Multiple Levels of Control Regulate the Yeast cAMP-response Element-binding Protein Repressor Sko1p in Response to Stress*

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The Sko1p transcriptional repressor regulates a subset of osmoinducible stress defense genes in Saccharomyces cerevisiae by binding to cAMP-responsive elements. We have reported previously that in response to stress Sko1p is phosphorylated by the stress-activated Hog1p mitogen-activated protein kinase, which disrupts its interaction with the Ssn6p-Tup1p corepressor. Here we report that other mechanisms are essential for the regulation of the Sko1p repressor activity upon stress. The nuclear localization of Sko1p depends on the stress-inhibited protein kinase A (PKA). Sko1p is localized in the nucleus of unstressed cells, and it redistributes to the cytosol upon severe salt stress (1 M NaCl). Yeast mutants with low PKA activity localize Sko1p to the cytoplasm in the absence of stress and exhibit deregulated expression of cAMP-responsive element-regulated genes. The central part (315–480) of Sko1p, containing the PKA phosphorylation sites and the basic domain-leucine zipper domain, is essential for its nuclear localization. Salt-induced export of Sko1p from the nucleus is independent of Hog1p and of the Bcy1p regulatory subunit of PKA. Furthermore, phosphorylation by PKA slightly enhances DNA binding affinity of Sko1p in vitro, whereas Sko1p dimerization in vivo is not regulated by stress. Sko1p repressor activity is associated to its binding to the Ssn6p-Tup1p complex. Interestingly, the Sko1p NH2 terminus (1–315), containing the Hog1p phosphorylation sites, associates in vivo with Tup1p in the absence of Ssn6p, suggesting that Sko1p represses gene transcription by interacting directly with the Tup1p subunit of the Ssn6p-Tup1p complex.

Cells respond to various environmental stresses by activating signal transduction pathways that culminate in modulating the activity of specific transcription factors. Very often transcription factors of the CREB family serve as direct effectors of signal transduction pathways and trigger changes in gene expression dependent on environmental stresses. All eukaryotic cells contain CREB factors that are characterized by their basic domain-leucine zipper (bZIP) structural motif (1, 2), which enables them to bind to cAMP-responsive elements (CRE). In higher eukaryotes CREB proteins stimulate transcription in response to cAMP- and calcium-activated protein kinases (3), and ATF-2 triggers stress-activated responses mediated by mitogen-activated protein (MAP) kinases (4, 5). As a consequence, CREB factors play critical roles in important biological functions such as memory (6, 7), circadian rhythm (8), and skeletal and neuronal development (9). The mammalian CREB factors are constitutively bound to their promoter elements, and phosphorylation modulates their interaction with other transcriptional regulators (reviewed in Ref. 3).

In the yeast Saccharomyces cerevisiae the function of the CREB repressor Sko1p (10, 11) has been identified recently. Sko1p represses gene expression from CRE promoter sequences of various osmoinducible genes such as ENA1 (Na+ extrusion ATPase) (12), HAL1 (ion homeostasis protein) (13), or GRE2 (homolog to plant isoflavonoid reductase) (14–16). Sko1p recruits the Ssn6p-Tup1p corepressor complex to a subset of stress defense gene promoters that are induced by hyperosmotic stress and thereby prevents their expression under normal growth conditions (12–15). Upon osmotic stress Sko1p is phosphorylated by the MAP kinase Hog1p, the interaction with Ssn6p/Tup1p is disrupted, and finally stress defense genes are up-regulated (15).

Apart from the HOG pathway, a second signal transduction pathway, the cAMP-activated protein kinase A (PKA) pathway, has been shown to broadly modulate stress-induced gene expression in yeast. More specifically, efficient adaptation to osmotic stress depends on the cellular PKA activity (17) that is inhibited by salt stress (18). One important mechanism by which PKA influences gene expression seems to be the modulation of nuclear-cytoplasmic trafficking of transcription factors. For example, under normal growth conditions, high PKA activity prevents the nuclear import of Msn2p and Msn4p, transcriptional activators that enter the nucleus upon various stress conditions and activate the expression of many defense genes (19, 20). Although it has been shown recently that protein kinase A phosphorylates Sko1p in vitro (15), the connection between PKA activity and Sko1p-mediated gene regulation remained unknown. In this work we show that nuclear localization of Sko1p requires high PKA activity and that salt stress rapidly causes the export of Sko1p from the nucleus.

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The abbreviations used are: CREB, cAMP-response element-binding protein; CRE, cAMP-responsive element; MAP, mitogen-activated protein; HOG, high osmolarity glycerol; PKA, protein kinase A; GST, glutathione S-transferase; HA, hemagglutinin; bZIP, basic domain-leucine zipper.
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**Fig. 1. Subcellular localization of GST-Sko1p.** Wild type yeast cells (W303–1A) transformed with GST-SKO1 expression plasmid pYEX-SKO1 were treated (+NaCl) or not (−NaCl) with 1 M NaCl for 5 min before fixing the cells. GST-Sko1p was detected by indirect immunofluorescence (GST), DNA was stained by 4,6-diamidino-2-phenylindole treatment (DAPI), and whole cells were visualized by differential interference contrast (DIC). wt, wild type.

**Fig. 2. Subcellular localization of GST-Sko1p is not altered in Δhog1 mutant cells.** GST-SKO1 was expressed in Δhog1 yeast mutants (MAP32), and GST-Sko1p was detected by indirect immunofluorescence as indicated in the legend for Fig. 1.

**Fig. 3. Nuclear accumulation of GST-Sko1p requires normal PKA activity.** Localization of GST-Sko1p in yeast cells with constitutively low (S13–58A,−tpk1Δtpk2,3 Δbcy1 TPK1) or constitutively high PKA activity (S13–58A, Δtpk2,3 Δbcy1 TPK1) was detected as described in the legend for Fig. 1.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—The following yeast strains were used: SP1, MATα ura3 leu2 trp1 ade8 can1; S13–58A, SP1 with tpk2ΔHIS3 tpk3ΔTRP1 bcy1ΔLEU2; S13–58A, SP1 with tpk1Δtpk2ΔHIS3 tpk3ΔTRP1 bcy1ΔLEU2; W303–1A, MATα, his3–11, 15 leu2–3, 112 trp1–1 ura3–1 ade2–1 can1–100; MAP6, W303–1A with tp1ΔloxP-KAN-loxP; MAP6, W303–1A with ssn6ΔloxP-KAN-loxP; MAP19, W303–1A with sko1ΔloxP-KAN-loxP; MAP32, W303–1A with hog1ΔΔΔTRP1; MAP37, W303–1A with chromosomal 3xHA-SKO1.

Synthetic medium contained 2% glucose, 0.67% yeast nitrogen base without amino acids (Difco), and the amino acids, purine and pyrimidine bases required by the strains. YPD medium contained 2% glucose, 2% peptone, and 1% yeast extract.

**Plasmids**—The yeast expression vector pYEX-4T (P_CUP1-GST, UR3A; 2 μm; AMRAD Biotech) was used to express GST-Sko1 fusion proteins. The full-length GST-SKO1 fusion plasmid pSKOYEX is described in Ref. 15. GST-Sko1p expressed from pSKOYEX is fully functional as it complements sko1Δ mutant strains (15). pSKOYEX(E) contains a triple amino acid substitution, Ser108→Thr113, and Ser126→Ala, and abolishes phosphorylation in vitro by Hog1p (15). Truncated GST-SKO1 fusions were constructed by inserting polymerase chain reaction-generated SKO1 fragments into pYEX-4T BonHIS1/San1 resulting in constructions pMP252 containing GST-SKO1 (315–647), pMP254 containing GST-SKO1 (315–647), and pMP255 containing GST-SKO1 (315–647). The 2xCRE_HAL1-CYC1-lacZ reporter was constructed by insertion of a tandem repetition of CRE_HAL1 (pPY17) (13) into the CYC1-lacZ reporter plasmid pMP206 (pCYC1-HAL1-lacZ, URA3; 2 μm) (12). β-Galactosidase Assay—Transformed yeast strains were grown selectively until saturation in the appropriate SD liquid medium and were then diluted in YPD. Logarithmically growing cells (optical density at 660 nm of 0.5 to 0.8) were treated or not with 0.4 mM NaCl for 20 min and permeabilized by ethanol/toluene treatment, and β-galactosidase activity was determined as described in Ref. 21.

**Microscopy**—GST-Sko1 fusion protein was detected by indirect im-
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Fig. 6. Nuclear accumulation of Sko1p depends on a central domain (315-486), and efficient interaction with Tup1p requires the Sko1p NH2-terminal region (1-315). A, schematic representation of Sko1p functional domains and the construction of truncated GST-Sko1 fusion proteins (left). Subcellular localization of the truncated GST-Sko1p versions was determined as described in the legend for Fig. 1 with nuc = nuclear, cyt = cytoplasmatic. B, Northern blot analysis of the Sko1p-dependent GIRE2 transcription. The empty expression vector (pYEX-4T) or full-length and truncated GST-SKO1 expression plasmids were transformed into yeast Δsko1 mutant (MAP19), and GIRE2 transcription was measured before and after 10 min of salt treatment (0.4 M NaCl). TBP1 was used as an internal loading control. Signals were quantified densitometrically, and GIRE2 mRNA levels corrected for the TBP1 control are given below the blot. C, Sko1p-Tup1p interaction assay. Immunoblot analysis of GST-Sko1p (full-length and truncated versions) in wild type and mutant yeast cells (data not shown). As a control, we expressed GST alone from the same expression vector and detected it distributed over the entire cell volume, whereas untransformed yeast cells showed only a very weak background fluorescence (data not shown). We next tested whether stress conditions altered Sko1p localization and applied mild hyperosmotic (0.4 M NaCl), hypo-osmotic (distilled water), heat (42 °C), oxidative (0.1 mM H2O2), and nutritional (SC medium without carbon source) stress. The GST-Sko1p did not change its nuclear localization under any of these conditions (data not shown). However, upon severe salt stress (1 M NaCl) GST-Sko1p was detected in the entire cell (Fig. 1), indicating that Sko1p is exported from the nucleus under these conditions. This change in localization occurs quickly (5 min after salt treatment), is maintained as long as cells are challenged with 1 M NaCl, and is fully reversible, as GST-Sko1p reconcentrates in the nucleus after washing the cells with normal medium (data not shown).

RESULTS

Sko1p Localizes to Yeast Nuclei under Normal Growth Conditions and to the Cytoplasm upon Severe Salt Stress—To detect the subcellular localization of the Sko1p transcription factor we expressed an NH2-terminal GST-Sko1 fusion protein in wild type and sko1Δ mutant yeast cells and followed its localization by indirect immunofluorescence using an anti-GST antibody. The GST-Sko1p protein has been demonstrated to be fully functional (15). When yeast cells were grown in SD medium, GST-Sko1p localized exclusively to the nuclei (Fig. 1). No differences were observed using Δsko1 mutant instead of wild type cells (data not shown). As a control, we expressed GST alone from the same expression vector and detected it distributed over the entire cell volume, whereas untransformed yeast cells showed only a very weak background fluorescence (data not shown). We next tested whether stress conditions altered Sko1p localization and applied mild hyperosmotic (0.4 M NaCl), hypo-osmotic (distilled water), heat (42 °C), oxidative (0.1 mM H2O2), and nutritional (SC medium without carbon source) stress. The GST-Sko1p did not change its nuclear localization under any of these conditions (data not shown). However, upon severe salt stress (1 M NaCl) GST-Sko1p was detected in the entire cell (Fig. 1), indicating that Sko1p is exported from the nucleus under these conditions. This change in localization occurs quickly (5 min after salt treatment), is maintained as long as cells are challenged with 1 M NaCl, and is fully reversible, as GST-Sko1p reconcentrates in the nucleus after washing the cells with normal medium (data not shown).

Nuclear Localization of Sko1p Depends on PKA Activity whereas Its Export Is Dependent on the Bec1p Regulatory Subunit of PKA and of the Hog1p MAP Kinase—Nuclear localization of transcription factors is often regulated by phosphorylation. Sko1p is phosphorylated by at least two different kinases, the Hog1p MAP kinase and protein kinase A (15). We tested whether the salt stress-regulated localization of Sko1p depends on either the HOG or the cAMP protein kinase A signaling pathway. The localization of GST-Sko1p in ∆hog1

Northern Blot Analysis—GIRE2 and TBP1 mRNA levels were determined by Northern blot analysis using total RNA from yeast cells as described in Ref. 15.
mutant cells under normal and severe salt stress conditions was indistinguishable from that of wild type cells (Fig. 2). Also, a point-mutated GST-Sko1p unphosphorylatable by Hog1p (GST-Sko1(E)) (15) was localized as the wild type protein (data not shown). PKA activity is essential for yeast growth, but mutants with high or low constitutive activity of this kinase are viable (22). In this study we used a yeast mutant strain with a deletion of the negative regulatory subunit Bcy1p and deletions in two of the three catalytic subunits (Tpk2, 3p) of PKA. This genetic manipulation completely disconnects the remaining Tpk1p catalytic subunit from its cAMP dependence and results in constitutive high PKA activity (23). By further introduction of the tpk1™ point-mutated allele, PKA activity is reduced dramatically (24). Interestingly, cells with low protein kinase A activity failed to localize GST-Sko1p to the nucleus so that the protein was cytoplasmic under normal, as well as salt stress conditions (Fig. 3). Furthermore, cells with constitutively high protein kinase A activity showed a normal localization pattern of Sko1p (Fig. 3).

**CRE-mediated Gene Expression Depends on PKA Activity**—Having found that normal PKA activity is necessary for the nuclear accumulation of Sko1p under normal growth conditions, we tested whether PKA activity is also needed for the regulated gene expression from CRE sites that are the natural DNA target sequences for the Sko1p repressor. We made use of a CYC1-lacZ-based reporter assay where we measured expression of lacZ driven by two CRE sequences from the Sko1p-regulated HAL1 gene (13). The CRE<sub>HAL1</sub>-CYC1-lacZ construct completely depends on Sko1p; therefore it can be used as a specific measure for Sko1p activity in vivo (13). As shown in Fig. 4, the reporter construct is repressed under normal conditions and activated upon salt shock. Normal PKA activity is required for repression from the CRE sites, because mutants with low PKA activity show constitutively activated reporter gene expression (Fig. 4). Up-regulated PKA activity did not seem to interfere with the repression/activation pattern of CRE-mediated expression (Fig. 4).

**Affinity of Sko1p to CRE in Vitro Is Not Modulated by Hog1p Phosphorylation and Slightly Enhanced by PKA Phosphorylation**—To test the possibility that the DNA binding capacity of Sko1p is regulated by Hog1p- or PKA-dependent phosphorylation, we performed in vitro binding assays using bacterially expressed and purified GST-Sko1p that was either unphosphorylated or phosphorylated in vitro by either one of the two kinases. As described in (15), GST-Sko1p is phosphorylated in vitro by the Hog1p MAP kinase (that is activated by the Pbs2p MAP kinase kinase) or by PKA at multiple residues. Measurements of incorporated radioactive phosphate into GST-Sko1p by either Hog1p or PKA showed that more than 60% of the sites used by either kinase were phosphorylated in the in vitro system used (data not shown).

We followed binding of GST-Sko1p to the CRE<sub>HAL1</sub> promoter element (13). GST-Sko1p bound specifically to CRE<sub>HAL1</sub>, but Hog1p-dependent phosphorylation did not change the affinity of Sko1p for CRE<sub>HAL1</sub> (Fig. 5, lanes 3 and 4). When GST-Sko1p was phosphorylated by PKA, it increased its affinity to CRE<sub>HAL1</sub> by about 2-fold (Fig. 5, lanes 6 and 7). **Deletion Analysis of Sko1p: A Central Region (315–486) Is Required for Nuclear Localization, and an NH<sub>2</sub>-terminal Region (1–314) Is Required for Efficient Association with Ssn6p**—To determine the function of different Sko1p domains for nuclear localization and in vivo repressor activity, we expressed truncated GST-Sko1p versions in yeast and followed their cellular localization by immunofluorescence and their capacity to repress gene expression by detecting mRNA levels of the Sko1p-dependent GRE2 gene (14–16). Deletion of the NH<sub>2</sub>-terminal cluster of Hog1 phosphorylation sites in GST-Sko1p (315–647) resulted in normal nuclear accumulation of the fusion protein and repression of GRE2 transcription (Fig. 6, A and B). An additional deletion of the Sko1p central domain containing the cluster of PKA phosphorylation sites and the adjacent bZIP motif in GST-Sko1p (486–647) did not allow nuclear localization of the protein, and accordingly, it did not complement a Δsko1 mutant for repression of GRE2 transcription (Fig. 6B). We followed additionally the interaction capacity of the different Sko1p truncations with the Ssn6p/Tup1p corepressor by GST pull down assays detecting coprecipitated Tup1p (see Fig. 6C). As reported recently (15), GST-Sko1p coprecipitates specifically and efficiently with Tup1p. However, the deletion of the Sko1p NH<sub>2</sub>-terminal domain (1–314) strongly diminished, but not abolished completely, the association with Tup1p (Fig. 6C). A further deletion of the central part of Sko1p expressing GST-Sko1p (486–647) nearly abolished all binding to Tup1p. The NH<sub>2</sub>-terminal region (1–315) alone was sufficient to associate strongly with Tup1p (Fig. 6C, last lane).
**motic Stress**—CREB transcription factors bind to their CRE recognition sequences as homo- or heterodimers (11, 14). To determine whether dimerization of Sko1p in vivo plays a role in the regulation of this transcriptional repressor by osmotic stress, we assayed for Sko1p-Sko1p interaction by coprecipitation experiments. We co-expressed GST-Sko1p and HA-Sko1p in yeast cells, purified GST-Sko1p, and detected the amount of copurified HA-Sko1p (Fig. 7). In these experiments HA-Sko1p specifically copurified with GST-Sko1p from yeast extracts. The amount of Sko1-Sko1 interaction, however, was not much affected by osmotic stress, even though there was a slightly enhanced interaction detectable after treatment with 0.4 M NaCl.

**Sko1p Coprecipitates with Tup1p in the Absence of Ssn6p**—We finally addressed the question whether Sko1p interacts with the Ssn6p or the Tup1p subunit of the Ssn6-Tup1p corepressor complex. We therefore expressed GST-SK01 in wild type, ssn6Δ, and tup1Δ cells. After purification of GST-Sko1p we tested for Ssn6p and Tup1p coprecipitation by Western blot. As shown in Fig. 8, interaction of Sko1p with Tup1p was not affected when Ssn6p was deleted. However, in the absence of Tup1p the association of Sko1p with Ssn6p was abolished. These results suggest that Sko1p recruits the Ssn6-Tup1p corepressor by interaction with the Tup1p subunit.

**DISCUSSION**

CREB transcription factors are downstream effectors that connect various signal transduction pathways with regulated gene expression. CREB factors receive inputs from upstream kinases and transduce them into specific gene expression patterns, a system that is conserved from yeast to man (for review see Ref. 3). Because one CREB factor can be phosphorylated by various signal kinases, it is important to find out at which level(s) distinct signaling pathways modulate CREB activity.

We have characterized the mechanisms of regulation by salt stress of the CREB repressor Sko1p of *S. cerevisiae*. Sko1p, by recruiting the Ssn6p-Tup1p corepressor, inhibits the transcription of genes important for ion homeostasis, like ENA1 (12) or HAL1 (13), and genes with protective function against oxidative stress (16). Here we characterize this recruitment more in detail by showing that Sko1p can interact in vivo with Tup1p in the absence of Ssn6p. It is therefore likely that Sko1p contacts directly the Tup1p subunit of the corepressor, which then recruits histone deacetylase activities as shown recently (25). However, both subunits of Ssn6p-Tup1p seem to be equally important for repression of Sko1p-regulated genes (15, 16), arguing for an essential role of Ssn6p in the corepressor complex although it is not directly recruited by Sko1p. The NH2-terminal (1–315) domain of Sko1p is required and sufficient for efficient interaction with Tup1p (Fig. 6C). However, the interaction is not completely abolished, explaining that a truncated Sko1p lacking that region still can repress GRE2 transcription. Interestingly, Sko1p domain (1–315) contains the three Hog1p phosphorylation sites (15), pointing to the possibility that modification of these sites by the Hog1p MAP kinase directly triggers changes in the affinity of Sko1p to Tup1p.

Activation of the HOG pathway upon osmotic stress disrupts the Sko1p-Ssn6p-Tup1p interaction (15), but here we present evidence that in addition to this mechanism, Sko1p activity is regulated at other levels. Favorable growth conditions lead to high protein kinase A activity and the subsequent accumulation of Sko1p in the nucleus (Fig. 1). This nuclear targeting is crucial for the repression of Sko1p-regulated genes, and we show that yeast mutants with low PKA activity are not able to target Sko1p to the nucleus and consequently fail to repress CREB-regulated gene expression (see Figs. 3 and 4). This observation is in agreement with previous findings showing that low PKA activity favors *HAL1* transcription even under non-stress conditions (26). We identify a central region of Sko1p (315–486) that is essential for its nuclear localization (Fig. 6). Within this domain a possible bipartite nuclear localization sequence is present that partially overlaps with the bZIP domain, RKKRKR (451). Whether this sequence is responsible for nuclear import of Sko1p will require further analyses, but for the plant CREB protein OPAQUE2 a very similar nuclear target sequence overlapping with bZIP has been functionally proven (27).

Additionally, under normal growth conditions high PKA activity seems to favor the binding of Sko1p to its CRE target sequences, because we show that phosphorylation of Sko1p by PKA stimulates the *in vitro* association of Sko1p to CRE (Fig. 5). This result is in agreement with stronger repression of CRE-driven reporter genes (reported here in Fig. 4) or natural Sko1p-regulated stress genes like *HAL1* (26) in cells with high PKA activity.

Under salt stress conditions the Sko1p repressor activity has to be decreased to allow defense gene expression. This release from repression seems to be dominantly regulated by the HOG pathway, because *hog1* mutants are completely unable to overcome Sko1p-mediated repression upon stress (12, 13, 15, 16). Mutants with high PKA activity, however, readily derepress Sko1p target genes upon stress (Fig. 4) (15, 28). We can summarize this stress-induced derepression process as coordination of several mechanisms; thus upon stress-activated Hog1p MAP kinase phosphorylates Sko1p and disrupts the interaction of Sko1p with the Ssn6p-Tup1p corepressor (15), but Hog1p neither interferes with the association of Sko1p with CRE nor with the subcellular localization of Sko1p. Additionally, the decrease of PKA activity results in decreased affinity of Sko1p for CRE sites. Finally, under severe salt stress conditions Sko1p is exported from the nucleus to the cytoplasm resulting in complete derepression of Sko1p-dependent genes.

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