unpublished data). Thus, Sir3p phosphorylation is neither dependent on transcriptionally silenced chromatin nor on *SIR1*, *SIR2*, or *SIR4* function. Determining whether the Sir3p that is found within silencing complexes is phosphorylated, and identifying which site(s) may be phosphorylated within such complexes, will be important for a refined understanding of the nature of silenced chromatin. Sir3p phosphorylation may precede assembly of a silencing complex to promote association of its components, or phosphorylation may occur after assembly to enable a complex to act more efficiently. Understanding Sir3p phosphorylation in the context of its physi-

cal interaction with other silencing proteins will supply important clues for how silenced chromatin assembles and functions.

DNA Replication and Phosphorylation of Chromatin-associated Proteins

Silenced transcriptional states are known to be mitotically stable and heritable (Pillus and Rine, 1989; Loo and Rine, 1995). Although DNA replication and the movement of replication forks through silenced chromatin may facilitate its assembly or remodeling (for reviews see Rivier and Rine, 1992; Loo and Rine, 1995), the precise connection between replication and silencing has not been defined. A provocative link between yeast transcriptional silencing and DNA replication is that Sir3p and Orc1p, the largest subunit of the Origin Recognition Complex, show a high degree of protein sequence identity within their first 200 amino acids and scattered similarity throughout the remainder of their sequences (Bell et al., 1995). It is not yet known whether Orc1p is phosphorylated or if it responds to any of the stimuli that induce Sir3p hyperphosphorylation. An additional connection between DNA replication and silencing is the observation that mutation of the *CDC7*-encoded protein kinase, active during DNA synthesis, can suppress silencing defects resulting from mutations in *cis*-regulatory elements. Likewise, increased *CDC7* gene dosage can interfere with normal silencing (Axelrod and Rine, 1991). It will be important to determine if any of the proteins involved in silencing, including Sir3p, are targets of Cdc7p activity.

Interestingly, at least one other yeast transcriptional silencing protein, Sir4p, is known to be phosphorylated (Kimmerly, 1988). Sir4p, which has limited sequence similarity to vertebrate nuclear lamins (Diffley and Stillman, 1989), contains two potential sites for MAP kinase recognition, LKSP at residues 387–390 and PSSP at 1132–1135 (Nigg, 1993). MAP kinases as well as the cdc2 kinase are known to act on the site important for chicken lamin B depolymerization (Peter et al., 1992), and lamin B phosphorylation at specific sites during mitosis results in nuclear lamina dissasembly and subsequent nuclear envelope breakdown (Peter et al., 1990). In *Drosophila*, the HP1 chromodomain protein becomes mutliply phosphorylated during heterochromatin assembly in the developing embryo. In this case, several different kinases are postulated to regulate HP1 phosphorylation (Eissenberg et al., 1994). Hyperphosphorylation thus occurs on proteins involved in both chromatin assembly and nuclear disassembly.

The phosphorylation of chromatin-associated proteins may prove to be a dynamic means of regulating silenced chromatin in yeast and in other organisms. Components of MAP kinase cascades might generally function in assembly and regulation of chromatin. Since MAP kinase cascades are key signal transducers for communicating and coordinating cellular responses, one aspect of this coordination may manifest itself in remodeling chromatin to mediate alterations in transcriptional regulation. Identification of the kinases involved and the specific target sites of Sir3p phosphorylation will enhance understanding the interplay between signal transduction, phosphorylation of silencing proteins, and nuclear organization in yeast.

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