Control of Cell Polarity in Fission Yeast by Association of Orb6p Kinase with the Highly Conserved Protein Methyltransferase Skb1p*

Received for publication, September 20, 2002, and in revised form, February 19, 2003
Published, JBC Papers in Press, March 19, 2003, DOI 10.1074/jbc.M209703200

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In the fission yeast Schizosaccharomyces pombe, proper establishment and maintenance of cell polarity require Orb6p, a highly conserved serine/threonine kinase involved in regulating both cell morphogenesis and cell cycle control. Orb6p localizes to the cell tips during interphase and to the cell septum during mitosis. To investigate the mechanisms involved in Orb6p function, we conducted a two-hybrid screen to identify proteins that interact with Orb6p. Using this approach, we identified Skb1p, a highly conserved protein methyltransferase that has been implicated previously in cell cycle control, in the coordination of cell cycle progression with morphological changes, and in hyperosmotic stress response. We found that Skb1p associates with Orb6p in S. pombe cells and that the two proteins interact directly in vitro. Loss of Skb1p exacerbates the phenotype of orb6 mutants, suggesting that Skb1p and Orb6p functionally interact in S. pombe cells. Our results suggest that Skb1p affects the intracellular localization of Orb6p and that loss of Skb1p leads to a redistribution of the Orb6p kinase away from the cell tips. Furthermore, we found that Orb6p kinase activity is strongly increased following exposure to salt shock, suggesting that Orb6p has a role in cell response to hyperosmotic stress. Previous studies have shown that Skb1p interacts with the fission yeast p21-activated kinase homologue Pak1p/Shk1p to regulate cell polarity and cell cycle progression. Our findings identify Orb6p as an additional target for Skb1p and suggest a novel function for Skb1p in the control of cell polarity by regulating the subcellular localization of Orb6p.

The establishment of cell polarity and the control of cell shape are fundamental to cell growth and function and are essential for intracellular transport, cell differentiation, and cell locomotion. Cell shape and cell polarity change at specific stages of the cell cycle, in response to extracellular signals and during tumorigenesis (1, 2). However, mechanisms responsible for regulating cell morphogenesis and integrating polarized cell growth with the cell cycle remain poorly understood.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† Supported by National Institutes of Health Grant RO1GM53239.
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The fission yeast Schizosaccharomyces pombe is an attractive model organism for studies of cell morphogenesis because it grows in a polarized fashion with a well-defined cylindrical shape. Polarized cell growth in S. pombe is regulated strictly during the cell cycle. Cells grow from only one tip (the older end) following mitosis, and when a minimal cell length has been achieved, the cells activate growth from the second end in a process referred to as New End Take-Off (3–5). We have previously identified 19 genes in fission yeast that have roles in various aspects of cell polarity control (6). One of these genes is orb6, which encodes an essential serine/threonine kinase required for maintenance of cell polarity during interphase as well as proper negative regulation of mitosis (7). Orb6p belongs to a conserved family of proteins in eukaryotic cells. Members of this family include Neurospora crassa Cot1 (8), Saccharomyces cerevisiae Cbk1p (9, 11), Caenorhabditis elegans SAX1 (12), Drosophila WARTS (13), and human NDR kinase (14). These kinases are related to the human myotonic dystrophy kinase (15) and to Rho kinase (16–18); however, they lack Rho binding motifs. Orb6p, Cot1, Cbk1p, and WARTS have a role in the regulation of cell morphology (7–9, 11, 13). Similarly, C. elegans SAX-1 has a role in regulating neuronal cell shape and neurite initiation (12).

To explore further the molecular mechanisms involved in Orb6p function, we conducted a two-hybrid screen to identify proteins that interact with Orb6p. One of the proteins identified from this screen is the highly conserved protein methyltransferase Skb1p (19). Skb1p was originally identified as a protein that interacts with the essential fission yeast p21-activated kinase homologue Pak1p/Shk1p (19). Like Orb6p, Pak1p/Shk1p is required for proper establishment and maintenance of cell polarity in fission yeast (7, 20, 21). However, unlike Orb6p and Pak1p/Shk1p, Skb1p is not essential for cell viability or polarity under normal growth conditions (19). Interestingly, in the absence of Skb1p, S. pombe cells are unable to maintain cell polarity following hyperosmotic shock (22), suggesting that Skb1p functions as a mediator of hyperosmotic stress response.

Skb1p also has been shown to act as a dosage-dependent mitotic inhibitor in fission yeast, and this function requires both Pak1p/Shk1p as well as the Cdc2p inhibitory kinase Wee1p (23). Interestingly, the S. cerevisiae homologue of Skb1p, Hsl7p, has been implicated in a morphological checkpoint that delays the cell cycle in response to polarity and bud formation defects (24, 25). Hsl7p localizes to the septin ring and mediates the interaction of the Wee1p homologue, SWe1p, with the protein Hsl1p. This interaction is thought to promote Swe1p phosphorylation and its degradation at the G2/M phase transition, thus promoting entry into mitosis (24, 25). Thus,
both Skb1p and Hls7p have a role in cell cycle control, although the molecular details of these controls may be different.

In this report, we characterize the interaction between Skb1p and Orb6p at the genetic and molecular level. Our results indicate that Skb1p interacts with Orb6p to regulate cell polarity in fission yeast by positively regulating Orb6p localization to the cell tips. We also report that Orb6p kinase activity is increased during hyperosmotic stress, suggesting that Orb6p may play an important role in hyperosmotic stress response. Our findings identify a novel function for Skb1p and suggest a wider role for this protein in regulating the spatial localization of protein kinases involved in the control of cell morphology in fission yeast.

### EXPERIMENTAL PROCEDURES

#### Strains and Cell Culture—S. pombe strains used in this study are listed in Table I. All strains are isogenic to the original strain h r72.

Cells were cultured at the indicated temperatures in either YE (0.5% (w/v) Difco yeast extract, 3.0% (w/v) glucose, pH 5.6, + supplements (225 mg/liter adenine, histidine, leucine, uracil, and lysine hydrochloride) or minimal medium plus required supplements. Standard techniques were used for genetic manipulation and analysis (25; also see www.bio.uva.nl/pombe/handbook/).

**S. cerevisiae** strain Y190 (MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-2 leu2-3,112, URA3::GAL-lacZ, LYS2::GAL(UAS)-HIS3 cyh2) was used as the host for the two-hybrid interaction experiments. Strain Y187 (MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-2 leu2-3,112 mut - URA3::GAL-lacZ) was used for mating experiments (27). Cells were cultured in YPAD (per liter 10.0 g of Difco yeast extract, 20.0 g of Difco peptone, 20.0 g of glucose, 100 mg of adenine hemisulfate) or selective SC (per liter 6.7 g of Difco yeast nitrogen base (without amino acids), 20 g of glucose, pH 5.6, + appropriate amino acids (40 mg of adenine hemisulfate, 20 mg of t-arginine (HCl), 100 mg of t-aspartic acid, 100 mg of t-glutamic acid (monosodium salt), 20 mg of t-histidine, 60 mg of t-leucine, 30 mg of t-lysine (nono-HCl), 20 mg of t-methionine, 50 mg of t-phenylalanine, 375 mg of t-serine, 200 mg of t-threonine, 40 mg of t-tryptophan, 30 mg of t-tyrosine, 150 mg of t-valine, 20 mg of uracil) at 30 °C. Aminotriazole was added to the plates when selecting for His prototrophy.

**Two-hybrid Screen for Identifying Proteins Interacting with Orb6—** The orb6 gene was fused to the DNA binding domain of Gal4p (GPD)1 in a plasmid carrying the trp1 marker (pAS1-orb6) and was used as the bait for a two-hybrid interaction screen. The S. pombe cDNA library was cloned into the pACT plasmid as cDNAs fused to the activation domain of Gal4p (GAD). These plasmids (pACT) carry the leu2 marker. The plasmids pAS1, pAC2, pS1-p53, pSE1111, and pSE1112 as well as the S. pombe cDNA library prepared in phase A vectors were kindly provided by Dr. S. Elledge. The histidine prototrophy assay and &beta-galactosidase assay were performed as described previously (27). cDNAs were isolated from strains showing histidine prototrophy and positive &beta-galactosidase activity and then tested for self-activation and specificity against control baits. Cells were exposed to X-gal for 1 h at 30 °C. For histidine auxotrophy assay, cells were grown at 30 °C on SC -Leu, His, plus 100 mg 3-aminotriazole. The cDNAs that did not self-activate and had specificity for interaction with Orb6p were sequenced at the Rockefeller University Protein/DNA Technology Center (PDTC) using the pACT forward 5′ primer (CTATCTTGGATGATGAAG) and/or the pACT reverse 3′ primer (ACACGGTAAAGACTTGCG). Five Orb6p-interacting proteins were identified from a screen of ~2 × 106 transformants.

**Purification of Orb6p and Skb1p—** For copurification of Orb6p and Skb1p from fission yeast extracts, we cultured h r67-704 ura4-D18 leu1-32 cells coexpressing either hemagglutinin (HA)-tagged Orb6p and glutathione S-transferase (GST)-tagged Skb1p (strain FV544) or HA-tagged Orb6p and GST alone as a control (strain FV545) for 15 h at 32 °C in the absence of thiamine. The orb6-HA construct was shown previously to rescue an orb6Δ strain when integrated in single copy (Ref. 7 and this study). Cells were then resuspended in HB buffer (25 mm MOPS, pH 7.2, 60 mm β-glycerophosphate, 15 mm Na-p-nitrophenyl phosphate, 15 mg MgCl2, 15 mg EGTA, 1 mg dithiothreitol, 0.1 mm sodium vanadate, 1% Triton X-100, 1 mm phenylmethylsulfonyl fluoride + protease inhibitors (complete EDTA-free protease inhibitor cocktail tablets (Roche Applied Science)) and broken using a FastPrep FP120 bead beater (Savant). Extracts were spun at 15,000 × g for 15 min. GST-tagged Skb1p or GST alone was purified using glutathione beads (Molecular Probes) for the experiment in Fig. 3A. HA-tagged Orb6p was immunoprecipitated using monoclonal anti-HA antibody-coupled resin ( Covance). When copurification of Orb6p and Skb1p was performed following hyperosmotic shock, cells were grown in the absence of thiamine for 15 h at 32 °C, diluted in a medium containing 1.5 m KCl for 20 min, and then broken as described previously.

To test the interaction of Orb6p and Skb1p in vitro, orb6 was cloned into the pCDNA1.1+ (Invitrogen) vector for expression of 35S-labeled Orb6p in the T7 T7 reticulocyte lysate system (Promega). GST and GST-Skb1p were expressed in BL21 bacterial cells from a pET15b vector and then purified by binding to glutathione resin (Molecular Probes). The GST- and GST-Skb1p-bound resin was incubated for 30 min with the reticulocyte lysate expressing 35S-labeled Orb6p, washed, and then eluted by boiling the resin in SDS loading buffer.

Immunoblot analysis then was performed using standard methodology. For Western blot analysis, monoclonal anti-HA antibody (Covance), monoclonal anti-tubulin (TAT1, a kind gift of Dr. K. Gull), and polyclonal anti-GST antibody (Molecular Probes) were used as primary antibodies. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were employed as secondary antibodies (ECL, Amer sham Biosciences).

**Expression of Orb6p-HA under the Control of the Endogenous orb6 Promoter—** We inserted one promoterless copy of the orb6+ gene, tagged at the C terminus with three copies of the HA epitope, in an integrative plasmid containing the sup3-5 marker. We then transformed h r67-704 leu1-32 ura4-D18 cells and selected for clones capable of growth in the absence of adenine. We checked by PCR that the integration had occurred at the orb6 locus and that the HA-tagged orb6+ gene was positioned correctly after the endogenous orb6 promoter. Three strains were used to produce cell extracts and to test on Western blots for the expression of a protein corresponding to the size of Orb6p-HA. In all

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1 The abbreviations used are: GBD, DNA binding domain of Gal4p; GAD, activation domain of Gal4p; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; HA, hemagglutinin; GST, glutathione S-transferase; MOPS, 4-morpholinopropanesulfonic acid.
cases, a single band of the correct size was recognized by the HA antibody (Covance). The strains used in our experiments were FV542 and its derivatives FV543, -521, and -539 (Table I).

**Determination of Orb6p Kinase Activity—** Orb6p expressed as a GST-tagged fusion protein from an orb6::his3 strain was purified from fission yeast extracts using glutathione resin (Molecular Probes). GST-Orb6p could rescue an orb6Δ strain when integrated in single copy under the control of the nmt1 promoter (data not shown). The GST-Orb6p-bound glutathione resin was washed with wash buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM MnCl₂). The resin then was resuspended in 25 µl of kinase buffer containing 10 µCi of [γ-32P]ATP (6000 Ci/mmol) and 20 µM ATP. The kinase reaction was terminated after 20 min at 30 °C and was analyzed on a gel.

Orb6p-HA, expressed from its endogenous promoter (strains FV521 and FV539, see above), was immunoprecipitated as a complex with Macs from a orb6::his3 strain, was suspended in 25 µl of kinase buffer containing 10 µCi of [γ-32P]ATP (6000 Ci/mmol) and 20 µM ATP. The kinase reaction was terminated after 20 min at 30 °C and was analyzed on a gel.

**Immunofluorescence and Fluorescence Microscopy—** Cells for immunofluorescence were grown exponentially in liquid for at least eight generations while keeping cell density below 10⁷ cells/ml before the start of the experiment. Standard immunofluorescence protocols were followed (26) after the cells were fixed in methanol. The primary antibodies utilized in this experiment were monoclonal anti-HA antibody and monoclonal anti-actin antibody (N350, Amersham Biosciences). The secondary antibody was Cy3-conjugated anti-mouse antibody (Covance). The strains used in our experiments were FV542–543, -521, and -539 (Table I). The kinase reaction was terminated after 20 min at 30 °C and was analyzed on a gel.

Orb6p-HA, expressed from its endogenous promoter (strains FV521 and FV539, see above), was immunoprecipitated as a complex with Macs from a orb6::his3 strain, was suspended in 25 µl of kinase buffer containing 10 µCi of [γ-32P]ATP (6000 Ci/mmol) and 20 µM ATP. The kinase reaction was terminated after 20 min at 30 °C and was analyzed on a gel.

**Results**

**A Two-hybrid Interaction Screen Identifies Skb1p as a Protein Interacting with Orb6p—** To gain insights into how Orb6p regulates cell polarity and mitotic progression, we conducted a two-hybrid screen to identify cDNAs that encode proteins interacting with Orb6p. One of the proteins identified from this screen is the conserved protein methyltransferase Skb1p (19, 22). Orb6p, fused to the DNA binding domain of Gal4p, was able to activate lacZ under the control of GAL4 promoters when coexpressed with Skb1p fused to the Gal4p activation domain (Fig. 1A). The positive control coexpressing Snf1p and Snf4p is shown in Fig. 1A. Orb6p was not able to activate lacZ when coexpressed with the Gal4p activation domain alone or with the Gal4p activation domain fused to Snf4p (Fig. 1A) or Pak1p/Skb1p (data not shown). Skb1p fused to the GAD was unable to support activation of lacZ when coexpressed with the GBD fused to Sds23p (Fig. 1A). Snf1p, Cdc23p, Cdt1p, or p53 (data not shown), suggesting that the interaction between Orb6p and Skb1p is specific.

Consistent with the lacZ data, Orb6p (fused to the DNA binding domain of Gal4p) activated HIS3 under the control of GAL4 promoter when coexpressed with Skb1p fused to the Gal4p activation domain (Fig. 1B). The positive control coexpressing Snf1p and Snf4p is shown in Fig. 1B. Orb6p was not able to activate HIS3 expression when coexpressed either with the Gal4p activation domain alone (data not shown) or with the Gal4p activation domain fused to Snf4p.
Fig. 4. Genetic interaction between orb6-25 and skb1Δ mutants. a, h− 972 control. b, orb6-25, c, skb1Δ, d, double mutant orb6-25 skb1Δ. e, graphical representation of the percentage of cells that become spherical at semipermissive temperature (33 °C). Cells were grown in YE at 33 °C for 5 h. WT, wild type.

Fig. 5. Skb1p does not affect Orb6p phosphorylation and kinase activity. A, in vitro autophosphorylation of GST-Orb6p. Lane 1, GST-Orb6p purification from skb1Δ cells. Lane 2, GST-Orb6p purification from skb1Δ::cald-Δ cells. B, in vitro autophosphorylation of Orb6p-HA. Lane 1, Orb6p-HA purification from skb1Δ cells. Lane 2, Orb6p-HA purification from skb1Δ cells. C, quantification of Orb6p kinase activity, expressed as a ratio to the activity measured in the control skb1Δ cells. The average of three independent experiments is shown. D, kinase assay control experiment showing that Orb6p-HA specifically coimmunoprecipitates with Mob2p-13Myc and that it becomes phosphorylated in vitro. Top membranes, cell extracts expressing Mob2p-13Myc and/or Orb6p-HA. Central membrane, Orb6p-HA is immunoprecipitated by anti-Myc antibody. Bottom membrane, the Orb6p-HA band becomes phosphorylated in vitro. Autoradiography of 32P-labeled Orb6p.

(d) data not shown) or Pak1p/Shk1p (Fig. 1B, right). Skb1p fused to the Gal4p activation domain did not activate the HIS3 GAL4 promoter when coexpressed with the Gal4p DNA binding domain fused to p53, Cdt1p, Snf1p (data not shown), or Cdc23p (Fig. 1B, left).

Orb6p Interacts with Skb1p in S. pombe Cells and in Vitro—To corroborate the two-hybrid interaction between Orb6p and Skb1p, we tested whether the two proteins interact in fission yeast cells. Orb6p was expressed as a HA epitope-tagged protein (Orb6p-HA), whereas Skb1p was fused to the GST protein (GST-Skb1p). As a negative control, Orb6p-HA was coexpressed with GST alone. Lysates were prepared from cells expressing Orb6p-HA with GST-Skb1p or GST (control). Orb6p-Skb1p or GST then were purified using glutathione-agarose beads. Orb6p-HA copurified with GST-Skb1p (Fig. 2A, lane 2) but not with GST alone (Fig. 2A, lane 1).

In a separate experiment we immunoprecipitated Orb6p-HA using an anti-HA antibody-coupled resin. GST-Skb1p commuinoprecipitated with Orb6p-HA (Fig. 2B, lane 2), whereas GST alone did not (Fig. 2B, lane 1). These results, taken together with previously published observations that both proteins localize similar to cell ends and septa in vivo (7, 22), provide strong evidence that Skb1p and Orb6p physically associate in fission yeast cells.

To test whether Orb6p and Skb1p can interact directly, we expressed 35S-labeled Orb6p in a rabbit reticulocyte system (Fig. 3A). Reticulocyte lysates containing 35S-Orb6p were incubated with GST-Skb1p expressed bacterially or GST alone, and then the GST complexes were precipitated using glutathione-agarose beads (Fig. 3B). We found that Orb6p coprecipitated with GST-Skb1p (Fig. 3A, lane 2) but not with GST (Fig. 3A, lane 1), suggesting that the interaction between Skb1p and Orb6p is direct.

Loss of Skb1p Exacerbates the Phenotypic Defects of orb6 Mutants—To determine the role of Skb1p in the control of Orb6p function, we tested if temperature-sensitive mutations in the orb6 gene displayed a synthetic phenotype with deletion mutants of skb1. Wild type 972 (Fig. 4A), single mutant orb6-25 (Fig. 4B), and double mutant orb6-25 skb1Δ (Fig. 4D) cells were allowed to grow exponentially for eight generations at the permissive temperature of 25 °C in YE medium and then were switched to the semipermissive temperature of 33 °C. The percentage of spherical cells is shown in Fig. 4E. After 5 h at 33 °C, 91% of orb6-25 skb1Δ double mutant cells exhibited a spheroidal morphology as compared with only 18% of orb6-25 mutants (Fig. 4, d and b, respectively). This is a notable difference, considering that single mutant skb1Δ cells are virtually indistinguishable from wild type cells under normal growth conditions (Fig. 4, a and c). Thus, the loss of Skb1p exacerbates the morphological defect resulting from partial loss of Orb6p (Fig. 4E).

Orb6p Kinase Activity Is Not Affected by Loss of Skb1p Function—To determine whether Skb1p affects Orb6p kinase activity as measured by autophosphorylation, we purified GST-Orb6p from wild type and skb1Δ cells and performed in vitro kinase assays. The level of GST-Orb6p autophosphorylation was the same for proteins isolated from wild type and skb1Δ cells, suggesting that Skb1p does not regulate Orb6p kinase activity.
activity in vivo (Fig. 5A). We also performed the same experiment using a strain expressing Orb6p-HA at the endogenous level. Because we found that Orb6p-HA kinase activity is inhibited when the anti-HA antibody is bound directly to it (data not shown), we immunoprecipitated the Orb6p protein using antibodies to Myc-tagged Mob2p, expressed from its endogenous promoter. As demonstrated previously (28), Orb6p-HA readily communoprecipitates with Mob2p-13Myc (Fig. 5D). When we performed the kinase assay, we found that the phosphorylated band shifts when Orb6p is untagged, consistent with the band being Orb6p (Fig. 5D, bottom membrane). Similar to the results obtained with the GST-Orb6p fusion, we found no difference in the Orb6p kinase activity associated with the Mob2p complex in the presence or absence of Skb1p (Fig. 5B).

Skb1p Is Required for Proper Localization of Orb6p to the Tips of S. pombe Cells—Like Orb6p, Skb1p is localized to the cell tips during interphase and to the cell septum during mitosis in S. pombe cells (7, 22). Using a strain carrying a green fluorescent protein-tagged Skb1p, we confirmed previously published observations using a HA-tagged Skb1p (22) and found that, similar to Orb6p (7), Skb1 localizes predominantly to the cell tips (data not shown). Because the above experiments suggested that loss of Skb1p does not affect the kinase activity of Orb6p, we investigated whether proper subcellular localization of Orb6p is dependent on Skb1p. To facilitate lo-
control studies, Orb6p, expressed under the control of the endogenous promoter, was fused to the HA epitope at its C terminus and was visualized using anti-HA antibodies (Fig. 6A) (see also "Experimental Procedures"). In wild type cells, Orb6p-HA was found localized to small dots concentrated at the cell tips (Fig. 6A, a and b). In contrast, in skb1Δ cells the Orb6p-HA dots were not found to localize preferentially at the cell tips and appeared distributed uniformly throughout the cell (Fig. 6A, c and d).

To observe changes of Orb6p-HA localization during the cell cycle and in the skb1Δ and skb1Δ cells, we followed the Orb6p protein in cdc25-22 mutant cells synchronously released from a G2 cell cycle block. Because the Orb6p-HA signal is barely detectable and the Orb6p localization at the tips is difficult to quantify when Orb6p is expressed at the endogenous level, we integrated a single copy of orb6-HA under the control of the nmt1 promoter into the temperature-sensitive cdc25-22 and cdc25-22 skb1Δ mutant strains. In the absence of thiamine, the nmt1 promoter expresses Orb6p-HA at levels that are ~8-fold higher than the endogenous promoter (data not shown). Exponentially growing cdc25-22 mutant cells were synchronized in G1 by shifting to the restrictive temperature of 36 °C for 4 h and subsequently were released from the cell cycle block. Samples were taken at the indicated times, and the nuclear content, septation index (Fig. 6D), and Orb6p-HA localization were observed (Fig. 6, B and C). The extent of Orb6p-HA localization to the tips was expressed as a ratio between the signal at the cell ends and an equivalent area at the center of the cell (Fig. 6C, Time 0 (min)) (see also "Experimental Procedures"). As expected, Orb6p-HA was found localized at the cell tips in skb1Δ control cells when arrested in G2 (Fig. 6B, a and b). Similar to the results shown in Fig. 6A for exponentially growing cells, we found that the localization of Orb6p-HA to the cell tips was strongly diminished in skb1Δ cells (Fig. 6B, e and f, and Fig. 6C) and diffusely distributed throughout the cell. 70 min after release from the cdc25 block, as cells were entering mitosis (Fig. 6D), Orb6p-HA disappeared from the cell tips and was found localized to the cell septum in both skb1Δ control cells (Fig. 6B, c and d) and skb1Δ cells (Fig. 6B, g and h). Although there was a significant decrease in the amount of Orb6p localized to the cell tips in skb1Δ cells, Orb6p still was able to associate normally to the cell septum during mitosis (Fig. 6C, Time 70 (min)). These observations suggest that the role of Skb1 in the localization of Orb6 is restricted to the cell ends.

Orb6p Localization Is Altered by Hyperosmotic Shock—Because Skb1p is thought to act as a mediator of hyperosmotic stress response in S. pombe (22), we analyzed the effect of salt exposure on Orb6p kinase localization. Cells expressing Orb6p-HA under the control of the endogenous promoter were exposed to 1.5 M KCl for 20 min, washed to remove excess salt, and allowed to recover for 70 min. In skb1Δ control cells, the localization of Orb6p-HA to the cell tips was lost 15 min after salt addition (Fig. 7A, b). Orb6p-HA returned to the cell tips 70 min after salt removal (Fig. 7A, c). In skb1Δ cells Orb6p dots were distributed uniformly at all of the times that were analyzed (Fig. 7A, d–f).

To quantify the effect of hyperosmotic shock on Orb6p localization, we examined Orb6p-HA localization in cell cycle-synchronized cultures of cdc25-22 and cdc25-22 skb1Δ cells containing an integrated copy of orb6-HA under the control of the nmt1 promoter. Cells were arrested at the G2/M boundary and exposed to salt as described (Fig. 7B) (see also "Experimental Procedures"). We found that upon exposure to salt, Orb6p-HA localization to the cell tips is disrupted within 15 min, is complete by 30 min in skb1Δ cells, and at this point looks identical to the localization of Orb6p in skb1Δ cells. Orb6p dots still are dispersed 60 min after the beginning of the experiment (40 min after salt removal) (Fig. 7B, b and e). The initial Orb6p-HA localization to the cell tips is restored completely 90 min after the beginning of the experiment (70 min after salt removal) (Fig. 7B, c and f, and Fig. 7C). Even after recovery from hyperosmotic shock, the localization of Orb6p-HA to the cell tips in the skb1Δ strain is reduced significantly as compared with the skb1Δ strain (Fig. 7C). We also tested the effects of leaving salt in the growth medium for the whole duration of the experiment (data not shown). We found that when salt is not removed, Orb6p is still predominantly delocalized 90 min after salt addition (data not shown).

In a separate experiment, we found that the interaction between Orb6p-HA and GST-Skb1p, as measured by coimmunoprecipitation, does not change following hyperosmotic shock (data not shown), suggesting the Skb1p-Orb6p interaction is
not regulated by salt exposure. Thus, our experiments show that hyperosmotic stress induces loss of Orb6p from the cell tips. These findings are consistent with previous observations, which showed that the Skb1p protein becomes transiently de-localized from the cell tips when cells are exposed to hyperosmotic stress (22).

The Kinase Activity of Orb6p Increases during Exposure to Salt—We investigated the effect of hyperosmotic shock on Orb6p kinase activity in the presence and absence of Skb1p. To perform this experiment, we used the strain expressing Orb6p-HA at the N terminus. Similarly, we observed an increase of Orb6p kinase activity during exposure to salt (data not shown).

These results show that Orb6p kinase activity is stimulated by exposure to salt and suggest that Orb6p has a role in the response to hyperosmotic stress. Our observations also indicate that Skb1p does not directly modulate Orb6p kinase activity.

DISCUSSION

Orb6p is a member of a conserved family of serine/threonine kinases involved in regulating cell morphogenesis in a number of eukaryotic organisms (7–9, 11–18). In this paper, we have provided evidence that Skb1p, a highly conserved protein methyltransferase implicated in the regulation of cell morphology in response to hyperosmotic stress (19, 22), interacts directly with Orb6p to regulate cell polarity in fission yeast. Orb6p associates with Skb1p in S. pombe cells, and both proteins localize to the cell tips during interphase and to the septum during mitosis (7, 22). Unlike Orb6p, Skb1p is not essential for cell viability or polarity under normal growth conditions and plays only a minor role as a negative regulator of mitosis (19, 23). However, the skb1Δ mutation greatly exacerbates the morphological defects caused by mutations in orb6, suggesting that the Orb6p and Skb1p proteins cooperate to regulate morphogenesis of S. pombe cells. Consistent with these observations, in the absence of Skb1p, the relative amount of Orb6p found at the cell tips is greatly diminished but not abolished. This observation may explain why skb1Δ cells are able to maintain cell polarity under normal growth conditions.

Skb1p has been proposed to act as a mediator of hyperosmotic stress response because it is required for normal growth and cell polarity under conditions of hyperosmotic stress (22). Following hyperosmotic shock, the Skb1p protein becomes transiently de-localized, and its protein methyltransferase activity is increased markedly (22). Furthermore, skb1Δ cells lose normal cell shape under conditions of hyperosmotic stress (22). Similar to these observations, we have found that Orb6p localization to cell tips is altered and that Orb6p dots are dispersed following salt exposure. We also have found that Orb6p kinase activity significantly increases when cells are subjected to hyperosmotic stress. These findings indicate that Orb6p has a role in cell response to hyperosmotic stress. Furthermore, although our experiments have not proven a stronger requirement for Skb1p in Orb6p localization during salt exposure as compared with normal growth conditions, our results cumulatively indicate that the association of Skb1p with Orb6p may contribute to cell robustness during recovery from hyperosmotic stress, possibly by stabilizing Orb6p localization at the cell tips.

In the budding yeast S. cerevisiae, the Skb1p homologue Hsl7p (29) is thought to function as a scaffold, allowing the interaction between the cell cycle regulatory kinases Hsl1p and Swe1p to occur. Because Hsl1p and Swe1p localize to the bud neck in a septin-dependent fashion, it has been proposed that Hsl1p, Hsl7p, and Swe1p form part of a regulatory module that monitors cell integrity and conveys this information to the cell cycle control machinery (30). Our observations indicate that Skb1p also may function as a scaffold in fission yeast, allowing efficient localization of Orb6p at the cell tips.

Interestingly, Orb6p is thought to function not only as a regulator of cell polarity but also as a dose-dependent inhibitor of mitosis. This is because decreased levels of Orb6p lead to a precocious entry into mitosis and overexpression of Orb6p induces G2 arrest (7). Thus, by modulating the amount of Orb6p present at the cell tips, Skb1p may have an effect on the timing of entry into mitosis. Experiments in our laboratory currently are testing this hypothesis.

In summary, our findings identify a novel function for a conserved methyltransferase, Skb1p, which is part of a newly identified family of enzymes for which physiological function is still largely unknown (31). In mammalian cells, the Skb1p homologue has been reported to bind to Jak2 kinase (32). Our findings, identifying Orb6p kinase as a target for Skb1p in the control of cell polarity, suggest that Skb1p and its homologues may have a wider role in regulating the spatial localization of protein kinases involved in signaling and cell morphogenesis. Future research on the function of Skb1p/Hsl7p-related methyltransferases is likely to shed new light on the complex mechanisms that monitor cellular response to stress and cellular damage.

Acknowledgment—We thank R. Rotundo for sharing the imaging facility and J. Ofengand for critical reading of the manuscript.

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J. Biol. Chem. 2003, 278:25256-25263.
doi: 10.1074/jbc.M209703200 originally published online March 19, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M209703200

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