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Possible Roles of LAMMER Kinase Lkh1 in Fission Yeast by Comparative Proteome Analysis

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To investigate the possible roles of LAMMER kinase homologue, Lkh1, in *Schizosaccharomyces pombe*, whole proteins were extracted from wild type and *lkh1*-deletion mutant cells and subjected to polyacrylamide gel electrophoresis. Differentially expressed proteins were identified by tandem mass spectrometry (MS/MS) and were compared with a protein database. In whole-cell extracts, 10 proteins were up-regulated and 9 proteins were down-regulated in the mutant. In extracellular preparations, 6 proteins were up-regulated in the *lkh1*$^+$ null mutant and 4 proteins successfully identified: glycolipid anchored surface precursor, β-glucosidase (Psu1), cell surface protein, glucan 1,3-β-glucosidase (Bgl2), and exo-1,3 β-glucanase (Exg1). These results suggest that Lkh1 is involved in regulating cell wall assembly.

KEYWORDS : Lkh1, Proteome analyses, *Schizosaccharomyces pombe*

Lkh1, the LAMMER kinase homolog in *Schizosaccharomyces pombe*, has dual-specific kinase activity. The *lkh1*$^+$ deletion mutant exhibits flocculation upon reaching stationary phase in liquid media and filamentous adhesion growth on solid media [1]. Furthermore, the *lkh1*$^+$ null mutant is sensitive to oxidative stresses, such as increased H_2O_2. This oxidant sensitivity is due to reduced levels of *atf1*$^+$ mRNA, which reduces expression of the antioxidant enzymes catalase (Ctt1) and superoxide dismutase (SOD1) [2]. Recently, the Csx1 protein, which contains three RNA-recognition motifs, has been reported to bind directly to *atf1*$^+$ mRNA in response to oxidative stress but not other stresses, and also to maintain normal levels of Atf1 by stabilizing *atf1*$^+$ mRNA under oxidative-stress conditions [3]. Csx1 is phosphorylated by Lkh1 in response to oxidative stress, and the stress-activated binding of Csx1 to *atf1*$^+$ mRNA is also affected by Lkh1 and Spc1 [4].

Previously, a parallel proteomic and metabolomic analysis of the Sty1p-dependent hydrogen peroxide stress response has been reported [5], but there has been no report focused on a proteome analysis of Lkh1-related proteins. The present report compares the cellular and extracellular proteomes of the wild type and *lkh1*$^+$ null mutant to estimate possible roles of the Lkh1 kinase in cellular responses.

Materials and Methods

Strains and culture conditions. The wild-type strain ED665 (*h*$^-$ *ade6-M210 leu-32 ura4-D18*) and lkh1 cells, PHM5L (*h*$^-$ *lkh1::leu2 ade6-M210 leu-32 ura4-D18*), were used in this study. The rich medium was YES, and the selective medium was Edinburgh synthetic minimal medium [6]. Cells were grown at 30°C.

Extraction of proteins. After cultivation, the yeast cells in the culture supernatant were removed by centrifugation at 12,000 ×g for 15 min 4°C. The extracellular proteins in the cell-free supernatant were precipitated for 30 min with ice-cold 10% (w/v) trichloroacetic acid [7]. The precipitates were then harvested by centrifugation at 12,000 ×g for 30 min at 4°C and the protein pellet washed three times with cold acetone. Thereafter, the pellet was vacuum-dried and solubilized in a lysis buffer containing 20 mM Tris-HCl (pH 7.6), 10 mM NaCl, 0.5 mM deoxycholate, and protease inhibitor cocktail (40 μL/mL). To extract intracellular proteins, the cells (500 mg) were suspended in the lysis buffer, as described for the extracellular protein fraction, and homogenized using a Mini Bead-Beater (Bio-Spec Products, Bartlesville, OK, USA). The homogenate was centrifuged in a microcentrifuge for 5 min at 15,000 rpm at 4°C, and the supernatant was used for further analyses.

Two-dimensional gel electrophoresis (2-DE). 2-DE was carried out essentially as previously described [5] using 13 cm pH 3-10 NL IPG strips (GE Healthcare Inc., Princeton, NJ, USA) and 10% homogenous SDS-PAGE. After electrophoresis, the gels were stained with 0.1% Coo-

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massie Brilliant Blue R-250.

Protein identification. All tandem mass spectrometry (MS/MS) experiments for peptide identification were performed using a linear trap quadrupole mass spectrometry (Thermo Finnigan, San Jose, CA, USA) equipped with a nano-ESI source. From each sample, 10 mL was loaded by the auto sampler (Surveyor, Thermo Finnigan) onto a C18 trap column (id 300 mm, length 5 mm, particle size 5 mm; LC Packings) for desalting and concentration at a flow rate of 20 mL/min. The trapped peptides were then back-flushed and separated on a homemade microcapillary column (150 mm in length), packed with C18 (particle size 5 mm) in 75-mm silica tubing (8 mm id orifice). The mobile phases A and B were composed of 0% and 80% ACN, respectively, each containing 0.1% formic acid. The gradient began at 5% B for 15 min, was ramped to 20% B for 3 min, to 60% for 45 min, to 95% for 2 min, and, finally, held at 95% B for 7 min. The column was equilibrated with 5% B for 10 min before the next run. MS and MS/MS spectra were obtained at a heated capillary temperature of 220°C, an ESI voltage of 2.5 kV, and a collision energy setting of 35%. Data-dependent peak selection of the nine most abundant MS ions from MS was used. The previously fragmented ions were excluded for 60 sec. The proteins were identified by searching the fungi subset (219981 entries) of the National Center for Biotechnology Information (NCBI) protein databases using the MASCOT 2.0 search algorithm (Matrix Science, London, UK). The general parameters for the search were considered to allow a maximum of one missed cleavage, 10 ppm of peptide mass tolerance, and modifications of N-terminal glutamines to pyroGlu, oxidation of methionines, acetylation of protein N termini, carbamidomethylation of cysteines, and acrylamide modified cysteines. A peptide charge state of 2 or 3 and a fragment mass tolerance of 0.5 Da were used for the MS/MS ion search.

Results and Discussion

Comparative analysis of intracellular proteomes. To understand the effects of Lkh1, the differential protein

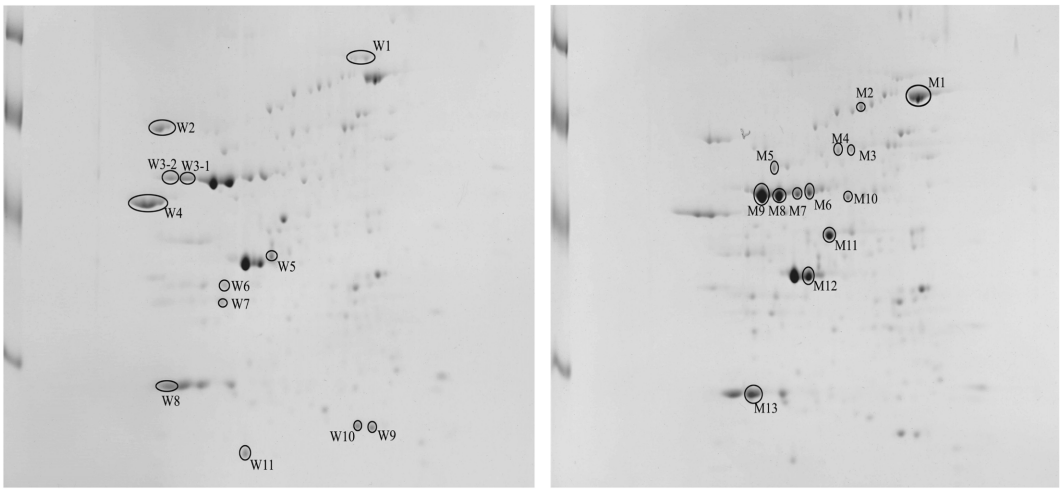

Fig. 1. 2-DE of soluble protein extracts of wild type (A) and *lkh1*$^{+}$ null mutant cells (B). The soluble protein fraction was separated on pH 3~10 NL IPG strips, followed by 10% SDS-PAGE gels.

Table 1. Identification of differentially regulated proteins in wild type *Schizosaccharomyces pombe* by peptide mass fingerprinting

Spot no.	Identification	pI	Mr	Matched peptides (%)	Sequence coverage (%)	Accession no.
W1	SPAC926.04c (swo1, heat shock protein 90 homolog)	4.89	80,546	32	24	P41887
W2	SPAC4H3.10c (pyk1, predicted pyruvate kinase)	8.18	55,479	57	55	Q10208
W3-1/3-2	eno1 (enolase)	6.23	47,406	47	51	
W4	SPBC14F5.04c (pgk1, phosphoglycerate kinase)	8.33	43,936	42	51	O60101
W5	SPBC1773.06c (alcohol dehydrogenase)	5.94	37,297	30	49	
W7	SPBC215.11c (aldo/keto reductase)	6.48	33,901	27	45	O94315
W8	gpm1 (phosphoglycerate mutase)	6.92	23,751	27	59	
W9/10	SPCC576.03c (thioredoxin peroxidase)	5.37	21,178	23	56	O74887
W11	SPAC3C7.14c (obr1, P25 protein)	6.29	21,885	MS/MS	27	P30821

expressions between wild type cells and an *lkh1*$^+$ null mutant were examined by 2D-PAGE. More than 200 reproducible protein spots were detected on the individual CBB R-250 stained 2D gels for each yeast strain within a range of pH 3.0~10 and molecular masses of 20~100 kDa (Fig. 1). Among these, 24 differentially expressed proteins spots with a relatively high abundance were focused on for the present study. We identified 11 protein spots that were down-regulated and 12 protein spots that were up-regulated in the *lkh1*$^+$ null mutant. Meanwhile, W3-1 and W3-2, W9 and W10, M3 and M4, and M7-9 spots were identified as the same proteins. Table 1 lists the theoretical pI, molecular mass, and identity of proteins whose expression levels were lower in the null mutant than in the wild type. Six proteins were related to carbohydrate metabolism: pyruvate kinase, enolase, phosphoglycerate kinase, alcohol dehydrogenase, aldo/keto reductase, and phosphoglycerate mutase. Heat shock protein 90 homolog, Swo1, is an abundant molecular chaperone essential to the establishment of many cellular regulation and signal transduction systems [8, 9]. Thioredoxin peroxidase (Tsa p) is required for the transcriptional response to oxidative stress (OS) and is an important conserved system for protection against OS by reducing peroxides such as H_2O_2 to harmless products [10].

The majorities of the up-regulated proteins were glycolytic enzymes and contained two molecular chaperones (a member of the heat shock protein 70 family and mitochondrial heat shock protein). Glycolytic enzymes can constitute up to 30% of the total soluble proteins in *Saccharomyces cerevisiae* [11]. Heat shock protein 70 can be induced by environmental stress and acts as a biochemical stress indicator [12]. The Hsp70s also play a role in protein folding, the translocation of proteins across membranes, and gene regulation [13-15]. The M11 spot was identified as a hypothetical serine-rich protein and the role of this protein was confirmed after further analysis (Table 2).

Comparative analysis of secretory proteomes. The SDS-PAGE pattern of the extracellular proteins clearly showed that 6 proteins (N1-6) were up-regulated in the *lkh1*$^+$ null mutant and only 1 protein spot was down-regulated (N7) (Fig. 2). Using matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF)-MS/MS, 7 protein spots with a relatively high abundance were analyzed and 4 proteins successfully identified (Table

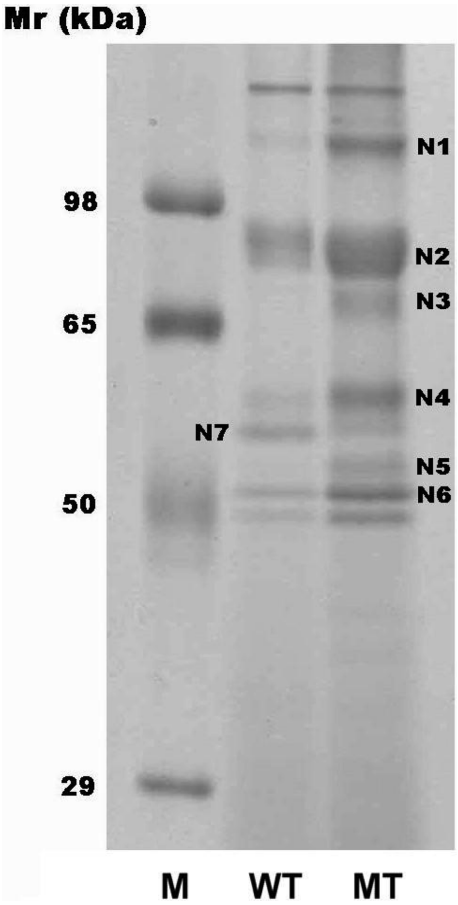

Fig. 2. SDS-PAGE pattern of extracellular proteins from wild type (WT) and *lkh1*$^+$ null mutant cells (MT). Proteins were visualized by Coomassie Brilliant Blue R staining. M, molecular size marker.

Table 2. Identification of differentially regulated proteins in the *lkh1*$^+$ null mutant of *Schizosaccharomyces pombe* by peptide mass fingerprinting

Spot no.	Identification	pI	Mr	Matched peptides (%)	Sequence coverage (%)	Accession no.
M1	SPCC1739.13 (heat shock protein 70 family)	5.13	70,190	50	43	O59855
M2	(hsp70, mitochondrial heat shock protein)	5.73	69,369	35	36	
M3/4	SPCC794.12c (mae2, NAD-dependent malic enzyme)	5.68	62,495	50	36	P40375
M5	SPAC9E9.09c (hypothetical aldehyde dehydrogenase)	6.29	54,734	51	53	O14293
M6	SPBC21C3.08c (probable ornithin aminotransferase)	5.94	48,217	40	46	Q9P7L5
M7/8/9	SPBC1815.01 (eno1, enolase)	6.23	47,406	44	45	P40370
M10	SPBPB21E7.01c (eno102, enolase1-2)	5.61	47,294	39	42	Q8NKC2
M11	SPAC23H3.15c (hypothetical serine-rich protein)	5.86	34,671	47	70	P78890
M12	SPBC19C2.07 (fba1, fructose-1,6-bisphosphate aldolase)	5.93	39,545	29	67	P36580
M13	SPAC26F1.06 (gpm1, phosphoglycerate mutase)	6.92	23,751	20	40	P36623

Table 3. Identification of extracellular proteins of *Schizosaccharomyces pombe* by MS/MS fragmentation

Spot no.	Amino acid sequences	Identification	Matched fragment ions (%)	Sequence coverage (%)	UniProt accession no.
N1	TIPVGYAGADIPVVR	Glycolipid anchored surface protein precursor	17	8	P22146
N2	TQWPSTQPSDGETR	Psu1 (predicted β-glucosidase)	12	5	Q9URO9
N4	QALMQYLPQYGVDHVR	Bgl2 (glucan 1,3-β-glucosidase precursor)	17	12	P15703
N6	LFIETQLDQFER	Exg1 (exo-1,3-β-glucanase precursor)	21	10	Q9URU6

3). When compared with the wild type, the levels of the proteins involved in cell wall synthesis and cell wall assembly, such as glycolipid anchored protein, predicted β-glucosidase (Psu1), glucan 1,3-β-glucosidase precursor (Bgl2), and exo-1,3-β-glucanase precursor (Exg1), were significantly increased in the *lkh1*$^+$ null mutant. The glycolipid anchored protein family is a group of yeast glycolipid proteins anchored to the membrane. It includes the *Candida albicans* pH-regulated protein, which is required for apical growth and plays a role in morphogenesis, and the *S. cerevisiae* Gas1 protein, which encodes a cell wall-bound 1,3-β-glucanosyltransferase involved in the formation and maintenance of 1,3-β-glucan, the major polysaccharide of the cell wall [16]. Psu1 is involved in cell wall synthesis and may be required for the activation of 1,3-β-glucan synthase [17]. *Bgl2* encodes an endo-β-1,3-glucanase that is abundant in the yeast cell wall; it introduces intrachain 1,6-β linkages into 1,3-β glucan, contributing to the rigid structure of the cell wall [18, 19]. Null *Bgl2* mutants are viable, but overproduction of Bgl2 protein leads to defects in the cell wall and sensitivity to osmotic stress [20]. Major exo-1,3-β-glucanase of the cell wall is involved in cell wall β-glucan assembly and exists as three differentially glycosylated isoenzymes [21, 22].

It had been suggested that Lkh1 has a negative role in flocculation and filamentous adhesion growth of the fission yeast by repressing expression of cell surface protein(s) [1], however, no direct evidences for this postulation has been provided. Recently activation of *S. pombe* transcriptoinal repressors, Tup11 and Tup12, by Lkh1-mediated phosphorylation and flocculation of Tup-deletion mutant cells were reported [23]. Our result presented here showed involvement of Lkh1 in cell wall assembly in *S. pombe* by regulating expression of cell wall proteins as well as affecting localization of cell wall protein(s). Further analysis of interaction between *lkh1*$^+$ and other genes should lead to better understanding of the mechanism for *S. pombe* cell wall biogenesis.

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