Plc1p Is Required for SAGA Recruitment and Derepression of Sko1p-regulated Genes

Nilanjan Guha, Parima Desai, and Ales Vancura

Department of Biological Sciences, St. John's University, Queens, NY 11439

Submitted October 25, 2006; Revised March 9, 2007; Accepted April 4, 2007

Monitoring Editor: Susan Wenthe

In *Saccharomyces cerevisiae*, many osmotically inducible genes are regulated by the Sko1p-Ssn6p-Tup1p complex. On osmotic shock, the MAP kinase Hog1p associates with this complex, phosphorylates Sko1p, and converts it into an activator that subsequently recruits Swi/Snf and SAGA complexes. We have found that phospholipase C (Plc1p encoded by *PLC1*) is required for derepression of Sko1p-Ssn6p-Tup1p-controlled osmoinducible genes upon osmotic shock. Although *plc1Δ* mutation affects the assembly of the preinitiation complex after osmotic shock, it does not affect the recruitment of Hog1p and Swi/Snf complex at these promoters. However, Plc1p facilitates osmotic shock-induced recruitment of the SAGA complex. Like *plc1Δ* cells, SAGA mutants are osmosensitive and display compromised expression of osmotically inducible genes. The reduced binding of SAGA to Sko1p-Ssn6p-Tup1p-repressed promoters in *plc1Δ* cells does not correlate with reduced histone acetylation. However, SAGA functions at these promoters to facilitate recruitment of the TATA-binding protein. The results thus provide evidence that Plc1p and inositol polyphosphates affect derepression of Sko1p-Ssn6p-Tup1p-controlled genes by a mechanism that involves recruitment of the SAGA complex and TATA-binding protein.

INTRODUCTION

In yeast cells, phospholipase C (Plc1p encoded by *PLC1*) and three inositol polyphosphate kinases (Ipk2p/Arg82p, Ipk1p, and Kcs1p) constitute a pathway responsible for synthesis of several inositol polyphosphates (InsPs). InsPs affect transcriptional control (Odom *et al.*, 2000), export of mRNA from the nucleus (York *et al.*, 1999), homologous DNA recombination (Luo *et al.*, 2002), cell death, and telomere length (Saiardi *et al.*, 2005; York *et al.*, 2005). Although Plc1p is required for the initial step of InsPs synthesis, it is not required for cell viability, but *plc1Δ* cells display a number of phenotypes, including slow growth, temperature sensitivity, osmosensitivity, defective utilization of carbon sources other than glucose, altered cell morphology, inability to complete cytokinesis, sporulation defect, and sensitivity to nitrogen starvation (Flick and Thorner, 1993; Yoko-o *et al.*, 1993).

Recently, InsPs have been shown to regulate activity of chromatin remodeling complexes in vivo and in vitro (Shen *et al.*, 2003; Steger *et al.*, 2003). Induction of the phosphate-responsive *PHO5* gene, chromatin remodeling of its promoter, as well as recruitment of Swi/Snf and Ino80 chromatin remodeling complexes are impaired in the *ipk2/arg82* mutant strain (Steger *et al.*, 2003). In vitro, nucleosome mobilization by the yeast Swi/Snf complex is stimulated by IP₄ and IP₅, whereas IP₆ inhibits nucleosome mobilization by yeast Isw2 and Ino80 complexes and by the *Drosophila* NURF complex (Shen *et al.*, 2003). Because recombinant Nurf and Isw1 proteins can bind IP₆, the possible mechanism by

which InsPs affects chromatin remodeling may involve effects on protein conformation of the chromatin remodeling complexes (Shen *et al.*, 2003). Alternatively, IP₄ or IP₅ might affect the interaction between chromatin-remodeling complexes and chromatin, as has been shown for PIP₂ and the Swi/Snf complex (Zhao *et al.*, 1998).

To learn more about the role of Plc1p and InsPs in transcriptional regulation, we analyzed regulation of expression of osmoinducible genes. We have shown previously that *plc1Δ* cells are defective in induction of the osmoinducible *GPD1* gene (Lin *et al.*, 2002), which is at least partly responsible for the osmosensitivity of *plc1Δ* cells (Flick and Thorner, 1993). The yeast responds to osmotic shock through an evolutionarily conserved MAP kinase pathway, the high-osmolarity glycerol (HOG) pathway (Brewster *et al.*, 1993) of which PBS2, a MAPKK, and HOG1, a MAPK, are the two most important regulators (Hohmann, 2002). Genome-wide analysis has shown that a large number of genes are regulated by osmotic stress in a HOG1-dependent manner (Posas *et al.*, 2000). On osmotic stress, Hog1p is phosphorylated by its upstream MAPKK, Pbs2p, which facilitates translocation of Hog1p to the nucleus (Reiser *et al.*, 1999). In the nucleus, Hog1p controls activity of different transcription factors and facilitates assembly of the preinitiation complex (PIC; Alepuz *et al.*, 2001, 2003). Sko1p is a bZIP transcriptional repressor that represses transcription of ~40 genes by recruiting the Ssn6p-Tup1p corepressor complex (Nehlin *et al.*, 1992; Vincent and Struhl, 1992; Rep *et al.*, 2001; Proft *et al.*, 2005). Sko1p is phosphorylated within its N terminal domain by Hog1p, and this phosphorylation event converts Sko1p from a repressor to an activator. The phosphorylated Sko1p then facilitates recruitment of Hog1p along with the SAGA histone acetylase complex and the Swi/Snf chromatin remodeling complex (Proft *et al.*, 2001; Proft and Struhl, 2002). The recruitment of the SAGA and Swi/Snf complexes is dependent on the presence of the Ssn6p-Tup1p complex, which remains bound to the promoter even under dere-

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06-10-0946>) on April 11, 2007.

Address correspondence to: Ales Vancura (vancuraa@stjohns.edu).

Abbreviations used: InsP_n, inositol polyphosphates; ChIP, chromatin immunoprecipitation; TBP, TATA binding protein.

Table 1. Yeast strains used in this study

Strain	Genotype	Source/reference
W303-1a	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 ssd1-d2 can1-100</i>	R. Rothstein
W303-1 α	<i>MATα ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 ssd1-d2 can1-100</i>	R. Rothstein
W303	<i>MATa/MATα ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 can1-100/can1-100</i>	R. Rothstein
HL1-1	W303-1 α <i>plc1::URA3</i>	Lin <i>et al.</i> (2000)
HL1-3	W303-1 α <i>plc1::URA3</i>	De Lillo <i>et al.</i> (2003)
WPA023	W303-1 α <i>plc1::TRP1</i>	De Lillo <i>et al.</i> (2003)
WPL046	W303-1 α <i>plc1::TRP1</i>	De Lillo <i>et al.</i> (2003)
A0004	W303-1 α <i>ipk1::kanMX</i>	York <i>et al.</i> (1999)
A0003	W303-1 α <i>ipk2::HIS3</i>	Odom <i>et al.</i> (2000)
FY50	<i>MATa spt8::LEU2 leu2Δ1 trp1Δ63 ura3-52 his4-917δ</i>	Roberts and Winston (1997)
ND687	W303-1 α <i>spt8::LEU2</i>	This study
FY405	<i>MATa spt3::TRP1 leu2Δ1 lys2-173 trp1Δ63 ura3-52 his4-917δ</i>	Roberts and Winston (1997)
ND703	W303-1 α <i>spt3::TRP1</i>	This study
FY1290	<i>MATa spt7::LEU2 leu2Δ1 lys2-173 trp1Δ63 ura3-52 his4-917δ</i>	Roberts and Winston (1997)
ND713	W303-1 α <i>spt7::LEU2</i>	This study
DY6707	W303-1 α <i>spt20::HIS3</i>	Yu <i>et al.</i> (2003)
DY5116	W303-1 α <i>gcn5::TRP1</i>	Yu <i>et al.</i> (2000)
DY2348	W303-1 α <i>swi2::HIS3</i>	Yu <i>et al.</i> (2000)
MAP5	W303-1 α <i>tup1::kanMX</i>	Proft and Serrano (1999)
MAP6	W303-1 α <i>ssn6::kanMX</i>	Proft and Serrano (1999)
DY7250	W303-1 α <i>GALp::CDC20::ADE2 SPT15-HA(3)::URA3</i>	Yu <i>et al.</i> (2003)
AD066	W303-1 α <i>SPT15-HA(3)::URA3</i>	This study
AD070	W303-1 α <i>plc1::TRP1 SPT15-HA(3)::URA3</i>	This study
DY6361	W303-1 α <i>SWI2-myc18::TRP1</i>	Yu <i>et al.</i> (2003)
NG022	W303-1 α <i>SWI2-myc18::TRP1</i>	This study
NG019	W303-1 α <i>plc1::URA3 SWI2-myc18::TRP1</i>	This study
MAP51	W303-1 α <i>HA(3)-HOG1</i>	Proft and Struhl (2002)
NG070	W303-1 α <i>plc1::URA3 HA(3)-HOG1</i>	This study
K8135	W303-1 α <i>ADA2-myc18::TRP1</i>	Cosma <i>et al.</i> (1999)
NG040	W303-1 α <i>plc1::URA3 ADA2-myc18::TRP1</i>	This study
SGY2	<i>MATa SPT20-myc::TRP1</i>	Bhaumik and Green (2001)
NG075	W303-1 α <i>SPT20-myc::TRP1</i>	This study
NG073	W303-1 α <i>plc1::URA3 SPT20-myc::TRP1</i>	This study
SGY4	<i>MATa GCN5-myc::TRP1</i>	Bhaumik and Green (2001)
AD099	W303-1 α <i>GCN5-myc::TRP1</i>	This study
AD100	W303-1 α <i>plc1::URA3 GCN5-myc::TRP1</i>	This study
NG088	W303-1 α <i>spt3::TRP1 SPT15-HA(3)::URA3</i>	This study
JC217-1	W303-1 α <i>sjl1::URA3</i>	Nguyen <i>et al.</i> (2005)
NG103	W303-1 α <i>sjl1::URA3 ADA2-myc18::TRP1</i>	This study
NG095	W303-1 α <i>ipk2::HIS3 ADA2-myc18::TRP1</i>	This study
NG097	W303-1 α <i>ipk1::kanMX ADA2-myc18::TRP1</i>	This study
NG106	W303-1 α <i>ADA2-myc18::TRP1 [pSH22]</i>	This study

pressing conditions (Proft and Struhl, 2002). Though the precise mechanism by which these two chromatin modifying complexes are recruited to Sko1p-Ssn6p-Tup1p-repressed promoters has not been studied in detail, the SAGA and Swi/Snf coactivators have been shown to be important for overcoming repression mediated by this complex (Papamichos-Chronakis *et al.*, 2002; Proft and Struhl, 2002).

The role of Plc1p in the process of cellular response to osmotic stress has not been studied in detail. We have shown previously that Plc1p is required for expression of the osmoinducible *GPD1* gene, for intracellular accumulation of glycerol, and for wild-type levels of osmoresistance. Our results also indicated that *hog1 Δ plc1 Δ* cells are more osmo-sensitive, synthesize less glycerol, and express lower levels of a *GPD1-lacZ* fusion than strains with either single deletion, suggesting that Plc1p and Hog1p contribute separate functions to the process of adaptation to increased extracellular osmolarity (Lin *et al.*, 2002).

In this study, we elucidate the molecular mechanism by which Plc1p and InsPs affect transcription of the Hog1p-regulated genes that are repressed by the Sko1p-Ssn6p-

Tup1p complex. We show that Plc1p and InsPs do not affect recruitment of Hog1p or the Swi/Snf complex but are required for efficient recruitment of the SAGA complex to the osmoinducible promoters, which in turn affects the recruitment of TATA-binding protein (TBP) and formation of the PIC.

MATERIALS AND METHODS

Strains and Media

All yeast strains are listed in Table 1. All the strains used in this study are isogenic to W303. Standard genetic techniques were used to manipulate yeast strains and to introduce mutations from nonW303 strains into the W303 background (Sherman, 1991). Cells were grown in rich medium (YPD; 1% yeast extract, 2% Bacto-peptone, 2% glucose) or under selection in synthetic complete medium (SC) containing 2% glucose and, when appropriate, lacking specific nutrients in order to select for a plasmid or strain with a particular genotype. Meiosis was induced in diploid cells by incubation in 1% potassium acetate.

S1 Nuclease Analysis

Oligonucleotides complementary to the genes assayed by S1 nuclease analysis are as follows: *ACT1*, 5'-gcttcagtcacaaagacagggtgtctctctggggcaactctcaattcggtga-

gaaggatacta-3'; *AHP1*, 5'-cctggaatgtgctgacagtacaggt tggggagaagcagctggagcac-cggtaatgtattgg-3'; *GRE2*, 5'-gggtcaaa tgcgtcagccttagatgtctgggacaacttcacatggagaa-ttttgggttgta cctttccg-3'; and *LACZ*, 5'-ccggcaccgcttctgtgcccgaaccaggcaagcgc-cattgccattcagctgcgttga-3'. Total RNA was isolated from cultures grown in YPD medium to optical density $A_{600\text{ nm}} = 1.0$ by the hot phenol method as described previously (Iyer and Struhl, 1996). S1 probes were end labeled in a 25- μ l reaction mixture (5 pmol oligonucleotide, 125 μ Ci [γ - 32 P]ATP [6000 Ci/mmol, Perkin Elmer, Norwalk, CT], 1 $\times$ T4 polynucleotide kinase buffer, and 20 U T4 polynucleotide kinase [New England Biolabs, Beverly, MA]) at 37°C for 1 h. The reaction mixture was diluted with 25 μ l of water, T4 polynucleotide kinase was inactivated at 65°C for 20 min, and the labeled oligonucleotides were purified using MicroSpin G-25 columns (Amersham Biosciences, Piscataway, NJ). The labeled oligonucleotides (0.5 pmol) were hybridized with 20–40 μ g of total RNA in a 50 μ l reaction mixture (0.3 M NaCl, 1 mM EDTA, 40 mM HEPES, pH 7.0, and 0.1% Triton X-100) for 12 h at 55°C and treated with S1 nuclease (Life Sciences, St. Petersburg, FL) as described previously (Iyer and Struhl, 1996). The samples were analyzed on 20% denaturing polyacrylamide gels, and quantification was performed using PhosphorImager (Perkin Elmer).

Chromatin Immunoprecipitation and Quantitative Real-Time PCR Analysis

In vivo chromatin cross-linking and immunoprecipitation were performed essentially as described (Geng *et al.*, 2001) with several minor modifications. Briefly, yeast cells were grown in 600 ml YPD to an $A_{600\text{ nm}} = 1.0$, at which point they were fixed for 15 min by the addition of formaldehyde to a concentration of 1%. Subsequently, the cells were converted to spheroplasts with zymolyase. Spheroplasts were washed in 40 ml of ice-cold TBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl) and subsequently with 1 ml of FA lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride [PMSF]) containing protease inhibitors (Roche; Complete protease inhibitors) for each 50-ml aliquot of the original culture. Finally, the spheroplasts were resuspended in 200 μ l of FA lysis buffer, 300 μ l of glass beads were added, and then the samples were vortexed 20 times in 15-s bursts at the highest setting. The samples were pooled, and the suspension was then sonicated 10 times for 10 s each to fragment chromosomal DNA to an average size ~500 base pairs. The suspension was centrifuged 30 min at 12,000 $\times g$, and the supernatant was diluted with FA buffer to provide 1-ml aliquots of the resultant solubilized chromatin solution per immunoprecipitation and 100 μ l for total input DNA. Each aliquot was precleared by adding 50 μ l of 50% protein A/G-agarose slurry (Santa Cruz Biotechnology, Santa Cruz, CA) and incubating 1 h at 4°C with gentle rocking. Beads were then harvested by centrifugation, and the supernatant was incubated with 100 μ l of 25% protein A/G slurry, which had been previously incubated for 8 h with 6 μ g of antibody (anti-myc polyclonal antibody A-14 or anti-HA mAb F7 from Santa Cruz Biotechnology, anti-acetyl-Histone H3 [Lys14] from Upstate Biotechnology [Lake Placid, NY] or anti-RNA polymerase II mAb 8WG16 from Covance [Madison, WI]). Beads were then harvested and washed, and the DNA was released and extracted as described (Geng *et al.*, 2001). Total input DNA and coimmunoprecipitated DNA were then analyzed by real-time PCR (25 μ l reaction mixture) using the iQ SYBR Green Supermix and the Bio-Rad MyiQ Single Color Real-Time PCR Detection System (Bio-Rad, Richmond, CA). Each PCR reaction mixture was used to detect the presence of a protein at a particular locus. The MyiQ software generates a threshold count for each reaction mixture, which can be used to determine the enrichment of a protein at a given locus. Each immunoprecipitation was performed at least three times using different chromatin samples, and the occupancy was calculated using the *POL1* coding sequence as a negative control and corrected for the efficiency of the primers. The levels of the tagged proteins used in this work were identical in wild-type and *plc1 Δ* cells as determined by Western blotting. Primers used for real-time PCR analysis are as follows: *AHP1* (5'-CGGACGGTATTCACATATTGTG-3' and 5'-GCTGGGAATTCTTGTTAACTAAGTC-3'), *GRE2* (5'-AACAAATTGGC-CCTCACCTCTTTG-3' and 5'-TATTTACGGGCGTGTGATACTGC-3'), *POL1* (5'-TCTGTGACAAAGAAGGCAATAGAAG-3' and 5'-TAAACACCCCTGAT-CCACCTCTG-3'). Sequences flanking the Sko1p binding site in plasmid pMP224 were amplified with primers 5'-AGGCGTGTATATATAGCGTG-GATG-3' and 5'-CAGGGTTTTCCAGTCACGAC-3'.

Ada2p-myc Immunoprecipitation and [3 H]InsP $_4$ Binding Assay

Yeast cells were grown in 200 ml YPD to an $A_{600\text{ nm}} = 1.0$ and were spheroplasted with zymolyase. Spheroplasts were washed in 40 ml of ice-cold TBS buffer and then with 4 ml of FA lysis buffer containing protease inhibitors (Roche; Complete protease inhibitors). Finally, the spheroplasts were resuspended in 200 μ l of FA lysis buffer containing the protease inhibitors, 300 μ l of glass beads were added, and the sample was vortexed five times in 15-s bursts at the highest setting. The suspension was centrifuged 30 min at 12,000 $\times g$, and the supernatant was precleared by adding 50 μ l of 25% protein A/G-agarose slurry (Santa Cruz Biotechnology) and incubated 1 h at 4°C with gentle rocking. Beads were then harvested by centrifugation, and the supernatant was incubated for 2 h with 100 μ l of 25% protein A/G slurry that had been previously incubated for 8 h with 6 μ g of anti-myc polyclonal

antibody (A-14; Santa Cruz Biotechnology). Beads were then harvested and washed three times with 1 ml FA lysis buffer and once with 50 mM Tris, pH 7.4, containing 50 mM NaCl. The beads were resuspended in binding buffer (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM dithiothreitol, 1 mM EDTA). Ten microliters of the bead suspension was assayed for the presence of the tagged protein by Western blot analysis using anti-myc polyclonal antibody A-14. To assay Ins(1,3,4,5)P $_4$ binding, 50 μ l of the bead suspension was incubated in a total volume of 100 μ l of the binding buffer with 10 nM Ins(1,3,4,5)P $_4$ (Echelon Biosciences, Salt Lake City, UT) and [3 H]Ins(1,3,4,5)P $_4$ (Perkin Elmer-Cetus; 4500 cpm per tube). The mixture was incubated for 15 min at 4°C with gentle rocking. The beads were harvested, washed with 1 ml of the binding buffer, and resuspended in a total volume of 100 μ l water. Radioactivity was measured by scintillation counting, and the total binding was expressed relative to the untagged strain.

RESULTS

Plc1p Is Required for Overcoming Repression Mediated by URS_{CRE-ENAI} and URS_{MIG-ENAI} Promoter Elements

The *ENAI* gene is highly expressed in response to osmotic stress and glucose starvation. The promoter contains two distinct elements that regulate transcription in response to these different environmental stimuli. The cyclic AMP (cAMP) response element (CRE)-like sequence (URS_{CRE-ENAI}) that is bound by the transcriptional repressor Sko1p regulates the osmotic shock-dependent transcription, whereas a Mig1/2p binding site (URS_{MIG-ENAI}) is responsible for the glucose mediated repression (Proft and Serrano, 1999).

We utilized the pMP222 and pMP224 reporter plasmids (Proft and Serrano, 1999) to examine whether Plc1p affects repression/derepression mediated by the URS_{CRE-ENAI} and URS_{MIG-ENAI} elements. Plasmid pMP224 contains the CRE element derived from the *ENAI* promoter inserted upstream of the CYC1[TATA]-*lacZ* sequence. Plasmid pMP222 contains the Mig1p-binding site from the *ENAI* promoter instead of the CRE element (Proft and Serrano, 1999). The expression was examined under repressing conditions (YPD medium) and after derepression (0.8 M NaCl for pMP224 and 0.05% glucose for pMP222). Both pMP224 and pMP222 yielded a strong increase in the β -galactosidase levels after osmotic shock and glucose derepression, respectively, in the wild-type strain (Figure 1). This increase in the β -galactosidase activity was completely lost in the *plc1 Δ* mutant, suggesting that Plc1p plays a key role in the regulation of both of these promoter elements. On the other hand, *lacZ* expression under repressing conditions was not significantly different in wild-type and *plc1 Δ* cells, indicating that the repressor function of Sko1p-Ssn6p-Tup1p and Mig1p-Ssn6p-Tup1p does not require Plc1p and InsPs. As has been previously shown, disruption of the Ssn6-Tup1p corepressor complex results in a complete loss of regulation, as *ssn6 Δ* and *tup1 Δ* mutants are defective in repression mediated by both URS_{CRE-ENAI} and URS_{MIG-ENAI} elements and express high levels of β -galactosidase under repressing and derepressing conditions (Proft and Serrano, 1999). The regulation of both URS_{CRE-ENAI} and URS_{MIG-ENAI} is dependent on functional SAGA, because the stress-mediated induction of *lacZ* is abolished in *spt20 Δ* and *spt3 Δ* strains, components that play a key role in SAGA function (Figure 1). The regulation is, however, not significantly affected in the *gcn5 Δ* strain, the histone acetylase component of the SAGA complex, suggesting that Gcn5p plays a more dispensable role in the control of both elements of the *ENAI* gene. The fact that *plc1 Δ* cells are defective in overcoming repression mediated by both the URS_{CRE-ENAI} and URS_{MIG-ENAI} regulatory elements indicates that Plc1p affects a common target involved in transcriptional derepression rather than influencing different components specific for response to osmotic shock or glucose starvation.

Figure 1. *plc1Δ* cells fail to relieve repression mediated by $URS_{CRE-ENAI}$ and $URS_{CRE-MIG1}$ promoter elements. (A) β -Galactosidase activity was measured under repressing (YPD) and derepressing conditions (YPD + 0.8 M NaCl) in cells transformed with the pMP224 plasmid ($URS_{CRE-ENAI}$ -CYC1-lacZ). (B) β -Galactosidase activity was measured under repressing (YPD) and derepressing conditions (0.05% glucose) in cells transformed with the pMP222 plasmid ($URS_{MIG-ENAI}$ -CYC1-lacZ). The assays were carried out as previously described (Choi *et al.*, 1998). The values were calculated from three independent experiments and represent means $\pm$ SD.

Plc1p Is Required for Derepression of Sko1p-regulated Genes GRE2 and AHP1

plc1Δ cells are osmosensitive and defective in overcoming repression mediated by the $URS_{CRE-ENAI}$ element in plasmid pMP224 (Figure 1). Because regulation of promoters fused to *lacZ* in a plasmid can differ significantly from regulation of promoters in their natural chromosomal locations, it was important to determine whether the expression of osmotically inducible chromosomally encoded genes that are repressed by the Sko1p-Ssn6p-Tup1p complex is affected in *plc1Δ* cells. Wild-type and *plc1Δ* cells were subjected to moderate osmotic stress (0.4 M NaCl), and expression of *GRE2* and *AHP1*, two prototypic osmotically inducible and Sko1p-Ssn6p-Tup1p-repressed genes (Proft and Struhl,

Figure 2. *plc1Δ* and *spt20Δ* cells are defective in the expression of *GRE2* and *AHP1*. The indicated strains were grown in YPD medium at 30°C to $A_{600\text{ nm}} = 1.0$, and the total RNA was harvested before (0 min) and after adding 0.4 M NaCl at the indicated times. S1 endonuclease protection assays were performed with probes specific for *AHP1*, *GRE2*, and *ACT1*. The experiment was repeated three times and representative results are shown.

2002), was determined at different time points. Both *GRE2* and *AHP1* were significantly induced by osmotic stress (Figure 2). As expected, expression of both these genes was significantly reduced in the *plc1Δ* strain after osmotic shock. The expression was also dependent on SAGA, as deletion of *SPT20*, the gene encoding the subunit that is required for structural integrity of the SAGA complex, results in reduced activation of expression, whereas deletion of *SWI2* had a lesser effect. Thus, the results suggest that Hog1p dependent gene expression at Sko1p-regulated promoters depends on Plc1p, and to a greater extent also on SAGA.

Recruitment of Hog1p and the Swi/Snf Complex Is Not Affected in plc1Δ Cells

To better understand the mechanism by which Plc1p regulates the transcription of Sko1-Ssn6-Tup1 regulated promoters *GRE2* and *AHP1*, we determined recruitment of transcriptional regulatory proteins to these promoters. We hypothesized that the reduced expression of the Hog1p-dependent genes in *plc1Δ* cells may be due to a defect in osmotically induced recruitment of Hog1p to the target promoters. Hog1p is phosphorylated by its upstream MAPKK, Pbs2p, after which Hog1p interacts with and phosphorylates Sko1p in the nucleus (Proft *et al.*, 2001), thereby promoting the assembly of the PIC (Alepuz *et al.*, 2001; Proft and Struhl, 2002). In addition, the recruitment of the RNA polymerase II (RNA Pol II) holoenzyme has been shown previously to depend on active Hog1p at the Hot1p-dependent promoter of *STL1* (Alepuz *et al.*, 2003). To examine the possibility that the defect in expression of *GRE2* and *AHP1* in the *plc1Δ* strain is due to compromised recruitment of Hog1p, we performed chromatin immunoprecipitation (ChIP) assay with HA-tagged Hog1p. We found that upon osmotic stress the recruitment of Hog1p to the osmoinducible promoters is not affected in *plc1Δ* cells (Figure 3A). The results thus suggest that Plc1p does not interfere with Hog1p's role in mediating the response to the osmotic stress and functions downstream of Hog1p in the regulation of the osmoinducible genes. The recruitment of Hog1p after osmotic shock is also dependent on Sko1p, as deletion of the latter abolishes the binding of Hog1p to the promoters of *GRE2* and *AHP1* (Proft and Struhl, 2002). The fact that Plc1p does not influence the binding of Hog1p after osmotic shock indicates that recruitment of Sko1p is also not affected in *plc1Δ* cells. This conclusion is also strongly supported by the fact that expression from the Hog1p-independent Mig1p-Ssn6p-Tup1p-regulated promoter element, $URS_{MIG-ENAI}$, is also defective in *plc1Δ* cells (Figure 1B).

The Swi/Snf chromatin remodeling complex is recruited to osmoinducible promoters in a Sko1p-Ssn6p-Tup1p-dependent manner (Proft and Struhl, 2002). To test whether the

Figure 3. Recruitment of Hog1p and Swi2p to *GRE2* and *AHP1* promoters is not affected in *plc1Δ* cells. ChIP was performed using chromatin from wild-type or *plc1Δ* cells subjected to either no treatment or treatment with 0.4 M NaCl for 5 min (osmotic shock). Each immunoprecipitation was performed at least three times using different chromatin samples, and the occupancy was calculated using the *POL1* coding sequence as a negative control. The data are presented as fold occupancy over the *POL1* coding sequence control and represent means $\pm$ SD. (A) Recruitment of Hog1p at the *GRE2* and *AHP1* promoters was assayed in *PLC1* (MAP51) and *plc1Δ* (NG070) strains expressing 3HA-Hog1p by immunoprecipitation with anti-HA antibody. (B) Swi2p occupancy was determined in *PLC1* (NG022) and *plc1Δ* (NG019) strains expressing Swi2p-myc18 using anti-myc antibody.

recruitment of this complex at these promoters is dependent on Plc1p, we performed ChIP using myc-tagged Swi2p. We found that *plc1Δ* cells show no defect in the osmotic shock-dependent recruitment of Swi2p at these promoters (Figure 3B). The results thus suggest that the recruitment of Swi/Snf at the osmoinducible promoters takes place independently of Plc1p and InsPs.

Plc1p Is Required for Osmotic Shock-dependent Recruitment of the SAGA Complex

Regulation of expression from the URS_{CRE-ENAI}-CYC1-*lacZ* construct (plasmid pMP224) shows that, in addition to Plc1p, stress-induced expression also requires the SAGA complex (Figure 1A). To address whether recruitment of SAGA is affected in *plc1Δ* cells, we performed ChIP assay using a myc-tagged version of Ada2p, one of the three Ada proteins of the SAGA complex. In wild-type cells, Ada2p is recruited in response to osmotic induction to both *GRE2* and *AHP1* promoters (Figure 4A). However, this recruitment is reduced to ~30% in *plc1Δ* cells. To see if Plc1p also affects other SAGA subunits, we determined recruitment of Gcn5p and Spt20p. Similarly to Ada2p, *plc1Δ* cells display a defect in recruitment of these subunits to *GRE2* and *AHP1* promoters in response to osmotic shock (Figure 4, B and C). Because the association of Spt20p, the subunit which maintains the structural integrity of the complex, is affected in the same way as recruitment of the other subunits, it appears that Plc1p affects the recruitment of SAGA as a whole complex and does not differentially affect individual subunits. The decreased recruitment of SAGA in *plc1Δ* cells is not due to the lower protein level of SAGA subunits, as indicated by Western blot analysis of Ada2p and Spt20p (Figure 4D). There is a high basal level of SAGA occupancy at the *AHP1* promoter in both wild-type and *plc1Δ* cells, which corresponds to high transcription of *AHP1* even in the absence of

osmotic stress (Figure 2). This could be due to other ATF/CREB activators such as Aca1p and Aca2p that regulate transcription independently of salt stress (Garcia-Gimeno and Struhl, 2000).

Because *plc1Δ* cells are completely devoid of all InsPs (York *et al.*, 1999; Odom *et al.*, 2000), we wanted to identify the specific inositol polyphosphate that is required for the recruitment of the SAGA complex. ChIP experiments in *ipk2Δ* and *ipk1Δ* cells expressing Ada2p-myc showed that, similar to *plc1Δ* cells, the *ipk2Δ* strain fails to recruit Ada2p to *GRE2* promoter after osmotic shock. However, Ada2p occupancy at the *GRE2* promoter in *ipk1Δ* cells was almost at the wild-type level (Figure 4E). Because Ipk2p converts IP₃ into IP₄ and IP₅ that is subsequently converted into IP₆ by Ipk1p, it appears that SAGA recruitment requires IP₄ and/or IP₅.

Because *AHP1* and *GRE2* were expressed from their genomic loci, it was possible that the reduced occupancy of the SAGA complex at these promoters in *plc1Δ* cells was not due to a decreased recruitment of SAGA to the Sko1p-Ssn6p-Tup1p complex but due to decreased occupancy of some transcriptional activator that is involved in recruitment of SAGA to the corresponding promoters. To exclude this possibility, we determined the recruitment of Ada2-myc to the pMP224 plasmid bearing minimal Sko1p-binding site and no other promoter elements (Proft and Serrano, 1999). The results show that similarly to *AHP1* and *GRE2* expressed from their genomic loci, the enhanced recruitment of Ada2p-myc to the Sko1p-binding site in response to osmotic shock is severely compromised in the *plc1Δ* strain (Figure 5A), thus confirming that Plc1p is required for the osmotic shock-dependent recruitment of SAGA and for overcoming repression mediated by the Sko1p-Ssn6p-Tup1p complex. Unlike *swi2Δ* cells, *plc1Δ* and *spt20Δ* cells are also defective in expression of *lacZ* from the pMP224 plasmid (Figure 5B), further suggesting that in the process of overcoming repression imposed by the Sko1p-Ssn6p-Tup1p complex, Plc1p and InsPs are required for the recruitment of SAGA, whereas recruitment of the Swi/Snf complex is relatively independent of Plc1p and InsPs (Figure 3B) and appears to be less important for derepression of transcription (Figure 2).

The recruitment of SAGA upon osmotic shock depends on Hog1p and Sko1p (Proft and Struhl, 2002). Our results indicate that the recruitment of Hog1p is independent of Plc1p and that only the recruitment of SAGA depends on Plc1p. However, this dependence of SAGA recruitment on Plc1p appears to be restricted only to Sko1p-Ssn6p-Tup1p-repressed promoters as Plc1p does not affect SAGA occupancy at the SAGA-dependent *ADH1* promoter (data not shown), where the binding of Ada2p-myc is comparable in both wild-type and *plc1Δ* strains.

Defect in SAGA Recruitment in plc1Δ Cells Is Not Caused by Increased PIP₂ Level

plc1Δ cells do not appear to accumulate PIP₂ under iso-osmotic conditions (Perera *et al.*, 2004). However, it is not known whether osmotic shock results in increased PIP₂ level in *plc1Δ* cells. To rule out possibility that the decreased recruitment of SAGA in *plc1Δ* strain is due to possible build up of the substrate PIP₂, we undertook two approaches to experimentally increase the cellular level of PIP₂. First, we determined the osmotic shock-induced recruitment of Ada2p to *GRE2* promoter in *sjl1Δ* strain. *SJL1* codes for phosphatidylinositol 4, 5-bisphosphate 5-phosphatase and plays a role in regulation of PIP₂ homeostasis. Deletion of *SJL1* causes an accumulation of PIP₂ (Stolz *et al.*, 1998). In our second approach, we overexpressed *MSS4*, which encodes phosphatidylinositol 4-phosphate kinase. Overexpression of

Figure 4. Plc1p is required for salt shock-mediated recruitment of the SAGA complex to *GRE2* and *AHP1* promoters. ChIP was performed using chromatin from *PLC1* or *plc1*Δ cells grown in YPD medium (0 min) and subjected to treatment with 0.4 M NaCl for 5 min. Each immunoprecipitation was performed at least three times using different chromatin samples, and the occupancy was calculated using the *POL1* coding sequence as a negative control. The data are presented as fold occupancy over the *POL1* coding sequence control and represent means $\pm$ SD. (A) Recruitment of Ada2p is hindered in the absence of Plc1p. *PLC1* (K8135) and *plc1*Δ (NG040) cells were assayed for recruitment of Ada2p-myc18 at the indicated promoters before and after osmotic shock. (B) Recruitment of Gcn5p is affected in *plc1*Δ cells. ChIP was performed using *PLC1* (AD099) and *plc1*Δ (AD100) cells expressing Gcn5-myc. (C) Osmotic shock-induced recruitment of Spt20p is dependent on Plc1p. ChIP was performed with *PLC1* (NG075) and *plc1*Δ (NG073) cells expressing Spt20p-myc. (D) *plc1*Δ cells have wild-type level of SAGA. Samples from *PLC1* and *plc1*Δ cells expressing Ada2p-myc and Spt20p-myc were analyzed by Western blotting with anti-myc polyclonal antibody (A-14; Santa Cruz Biotechnology). To confirm equivalent amounts of loaded proteins, the membrane was stripped and incubated with anti-actin mAb (clone C4, MP Biochemicals). The experiment was performed three times and representative results are shown. (E) *InsP*₄ and/or *InsP*₅ are required for SAGA recruitment upon salt shock. ChIP experiments were performed

Figure 5. Plc1p is required for recruitment of SAGA to a minimal Sko1p binding site. (A) *PLC1* (K8135) and *plc1*Δ (NG040) strains expressing Ada2p-myc18 were transformed with plasmid pMP224 (*URS*_{CRE-ENAI}-*CYC1-lacZ*) or control plasmid pMP206 that does not contain the CRE site (*CYC1-lacZ*). The cells were grown in YPD medium and subjected to treatment with 0.4 M NaCl for 5 min. ChIP was performed with anti-myc antibody utilizing primers that are adjacent to the CRE site in the pMP224 plasmid. The values indicate the fold occupancy of Ada2p at the CRE site in pMP224 over the *POL1* coding sequence control and relative to that of the control plasmid pMP206. The values represent means $\pm$ SD of three independent immunoprecipitations. (B) Reduced expression of *lacZ* in *plc1*Δ and *spt20*Δ cells after osmotic stress. The indicated strains transformed with plasmid pMP224 were grown in YPD medium at 30°C to $A_{600\text{ nm}} = 1.0$ and the total RNA was harvested before (0 min) and after adding NaCl (0.4 M) at the indicated times. S1 nuclease protection assays were performed using *LACZ* and *ACT1* probes. The experiment was repeated three times and a representative result is shown.

MSS4 also results in increased level of *PIP*₂ (Desrivieres *et al.*, 1998). In both strains, we observed increased recruitment of Ada2p upon osmotic stress, comparable to the wild-type strain (Figure 6A). Our results thus suggest that increased level of *PIP*₂ is not responsible for decreased SAGA recruitment in *plc1*Δ cells after salt stress.

Ada2p was found to bind phosphatidylinositolphosphates (C. Brandl, University of Western Ontario, London, Canada, personal communication). The fact that *InsP*₄ and/or *InsP*₅ affect the osmotic shock-mediated recruitment of Ada2p prompted us to test whether these molecules also bind Ada2p and thereby modulate Ada2p's recruitment to the osmoinducible promoters. To test this possibility, we assayed binding of *InsP*₄ to Ada2p-myc immunoprecipitated from wild-type strain with anti-myc antibody, whereas an untagged strain was used as a control. As confirmed by Western blot analysis of the immunoprecipitates, Ada2p-myc was only detected in the tagged strain (Figure 6B). The immunoprecipitated Ada2p-myc was then examined for the binding of *InsP*₄ using [³H]*InsP*₄. Compared with the untagged strain, Ada2p-myc immunoprecipitate was able to bind of [³H]*InsP*₄ to a level significantly above the control (Figure 6C). Though the functional significance of this bind-

using chromatin from *PLC1*, *plc1*Δ, *ipk2*Δ, and *ipk1*Δ cells expressing Ada2p-myc18 (strains K8135, NG040, NG095, and NG097, respectively). Each immunoprecipitation was performed at least three times using different chromatin samples, and the occupancy was calculated using the *POL1* coding sequence as a negative control. The data are presented as fold occupancy over the *POL1* coding sequence control and represent means $\pm$ SD.

Figure 6. Increased PIP_2 level is not responsible for decreased recruitment of Ada2p to *GRE2* promoter. (A) ChIP was performed with chromatin from *PLC1*, *sjl1Δ*, and *pMSS4* cells (*PLC1* cells transformed with pSH22 plasmid; 2μ *URA3*, *MSS4*) and *plc1Δ* cells, all expressing Ada2p-myc18 (strains K8135, NG103, NG106, and NG040, respectively), before and after salt shock. Each immunoprecipitation was performed at least three times using different chromatin samples, and the occupancy was calculated using the *POL1* coding sequence as a negative control. The data are presented as fold occupancy over the *POL1* coding sequence control and represent means $\pm$ SD. (B) Ada2p-myc was immunoprecipitated from cells expressing Ada2p-myc18 (strain K8135) using anti-myc antibody. Untagged strain (*ADA2*) was used as a negative control. Immunoprecipitates were analyzed by Western blotting. (C) Immunoprecipitated material from both strains was tested for [^3H]Ins(1,3,4,5) P_4 binding as described in *Materials and Methods*. The [^3H]Ins(1,3,4,5) P_4 binding in Ada2p-myc immunoprecipitate was arbitrarily set to 100%. The values were calculated from three independent experiments and represent means $\pm$ SD.

ing is yet to be determined, our results suggest presence of a receptor domain for InsP_4 in Ada2p. However, we cannot exclude a possibility that InsP_4 binds to a different protein, most likely subunit of SAGA, that coimmunoprecipitates with Ada2p-myc.

SAGA Is Required for TBP Recruitment at the *GRE2* and *AHP1* Promoters

To determine whether the difference between the wild-type and *plc1Δ* cells in SAGA occupancy at the *GRE2* and *AHP1* promoters is reflected in the level of histone H3 acetylation, we performed ChIP assay with antibodies against histone H3 acetylated at K14. Interestingly, increased recruitment of Gcn5p at the osmoinducible promoters in wild-type cells (Figure 4B) does not correlate with increased histone H3 acetylation (Figure 7A). In both wild-type and *plc1Δ* cells,

Figure 7. SAGA occupancy at *GRE2* and *AHP1* promoters is not associated with increased histone acetylation but is required for TBP recruitment. (A) Activation of *GRE2* and *AHP1* is associated with decreased histone H3 acetylation. *PLC1* (AD099) and *plc1Δ* (AD100) cells were grown in YPD medium and subjected to treatment with 0.4 M NaCl for 5 min. ChIP was performed with antibodies against acetylated histone H3 tail (Upstate Biotechnology). The level of histone acetylation before stress is arbitrarily set to 1. (B) *plc1Δ* and *spt3Δ* cells exhibit decreased recruitment of TBP to *GRE2* and *AHP1* promoters. ChIP was performed using anti-HA antibody and chromatin from *PLC1*, *plc1Δ*, and *spt3Δ* cells expressing Spt15p-3HA (strains AD066, AD070, and NG088, respectively). (C) *plc1Δ* cells exhibit decreased recruitment of RNA Pol II to *GRE2* and *AHP1* promoters in response to osmotic shock. RNA Pol II occupancy was determined by immunoprecipitation of Rpb1p (the largest subunit of RNA polymerase II) with 8WG16 mAb (Covance). Each immunoprecipitation (A–C) was performed at least three times using different chromatin samples, and the occupancy was calculated using the *POL1* coding sequence as a negative control. The data are presented as fold occupancy over the *POL1* coding sequence control and represent means $\pm$ SD.

the osmotic shock results in a somewhat reduced level of histone H3 acetylation. This could be attributed to the activity of the histone deacetylase complex Rpd3p-Sin3p, which is also recruited in a Hog1p-dependent manner to activate expression of osmotically inducible genes. The expression of these genes was shown to depend on the extent of histone deacetylation (De Nadal *et al.*, 2004). Our results thus suggest that SAGA regulates the expression of Sko1p-Ssn6p-Tup1p-repressed genes by a mechanism that is distinct from altering the pattern of histone H3 acetylation.

The fact that the level of histone H3 acetylation does not correlate with SAGA occupancy at the *GRE2* and *AHP1* promoters suggests that in addition to the histone acetyltransferase activity of Gcn5p, SAGA provides additional function at the osmotically inducible promoters. The Spt3p subunit of SAGA has been previously reported to play a role in the recruitment of TBP at different SAGA dependent promoters (Bhaumik and Green, 2002). To test the possibility that Spt3p is required for recruitment of TBP at the Sko1p-

Figure 8. Osmosensitivity of SAGA mutants. The indicated strains were grown to log phase at 30°C and 10-fold serial dilutions were spotted onto YPD plates containing different concentrations of NaCl. The strains were allowed to grow for 48 h.

Ssn6p-Tup1p-repressed promoters, we analyzed the recruitment of TBP in wild-type, *plc1Δ*, and *spt3Δ* strains before and after osmotic shock. Osmotic shock increases TBP occupancy at *GRE2* and *AHP1* promoters in wild-type cells 4–6 times (Figure 7B). However, in agreement with reduced transcription, *plc1Δ* cells display a significant reduction in the recruitment of TBP after salt induction. The recruitment of TBP is completely abolished in *spt3Δ* cells at *GRE2* and *AHP1* promoters, thus indicating that the Spt3p component of SAGA is indispensable for the osmotic shock-induced recruitment of TBP. The enhanced TBP recruitment at the *AHP1* promoter in the absence of stress in wild-type and *plc1Δ* cells is also diminished in *spt3Δ* cells, suggesting that SAGA is involved in TBP recruitment for transcription under normal conditions. The binding of RNA Pol II at *GRE2* and *AHP1* promoters was also severely affected in *plc1Δ* cells after salt shock (Figure 7C). Our results thus demonstrate that under conditions of osmotic shock, Plc1p facilitates recruitment of the SAGA coactivator, which in turn stimulates PIC assembly by facilitating Spt3p-dependent TBP recruitment.

SAGA Mutants Are Osmosensitive

Our results show that the osmotic shock-induced expression of *GRE2* and *AHP1* is SAGA dependent. We assessed the osmosensitivity of different SAGA mutants by spotting *spt20Δ*, *spt7Δ*, *spt8Δ*, *spt3Δ*, and *gcn5Δ* strains on YPD medium containing 0.4 M or 0.8 M NaCl. Spt7p along with Spt20p are required to maintain the structural integrity and function of the SAGA complex (Grant *et al.*, 1997), whereas Spt3p is a transcriptional regulator essential for recruitment of TBP at SAGA-dependent promoters (Eisenmann *et al.*, 1992; Bhaumik and Green, 2002). Spt8p, previously shown to interact with Spt3p and TBP (Eisenmann *et al.*, 1994), is also required for TBP recruitment at some SAGA-dependent promoters (Bhaumik and Green, 2002). Among the different SAGA mutants, *spt20Δ* and *spt3Δ* are the most sensitive to high salt concentration, followed by *spt7Δ* and *spt8Δ* (Figure 8). However, deletion of *Gcn5*, which encodes the subunit of SAGA with histone acetyltransferase activity, did not result in increased osmosensitivity, suggesting that only the subunits of SAGA that are involved in TBP recruitment are important for proper transcriptional response to osmotic shock.

Before osmotic shock

After osmotic shock

Figure 9. Model depicting the role of Plc1p and InsPs in derepression of Sko1p-Tup1-Ssn6p-regulated promoters. Before osmotic shock, the promoters of *GRE2* and *AHP1* are repressed by the Sko1p-Tup1-Ssn6p repressor complex that is bound to the upstream CRE site (Proft and Struhl, 2002), and Hog1p is located mainly in the cytoplasm (Reiser *et al.*, 1999). After osmotic shock, the phosphorylated Hog1p is translocated to the nucleus and converts the Sko1p-Tup1-Ssn6p repressor complex into an activator by phosphorylating Sko1p (Proft *et al.*, 2001). The activated repressor complex then recruits Swi/Snf and SAGA complexes (Proft and Struhl, 2002). The Plc1p-dependent recruitment of SAGA is required for TBP binding and PIC formation.

The osmosensitivity of *plc1Δ* and *ipk2Δ* strains correlates well with the defect in Ada2p recruitment, whereas *ipk1Δ* cells are not osmosensitive and display almost wild-type level of Ada2p recruitment to the *GRE2* promoter (Figure 4E). The greater level of osmosensitivity of *plc1Δ* and *ipk2Δ* strains compared with *spt20Δ* and *spt3Δ* strains is likely due to the role of InsPs in expression of Sko1p-Ssn6p-Tup1p- and SAGA-independent genes that are involved in osmore-sistance (Lin *et al.*, 2002).

DISCUSSION

Chromatin structure can be altered either by ATP-dependent chromatin remodeling complexes such as Swi/Snf or by covalent modification of histone proteins by complexes such as SAGA. The role of InsPs in the regulation of recruitment and activity of ATP-dependent chromatin remodeling complexes is well established (Shen *et al.*, 2003; Steger *et al.*, 2003). Here we present data that suggest that Plc1p and InsPs are also required for the recruitment of the SAGA complex to promoters regulated by the Sko1p-Ssn6p-Tup1p complex (Figure 9). *GRE2* and *AHP1* are regulated by Sko1p, which recruits the Ssn6p-Tup1p corepressor complex. Sko1p is activated by the Hog1p MAP kinase by direct phosphorylation that converts Sko1p into an activator that recruits SAGA and Swi/Snf in an Ssn6p-Tup1p-dependent manner (Proft and Struhl, 2002). Because InsPs were shown to be important for recruitment of chromatin remodeling complexes (Steger *et al.*, 2003),

we expected that *plc1Δ* cells that are completely devoid of InsPs (York *et al.*, 1999; Odom *et al.*, 2000) would fail to recruit the Swi/Snf complex to Sko1p-Ssn6p-Tup1p-repressed promoters. However, *plc1Δ* cells do not display any defect in osmotically induced recruitment of Swi2p to *GRE2* and *AHP1* promoters (Figure 3B), whereas recruitment of SAGA is severely compromised (Figures 4 and 9).

Because Plc1p is required for the recruitment of Spt20p, the subunit essential for SAGA integrity and function, we conclude that *plc1Δ* cells fail to recruit the entire SAGA complex to Sko1p-Ssn6p-Tup1p-repressed promoters that results in failure to overcome repression imposed by the Sko1p-Ssn6p-Tup1p complex. This conclusion is further supported by the fact that *plc1Δ* cells are not able to recruit SAGA to a minimal Sko1p-binding site that regulates expression of a plasmid-encoded *CYC1-lacZ* reporter. Decreased SAGA occupancy then leads to compromised transcription (Figure 5B). The role of SAGA in derepression of Sko1p-Ssn6p-Tup1p-repressed promoters does not seem to depend on Gcn5p and increased histone H3 acetylation (Figure 7A). Rather, SAGA facilitates recruitment of TBP in a Spt3p-dependent manner (Figures 7B and 9). The less important role of Gcn5p in the process of osmotic derepression of Sko1p-Ssn6p-Tup1p-regulated promoters is also suggested by the fact that, in wild-type cells, the osmotically induced recruitment of SAGA is not accompanied by increased histone H3 acetylation (Figure 7). This result is in agreement with a finding that the expression of *GRE2* is severely impaired in cells lacking the Rpd3p-Sin3p histone deacetylase complex that is recruited in a Hog1p-dependent manner to osmotically inducible promoters (De Nadal *et al.*, 2004). Our results are thus in agreement with the model in which decrease in histone acetylation of these promoters is associated with increased transcription. This conclusion is indirectly supported by the fact that unlike *spt20Δ*, *spt3Δ*, and *spt7Δ* cells, *gcn5Δ* cells are not osmosensitive (Figure 8).

Spt3p functions by facilitating recruitment of TBP in a manner largely independent of Gcn5p (Lee *et al.*, 2000). Genome-wide computational approaches classified yeast promoters as TATA box-containing (20%) or TATA-less (80%; Basehoar *et al.*, 2004). In comparison to TATA-less genes, TATA box-containing genes are highly regulated, often involved in stress response, and utilize SAGA rather than TFIID. Spt3p-dependent genes belong to the group of TATA box-containing genes. Both *GRE2* and *AHP1* were classified as TATA box-containing and SAGA-regulated (Basehoar *et al.*, 2004). The defect in TBP recruitment to *GRE2* and *AHP1* promoters in the *spt3Δ* mutant (Figure 7B) is in agreement with this classification.

The recruitment of both SAGA and Swi/Snf complexes in response to osmotic stress is dependent on the Ssn6p-Tup1p corepressor complex, which has been demonstrated to co-occupy the activated *GRE2* promoter along with these complexes (Proft and Struhl, 2002). The fact that the recruitment of Swi/Snf is not affected in *plc1Δ* cells rules out the possibility that *plc1Δ* cells are defective in binding of Ssn6p-Tup1p. In addition, because recruitment of Hog1p requires Sko1p (Proft and Struhl, 2002) and we did not observe any noticeable differences in Hog1p occupancy in wild-type and *plc1Δ* cells (Figure 3A), we conclude that Sko1p binding is not affected in *plc1Δ* cells.

Our results do not rule out a possibility that Plc1p-dependent recruitment of SAGA is mediated by other transcriptional factor(s) that interact(s) with SAGA. Cti6p (Cyc8-Tup1-interacting protein 6) has been shown to specifically interact with the Ssn6p subunit of the Ssn6p-Tup1p corepressor (Papamichos-Chronakis *et al.*, 2002). The protein

contains a PHD finger that is common to a number of chromatin regulatory proteins. The PHD finger of ING2, a candidate tumor suppressor protein in mammals was demonstrated to be a nuclear PtdInsPs receptor (Gozani *et al.*, 2003). Though a specific function of the PHD motif is yet to be found in yeast, Cti6p has been demonstrated to be involved in recruitment of SAGA at the *GAL1* promoter under inducing conditions (Papamichos-Chronakis *et al.*, 2002). At the *GAL1* promoter, Cti6p links the SAGA coactivator with the Ssn6p-Tup1p corepressor, thus facilitating its transcription in the presence of galactose (inducing condition). Cti6p is also required for overcoming Tup1p repression at the *ARN1* promoter (Crisp *et al.*, 2006) and was found to associate with the Rpd3p-Sin3p histone deacetylase complex (Puig *et al.*, 2004).

To test the possibility that Cti6p is recruited to the *GRE2* and *AHP1* promoters upon osmotic stress and facilitates recruitment of SAGA to the Ssn6p-Tup1p complex to overcome the repression, we determined Cti6p occupancy at both promoters. In both wild-type and *plc1Δ* cells, we failed to detect any Cti6p recruitment to *GRE2* and *AHP1* promoters before or after osmotic induction (data not shown). However, recruitment of SAGA to *GRE2* and *AHP1* was not affected in *cti6Δ* cells. Also, in contrast to different SAGA mutants (Figure 8), *cti6Δ* cells are not osmosensitive (data not shown), suggesting that Cti6p does not mediate recruitment of the SAGA complex to Sko1p-Ssn6p-Tup1p-repressed promoters.

The mechanism for the role of InsPs in the recruitment of the SAGA complex to Sko1p-Ssn6p-Tup1p-regulated promoters very likely involves binding of InsP₄ and/or InsP₅ by one or more subunits of the SAGA complex. This binding perhaps makes SAGA more competent for interaction with the Sko1p-Ssn6p-Tup1p repressor complex. Interestingly, the Ada2p subunit of SAGA was recently found to bind phosphatidylinositolphosphates (C. Brandl, personal communication), and we found that immunoprecipitated Ada2p binds also InsP₄ (Figure 6C). Detailed analysis of InsPs binding to the components of the SAGA complex and the role of InsPs in recruitment of SAGA will require additional work. An important conclusion of this work is that osmotic shock-dependent recruitment of SAGA to Sko1p-Ssn6p-Tup1p-repressed promoters is dependent on the presence of Plc1p and InsPs. Though InsPs have been shown to play a role in recruitment of ATP-dependent chromatin-remodeling complexes (Steger *et al.*, 2003), our results show that they also affect recruitment of other coactivator complexes, such as SAGA.

ACKNOWLEDGMENTS

We thank Drs. Green, Hall, Nasmyth, Proft, Ronne, Stillman, Struhl, Winston, and York for strains and plasmids, Dr. Brandl for communicating results before publication, and members of Vancura lab and Dr. Vancurova for helpful comments. This work was supported by grants from the National Institutes of Health (GM62183) and the American Cancer Society (RSG-01-145-01-CCG) to A.V.

REFERENCES

- Alepuz, P. M., de Nadal, E., Zapater, M., Ammerer, G., and Posas, F. (2003). Osmotically-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA Pol II. *EMBO J.* 22, 2433-2442.
- Alepuz, P. M., Jovanovic, A., Reiser, V., and Ammerer, G. (2001). Stress-induced map kinase Hog1 is part of transcription activation complexes. *Mol. Cell* 7, 767-777.
- Basehoar, A. D., Zanton, S. J., and Pugh, B. F. (2004). Identification and distinct regulation of yeast TATA box-containing genes. *Cell* 116, 699-709.
- Bhaumik, S. R., and Green, M. R. (2001). SAGA is an essential in vivo target of the yeast acidic activator Gal4p. *Genes Dev.* 15, 1935-1945.

- Bhaumik, S. R., and Green, M. R. (2002). Differential requirement of SAGA components for recruitment of TATA-box-binding protein to promoters in vivo. *Mol. Cell. Biol.* 22, 7365–7371.
- Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E., and Gustin, M. C. (1993). An osmosensing signal transduction pathway in yeast. *Science* 259, 1760–1763.
- Choi, J. H., Lou, W., and Vancura, A. (1998). A novel membrane-bound glutathione S-transferase functions in the stationary phase of the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* 273, 29915–29922.
- Cosma, M. P., Tanaka, T., and Nasmyth, K. (1999). Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. *Cell* 97, 299–311.
- Crisp, R. J., Adkins, E. M., Kimmel, E., and Kaplan, J. (2006). Recruitment of Tup1p and Cti6p regulates heme-deficient expression of Aft1p target genes. *EMBO J.* 25, 512–521.
- De Nadal, E., Zapater, M., Alepuz, P. M., Sumoy, L., Mas, G., and Posas, F. (2004). The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmo-responsive genes. *Nature* 427, 370–374.
- DeLillo, N., Romero, C., Lin, H., and Vancura, A. (2003). Genetic evidence for a role of phospholipase C at the budding yeast kinetochore. *Mol. Genet. Genomics* 269, 261–270.
- Desrivieres, S., Cooke, F. T., Parker, P. J., and Hall, M. N. (1998). MSS4, a phosphatidylinositol-4-phosphate 5-kinase required for organization of the actin cytoskeleton in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 273, 15787–15793.
- Eisenmann, D. M., Arndt, K. M., Ricupero, S. L., Rooney, J. W., and Winston, F. (1992). SPT3 interacts with TFIID to allow normal transcription in *Saccharomyces cerevisiae*. *Genes Dev.* 6, 1319–1331.
- Eisenmann, D. M., Chapon, C., Roberts, S. M., Dollard, C., and Winston, F. (1994). The *Saccharomyces cerevisiae* SPT8 gene encodes a very acidic protein that is functionally related to SPT3 and TATA-binding protein. *Genetics* 137, 647–657.
- Flick, J. S., and Thorner, J. (1993). Genetic and biochemical characterization of a phosphatidylinositol-specific phospholipase C in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 13, 5861–5876.
- Garcia-Gimeno, M. A., and Struhl, K. (2000). Aca1 and Aca2, ATF/CREB activators in *Saccharomyces cerevisiae*, are important for carbon source utilization but not the response to stress. *Mol. Cell. Biol.* 20, 4340–4349.
- Geng, F., Cao, Y., and Laurent, B. C. (2001). Essential roles of Snf5p in Snf-Swi chromatin remodeling in vivo. *Mol. Cell. Biol.* 21, 4311–4320.
- Gozani, O. *et al.* (2003). The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. *Cell* 114, 99–111.
- Grant, P. A. *et al.* (1997). Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA (Spt/Ada) complex. *Genes Dev.* 11, 1640–1650.
- Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol. Mol. Biol. Rev.* 66, 300–372.
- Iyer, V., and Struhl, K. (1996). Absolute mRNA levels and transcriptional initiation rates in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 93, 5208–5212.
- Lee, T. I., Causton, H. C., Holstege, F. C., Shen, W. C., Hannett, N., Jennings, E. G., Winston, F., Green, M. R., and Young, R. A. (2000). Redundant roles for the TFIID and SAGA complexes in global transcription. *Nature* 405, 701–704.
- Lin, H., Choi, J. H., Hasek, J., DeLillo, N., Lou, W., and Vancura, A. (2000). Phospholipase C is involved in kinetochore function in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 20, 3597–3607.
- Lin, H., Nguyen, P., and Vancura, A. (2002). Phospholipase C interacts with Sgd1p and is required for expression of GPD1 and osmo-resistance in *Saccharomyces cerevisiae*. *Mol. Genet. Genomics* 267, 313–320.
- Luo, H. R., Saiardi, A., Yu, H., Nagata, E., Ye, K., and Snyder, S. H. (2002). Inositol pyrophosphates are required for DNA hyperrecombination in protein kinase c1 mutant yeast. *Biochemistry* 41, 2509–2515.
- Nehlin, J. O., Carlberg, M., and Ronne, H. (1992). Yeast SKO1 gene encodes a bZIP protein that binds to the CRE motif and acts as a repressor of transcription. *Nucleic Acids Res.* 20, 5271–5278.
- Nguyen, P. H., Hasek, J., Kohlwein, S. D., Romero, C., Choi, J. H., and Vancura, A. (2005). Interaction of Pik1p and Sjl proteins in membrane trafficking. *FEMS Yeast Res.* 5, 363–371.
- Odom, A. R., Stahlberg, A., Wente, S. R., and York, J. D. (2000). A role for nuclear inositol 1,4,5-trisphosphate kinase in transcriptional control. *Science* 287, 2026–2029.
- Papamichos-Chronakis, M., Petrakis, T., Ktistaki, E., Topalidou, I., and Tzamarias, D. (2002). Cti6, a PHD domain protein, bridges the Cyc8-Tup1 corepressor and the SAGA coactivator to overcome repression at GAL1. *Mol. Cell* 9, 1297–1305.
- Perera, N. M., Michell, R. H., and Dove, S. K. (2004). Hypo-osmotic stress activates Plc1p-dependent phosphatidylinositol 4,5-bisphosphate hydrolysis and inositol hexakisphosphate accumulation in yeast. *J. Biol. Chem.* 279, 5216–5226.
- Posas, F., Chambers, J. R., Heyman, J. A., Hoeffler, J. P., de Nadal, E., and Arino, J. (2000). The transcriptional response of yeast to saline stress. *J. Biol. Chem.* 275, 17249–17255.
- Proft, M., Gibbons, F. D., Copeland, M., Roth, F. P., and Struhl, K. (2005). Genomewide identification of Sko1 target promoters reveals a regulatory network that operates in response to osmotic stress in *Saccharomyces cerevisiae*. *Eukaryot. Cell* 4, 1343–1352.
- Proft, M., Pascual-Ahuir, A., de Nadal, E., Arino, J., Serrano, R., and Posas, F. (2001). Regulation of the Sko1 transcriptional repressor by the Hog1 MAP kinase in response to osmotic stress. *EMBO J.* 20, 1123–1133.
- Proft, M., and Serrano, R. (1999). Repressors and upstream repressing sequences of the stress-regulated ENA1 gene in *Saccharomyces cerevisiae*: bZIP protein Sko1p confers HOG-dependent osmotic regulation. *Mol. Cell. Biol.* 19, 537–546.
- Proft, M., and Struhl, K. (2002). Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. *Mol. Cell* 9, 1307–1317.
- Puig, S., Lau, M., and Thiele, D. J. (2004). Cti6 is an Rpd3-Sin3 histone deacetylase-associated protein required for growth under iron-limiting conditions in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 279, 30298–30306.
- Reiser, V., Ruis, H., and Ammerer, G. (1999). Kinase activity-dependent nuclear export opposes stress-induced nuclear accumulation and retention of Hog1 mitogen-activated protein kinase in the budding yeast *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 10, 1147–1161.
- Rep, M., Proft, M., Remize, F., Tamas, M., Serrano, R., Thevelein, J. M., and Hohmann, S. (2001). The *Saccharomyces cerevisiae* Sko1p transcription factor mediates HOG pathway-dependent osmotic regulation of a set of genes encoding enzymes implicated in protection from oxidative damage. *Mol. Microbiol.* 40, 1067–1083.
- Roberts, S. M., and Winston, F. (1997). Essential functional interactions of SAGA, a *Saccharomyces cerevisiae* complex of Spt, Ada, and Gcn5 proteins, with the Snf/Swi and Srb/mediator complexes. *Genetics* 147, 451–465.
- Saiardi, A., Resnick, A. C., Snowman, A. M., Wendland, B., and Snyder, S. H. (2005). Inositol pyrophosphates regulate cell death and telomere length through phosphoinositide 3-kinase-related protein kinases. *Proc. Natl. Acad. Sci. USA* 102, 1911–1914.
- Shen, X., Xiao, H., Ranallo, R., Wu, W. H., and Wu, C. (2003). Modulation of ATP-dependent chromatin-remodeling complexes by inositol polyphosphates. *Science* 299, 112–114.
- Sherman, F. (1991). Getting started with yeast. *Methods Enzymol.* 194, 3–21.
- Steger, D. J., Haswell, E. S., Miller, A. L., Wente, S. R., and O'Shea, E. K. (2003). Regulation of chromatin remodeling by inositol polyphosphates. *Science* 299, 114–116.
- Stolz, L. E., Kuo, W. J., Longchamps, J., Sekhon, M. K., and York, J. D. (1998). INP51, a yeast inositol polyphosphate 5-phosphatase required for phosphatidylinositol 4,5-bisphosphate homeostasis and whose absence confers a cold-resistant phenotype. *J. Biol. Chem.* 273, 11852–11861.
- Vincent, A. C., and Struhl, K. (1992). ACR1, a yeast ATF/CREB repressor. *Mol. Cell. Biol.* 12, 5394–5405.
- Yoko-o, T., Matsui, Y., Yagisawa, H., Nojima, H., Uno, I., and Toh-e, A. (1993). The putative phosphoinositide-specific phospholipase C gene, PLC1, of the yeast *Saccharomyces cerevisiae* is important for cell growth. *Proc. Natl. Acad. Sci. USA* 90, 1804–1808.
- York, J. D., Odom, A. R., Murphy, R., Ives, E. B., and Wente, S. R. (1999). A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. *Science* 285, 96–100.
- York, S. J., Armbruster, B. N., Greenwell, P., Petes, T. D., and York, J. D. (2005). Inositol diphosphate signaling regulates telomere length. *J. Biol. Chem.* 280, 4264–4269.
- Yu, Y., Eriksson, P., Bhoite, L. T., and Stillman, D. J. (2003). Regulation of TATA-binding protein binding by the SAGA complex and the Nhp6 high-mobility group protein. *Mol. Cell. Biol.* 23, 1910–1921.
- Yu, Y., Eriksson, P., and Stillman, D. J. (2000). Architectural transcription factors and the SAGA complex function in parallel pathways to activate transcription. *Mol. Cell. Biol.* 20, 2350–2357.
- Zhao, K., Wang, W., Rando, O. J., Xue, Y., Swiderek, K., Kuo, A., and Crabtree, G. R. (1998). Rapid and phosphoinositide-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. *Cell* 95, 625–636.