The Highly Conserved Protein Methyltransferase, Skb1, Is a Mediator of Hyperosmotic Stress Response in the Fission Yeast Schizosaccharomyces pombe*

Received for publication, February 20, 2001
Published, JBC Papers in Press, March 9, 2001, DOI 10.1074/jbc.C100096200

Shilai Bao‡‡, Yibing Qyang§§, Peirong Yang§§, HyeWon Kim‡, Hongyan Du‡, Geoffrey Bartholomeusz‡, Jenny Henkel‡, Ruth Pimental§, Fulvia Verde§, and Stevan Marcus‡‡

From the ‡Department of Molecular Genetics and the Graduate Program in Genes and Development, University of Texas, M. D. Anderson Cancer Center, Houston, Texas 77030 and the *Department of Biochemistry and Molecular Biology, University of Miami, School of Medicine, Miami, Florida 33136-1015

The p21-activated kinase, Shk1, is required for cell viability, establishment and maintenance of cell polarity, and proper mating response in the fission yeast, Schizosaccharomyces pombe. Previous genetic studies suggested that a presumptive protein methyltransferase, Skb1, functions as a positive modulator of Shk1. However, unlike Shk1, Skb1 is not required for viability or mating of S. pombe cells and contributes only modestly to the regulation of cell morphology under normal growth conditions. Here we demonstrate that Skb1 plays a more significant role in regulating cell growth and polarity under conditions of hyperosmotic stress. We provide evidence that the inability of skb1Δ cells to properly maintain cell polarity in hyperosmotic conditions results from inefficient subcellular targeting of F-actin. We show that Skb1 localizes to cell ends, sites of septation, and nucleoli of S. pombe cells. Hyperosmotic shock results in substantial delocalization of Skb1 from cell ends and nucleoli, as well as stimulation of Skb1 protein methyltransferase activity. Taken together, our results demonstrate a new role for Skb1 as a mediator of hyperosmotic stress response in fission yeast. We show that the protein methyltransferase activity of the human Skb1 homolog, Skb1Hs, is also stimulated by hyperosmotic stress in fission yeast, providing evidence for evolutionary conservation of a role for Skb1-related proteins as mediators of hyperosmotic stress response, as well as mechanisms involved in regulating this novel class of protein methyltransferases.

Skb1/Hsl7-related proteins have been highly conserved through evolution, with homologs identified in eukaryotes from yeast to human (1–3). The fission yeast Skb1 protein was identified from a two-hybrid screen for proteins that interact with the p21-activated kinase (PAK) homolog, Shk1 (also known as Pak1 and Orb2; Refs. 1, 4, 5). Skb1 is essential for cell viability, establishment and maintenance of cell polarity, and normal mating response in fission yeast (1, 2, 4–6). Genetic and molecular data suggest that Shk1 is a critical effector for the Rho-type p21 GTPase Cdc42, which like Shk1 is required for viability, morphological polarity, and normal mating of Schizosaccharomyces pombe cells (4–6). Cdc42 and Shk1 interact functionally with Ras1, the single known fission yeast homolog of the mammalian Ras p21 GTPase (1, 4, 5, 7–9). Ras1 is required for normal morphology and mating of S. pombe cells, but unlike Cdc42 and Shk1, it is not essential for cell viability (7, 8). Skb1 interacts with the amino-terminal regulatory domain of Shk1, and co-overexpression of the two proteins suppresses the morphological defect of ras1Δ cells (1). These and additional genetic data implicate Skb1 as a positive modulator of Shk1. However, unlike Shk1, Skb1 is not required for cell viability, morphological polarity, or mating of S. pombe cells (1). Indeed, the only defect previously attributed to skb1Δ mutants under normal growth conditions is that they divide at a length slightly shorter than that of wild-type S. pombe cells (1, 2). In contrast, overexpression of Skb1 results in a substantial delay in G2/M progression, suggesting that Skb1 has a dose-dependent mitotic inhibitory function. The G2/M delay caused by Skb1 overexpression is dependent on both Skb1 and the Cdc2 inhibitory kinase Wee1 (2).

The budding yeast homolog of the skb1 gene, HSL7, was discovered from a screen for mutations that are synthetically lethal in combination with a deletion of the amino terminus of histone H3 (3). The same screen resulted in the identification of mutations in genes encoding budding yeast homologs of the fission yeast cyclin-dependent kinase Cdc2 (Cdc28) and the Wee1 inhibitory kinase Nim1 (Hsl1). Loss-of-function of Hsl7 results in a delay in G2/M progression, suggesting that Hsl7 is required for cell viability, morphological polarity, and normal mating of S. pombe cells (1). This role for Hsl7 is supported by the findings of McMillan et al. (10), who provided evidence that Hsl7 acts in concert with Hsl1 to target Swe1 for degradation in S. cerevisiae, and by those of Shulewitz et al. (11), who showed that phosphorylation and ubiquitinylation of Swe1, modifications that target Swe1 for degradation, are substantially reduced in cells lacking Hsl7.

The cellular functions of metazoan Skb1/Hsl7-related proteins have yet to be defined, however, a human Skb1/Hsl7 homolog, Skb1Hs (also known as IBP72 (13) and JBP1 (14)), can substitute for Skb1 in fission yeast, suggesting that Skb1 protein function has been substantially conserved through evolution (2). Skb1Hs has been shown to associate with several different proteins in mammalian cells, including the tyrosine kinase JAK2 (14), the subtype 1 somatostatin receptor (15), and a protein of unclarified function, pICln (13). The biological
significance of the interactions between Skb1Hs and these various proteins has not yet been established.

Skb1/Hsl7-related proteins lack significant structural homology to any other characterized proteins. However, Pollack et al. (14) noted that among the proteins with which Skb1Hs exhibits relatively weak homology (E > 10^-5) were several related protein arginine methyltransferases. These investigators also demonstrated that immunoprecipitates of Skb1Hs contain protein methyltransferase activity, suggesting that Skb1Hs either possesses an intrinsic protein methyltransferase function or associates with a protein methyltransferase (14).

In this report, we show that Skb1, which is largely dispensable for the regulation of cell morphology under normal conditions, is a hyperosmotic shock stimulated enzyme required for normal cell viability and morphological polarity under conditions of hyperosmotic stress. We also provide molecular evidence for evolutionary conservation of a role for Skb1/Hsl7-related proteins as mediators of hyperosmotic stress response, as well as Skb1/Hsl7 regulatory mechanisms in eukaryotic organisms. Our results provide important new insights into the cellular roles of this novel class of protein methyltransferases.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Manipulation, and Plasmids—**S. pombe strains used in this study were SP870 (a ade6-M210 leu1–32 ura4-D18) (D. Beach), SKB1U (a ade6-M210 leu1–32 ura4-D18 skb1::ura4) (1), CHP428 (a ade6-M210 his7–368 leu1–32 ura4-D18) (from C. Hoffman), and SMM100 (a ade6-M210 his7–368 leu1–32 ura4-D18 skb1::ura4) (1). S. pombe cultures were grown in either rich medium (YEA) or in synthetic minimal medium (EMM) with appropriate auxotropic supplements (16). The plasmids pAAUGST (2), pAAUGST-Skb1 (2), pART1CM, pART1CMSkb1 (2), and pART1CMSkb1Hs (2) have been described. pREPUBA-Skb1 was constructed by cloning a 2.4-kilobase BamHI fragment of the Skb1 protein coding sequence isolated from pART1CMSkb1 into the urad-based plasmid, pREP4XHA (a gift from E. Chang). This plasmid allows for expression of a triple HA epitope-tagged Skb1 protein from the nmt1 promoter (17).

**F-actin Staining and Indirect Immunofluorescence Microscopy—**F-actin was visualized using rhodamine-phalloidin as described (16). Indirect immunofluorescence microscopy of HA-Skb1 was performed using monoclonal anti-HA antibody 12CA5 (19) and goat anti-mouse fluorescein isothiocyanate conjugated secondary antibody (Pierce), essentially as described (18).

**Protein Methylation Assays—**S. pombe cells transformed with the plasmids pART1CM, pART1CMSkb1, or pART1CMSkb1Hs were grown in 200 ml of EMM to about 10^7 cells/ml prior to harvesting of cells by centrifugation. Cultures were diluted with an equal volume of either EMM or EMM containing 3 M KCl and grown for 15–60 min prior to harvesting. Cell lysates were prepared as described (6). Immunoprecipitations were performed by incubating extract volumes containing 2 mg of protein with 5 ml of anti-c-Myc monoclonal antibody 9E10 ascites (20) and 25 ml of protein A-agarose beads (Roche Molecular Biochemicals) for 2 h at 4°C. Immune complexes were pelleted by centrifugation and washed three times with 1 ml of yeast lysate buffer and then twice with 1 ml of methylation assay buffer (50 mM Tris- HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA). Samples were divided into 2 equal volumes, one of which was pelleted and resuspended in 40 ml of methylation buffer and the other pelleted and resuspended in SDS-PAGE sample buffer. GST-Skb1 was expressed in the E. coli host strain BL21 from the plasmid pRP259 (a gift from E. Chang), a derivative of pGEX-1 (Amersham Pharmacia Biotech). GST-Skb1 was purified from bacterial cell lysates using glutathione-agarose beads following the manufacturer’s recommendations (Amersham Pharmacia Biotech). Beads containing GST-Skb1 were resuspended in methylation buffer. Methylation assays were performed essentially as described (14). To initiate the methylation reaction, 5 ml of [3H]adenosine methyl donor (specific activity, 78 Ci/mmol) (PerkinElmer Life Sciences) and 5 ml of myelin basic protein solution (12 mg/ml in methylation buffer) were added to each 40-ml volume of immune complex or GST-Skb1 beads in methylation buffer and incubated 30 min at 30°C. Reactions were terminated by placing on ice, adding SDS-PAGE sample buffer, and boiling for 5 min. Reactions were resolved by SDS-PAGE and exposed to x-ray film for 3–6 days. The remaining portion of each immune complex was boiled for 5 min and then subjected to SDS-PAGE and subsequent Western blot analysis to measure the relative amount of Skb1 protein isolated from each GST precipitation or immunoprecipitation.

**RESULTS AND DISCUSSION**

**Skb1 Is Required for Normal Cell Viability and Polarity in Hyperosmotic Medium—**Under normal growth conditions, S. pombe skb1Δ mutants are only modestly defective in cell growth and divide at a length just slightly shorter than that of wild-type S. pombe cells. We demonstrated previously that S. pombe mutants carrying a null mutation in the skb5 gene, which encodes an SH3 domain protein that directly activates Shk1, are unable to maintain cell polarity under conditions of hyperosmotic stress (21). Furthermore, PAKs have been implicated in the regulation of osmotic stress response in budding yeast and mammalian cells (22–24). We therefore determined whether Skb1 might play a more prominent role in regulating cell viability or morphology when S. pombe cells are subjected to hyperosmotic stress. Although not completely inhibited for growth, skb1Δ cells grew substantially slower than wild-type S. pombe cells on minimal medium containing 1.5 M KCl (Fig. 1A). We observed further that skb1Δ cells exhibited a marked defect in the ability to maintain cell polarity in hyperosmotic medium, becoming stubby to round in appearance at a high frequency when compared with wild-type S. pombe cells, which retained a primarily rod-like appearance under the same conditions (Fig. 1B). These observations demonstrate that Skb1, although largely dispensable for growth and maintenance of cell morphology under normal growth conditions, plays a more significant role in regulating cell growth and polarity under conditions of hyperosmotic stress.

**skb1Δ Mutants Are Defective in Restoring Localization of F-actin Patches to Cell Ends After Hyperosmotic Shock—**During interphase, cortical F-actin patches are concentrated at the growing ends of S. pombe cells (25). When S. pombe cells are subjected to hyperosmotic shock, F-actin patches become transiently delocalized from the cell ends and randomly distributed but eventually redistribute to the cell ends after continued incubation in hyperosmotic medium (26). To determine whether the inability of skb1Δ mutants to properly maintain cell polarity in hyperosmotic medium might correlate with a defect in regulating the polarization of F-actin patches, we examined the appearance of F-actin in wild-type and skb1Δ S. pombe cells after subjecting them to hyperosmotic stress. Consistent with previously reported observations (26), we found that F-actin patches became delocalized from cell ends and randomly distributed in both wild-type and skb1Δ cells within 30 min of exposure to EMM containing 1 M KCl (Fig. 2, EMM + KCl). After 2.5 h of incubation in EMM + 1 M KCl, F-actin patches became substantially redistributed to the cell ends in wild-type S. pombe cultures but remained depolarized.
in \( skb1\Delta \) cells (Fig. 2). After 3.5 h of incubation in 1 M KCl, cortical F-actin was redistributed to the ends of \( skb1\Delta \) cells but at a lower frequency than in cultures of wild-type cells. These results suggest that the failure of \( skb1\Delta \) mutants to properly maintain morphological polarity in hyperosmotic medium is likely to be due, at least in part, to a defect in effectively relocalizing the localization of F-actin patches to the cell ends, a process viewed as essential for establishing and maintaining morphological polarity in \( S.\ pombe \) cells (25).

**Skb1 Localizes to Cell Ends, Sites of Septation, and Nuclei in \( S.\ pombe \) Cells**—To obtain additional insights into the role of Skb1 as a morphological regulator, we performed experiments to examine its subcellular localization. To do this, we constructed the plasmid pREP/HASKb1 for expressing Skb1 as a triple hemagglutinin epitope-tagged protein (HA-Skb1) from the thiamine-repressible \( nmt1 \) promoter (17). \( S.\ pombe \) cells transformed with pREP/HASKb1 were grown in medium containing thiamine to repress expression of HA-Skb1, then transferred to medium lacking thiamine, and grown for 11 h to derepress HA-Skb1 expression prior to immunofluorescence microscopy. HA-Skb1 protein was detected at either one or both cell ends in interphase cells and at what appeared to be the nuclear periphery in both interphase and mitotic cells (Fig. 3, A and B). In a small percentage of dividing cells, we were able to detect HA-Skb1 at the septum-forming region (Fig. 3B). The localization of Skb1 to cell ends is consistent with its role as a regulator of morphological polarity, because a number of \( S.\ pombe \) proteins required for proper control of cell polarity has been shown to localize to the cell ends (27, 28).

**Hyperosmotic Shock Induces Rapid De-localization and Enzymatic Stimulation of Skb1**—To obtain additional molecular evidence of a role for Skb1 as a mediator of hyperosmotic stress response, we determined whether its subcellular localization or protein methyltransferase activity is affected by hyperosmotic shock. \( S.\ pombe \) cells expressing HA-Skb1 were grown in EMM and then shifted to EMM containing 1.5 M KCl. Culture samples were fixed at the indicated times for detection of HA-Skb1 protein by indirect immunofluorescence microscopy. The percentage of cells exhibiting HA-Skb1 localization at cell ends (black bars) and nuclei (gray bars) is indicated in the graph (Fig. 4). Hyperosmotic shock triggers a rapid delocalization of Skb1 from cell ends and nuclei. HA-Skb1 expressing \( S.\ pombe \) cells were grown in EMM and then were shifted to EMM containing 1.5 M KCl. Culture samples were fixed at the indicated times for detection of HA-Skb1 protein by indirect immunofluorescence microscopy. The percentage of cells exhibiting HA-Skb1 localization at cell ends (black bars) and nuclei (gray bars) is indicated in the graph (Fig. 5). The protein methyltransferase activities of \( S.\ pombe \) and human Skb1 proteins are rapidly stimulated by hyperosmotic shock of \( S.\ pombe \) cells. A, fission yeast Skb1 possesses intrinsic protein methyltransferase activity. The panel at the far left shows MBP methylation (top) and immunoblot analysis (bottom) of GST and GST-Skb1 isolated from \( S.\ pombe \) cells. The adjacent panel shows results of the same analyses for GST and GST-Skb1 isolated from \( E.\ coli \) cells. The far right panels show MBP methylation (top) and immunoblot analysis of c-Myc epitope-tagged \( S.\ pombe \) (CM-Skb1) and human (CM-Skb1Hs) proteins expressed and purified from \( S.\ pombe \) cells. B, \( S.\ pombe \) cells expressing CM-Skb1 were grown in EMM and were then shifted to EMM containing 1.5 \( M \) KCl and incubated for the indicated times before assaying for CM-Skb1 methyltransferase activity. C, \( S.\ pombe \) cells expressing CM-Skb1Hs were subjected to hyperosmotic shock and assayed for protein methyltransferase activity as described in B.
To examine whether Skb1 methyltransferase activity is stimulated by hyperosmotic shock, cells expressing a c-Myc epitope-tagged Skb1 protein were grown in EMM and were then shifted to either EMM or EMM + 1.5 M KCl and incubated for 15 to 60 min prior to lysing the cells and assaying for Skb1 protein methyltransferase activity. As shown in Fig. 5B, Skb1 methyltransferase activity was stimulated within 15 min of hyperosmotic shock. We conclude from these experiments that hyperosmotic shock induces both a delocalization of Skb1 protein from cell ends and nuclei, as well as a concomitant increase in Skb1 protein methyltransferase activity.

The Human Skb1 Homolog, Skb1Hs, Is Stimulated by Hyperosmotic Shock in Fission Yeast—To address whether the methyltransferase activity of Skb1Hs is stimulated by hyperosmotic shock in fission yeast cells, we subjected cells expressing Skb1Hs to hyperosmotic shock and then prepared cell lysates and assayed Skb1Hs protein methyltransferase activity. As shown in Fig. 5C, Skb1Hs methyltransferase activity, similar to that of fission yeast Skb1, was rapidly stimulated by hyperosmotic shock, suggesting that mediation of hyperosmotic stress response is likely to represent a conserved function of Skb1-related proteins.

In conclusion, we have demonstrated that a highly conserved protein methyltransferase, Skb1, is required for normal growth and maintenance of cell polarity under conditions of hyperosmotic stress in fission yeast. We have shown that skb1Δ mutants are defective in redistributing F-actin patches to cell ends after hyperosmotic stress-induced F-actin depolarization. It is likely that the failure of skb1Δ cells to properly maintain cell polarity in hyperosmotic medium is caused, at least in part, by this defect. Correlating with these findings, we found that in response to hyperosmotic stress, the Skb1 protein delocalizes from cell ends and nuclei and becomes markedly stimulated from cell ends and nuclei, as well as a concomitant increase in hyperosmotic shock. We conclude from these experiments that hyperosmotic shock is likely to represent a conserved function of these highly conserved proteins may have also been substantially conserved through evolution.

Protein sequence analyses suggest that Skb1Hs/Hsl7-related proteins belong to the protein arginine methyltransferase family (14, 29). A variety of eukaryotic proteins of diverse function have been shown to undergo methylation on arginine or lysine residues (30). Although the functional significance of this modification remains ill defined in all but a handful of cases, recent studies have implicated arginine methylation as being of potential significance in signal transduction (31), transcriptional regulation (32), and RNA processing (33, 34). The results presented in this report suggest that protein methylation is likely to play a role in cellular response to hyperosmotic stress. The continued characterization of Skb1/Hsl7-related protein methyltransferases and in particular the identification of Skb1 substrates in the evolutionarily distant fission and budding yeasts will undoubtedly shed substantial new insights into roles for protein methylation in eukaryotic organisms.

REFERENCES

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