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Molecular Characterization of the *USO1* Gene Product which is Essential for Vesicular Transport in *Saccharomyces cerevisiae*

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SUMMARY: We have previously shown that *USO1* gene required in the protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus encodes a hydrophilic protein of 1790 amino acids. The sequence of carboxyl-terminal 1010 amino acids was predicted to have an α-helical structure characteristic of the coiled-coil rod region of the cytoskeleton-related proteins. Antibodies raised against partial sequences of the Uso1 polypeptide reacted with a 200 kDa protein in Western blots of the wild-type yeast proteins. The Uso1 protein was found predominantly in the soluble fraction and displayed a molecular mass of 800-900 kDa in gel filtration when globular protein were used as molecular mass standards. In sucrose density gradient centrifugation, however, the Uso1 protein cosedimented with a globular 6S marker protein, horseradish peroxidase (44 kDa). These results suggest that, in its native state, the Uso1 protein forms a nonglobular oligomer.

In eucaryotic cells, protein destined for the extracellular environment, plasma membrane, or lysosomes (vacuoles) are first synthesized as precursor polypeptides with signal peptides in the cytoplasm. They are translocated across the ER membrane and transported along the membrane-enclosed organelles (1, 2). Transport from the ER to the Golgi apparatus is the first step of intracellular protein traffic, and requires more than 22 genes in *Saccharomyces cerevisiae* (3, 4, 5, 6, 7, 8, 9). *USO1* is one of these genes, as evidenced by the accumulation of the core-glycosylated secretory proteins in *uso1-1* temperature-sensitive mutants at the restrictive temperature (3). We have previously predicted that *USO1* gene encodes a hydrophilic protein of 1790 amino acids with a carboxyl-terminal 1010 amino acid-long α-helical structure characteristic of coiled-coil rod regions of the cytoskeleton-related proteins (3). In this study, we report identification, localization and structural characteristics of the Uso1 gene product.

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MATERIALS AND METHODS

Strains, culture conditions and chemicals: S. cerevisiae X2180-1A (*MATa, gal2*) and MK1 (*MATα, his3 leu2 trp1 ura3*) were grown in rich medium (YEPD) containing 1% Bacto Yeast Extract, 2% Bactopeptone (Difco) and 2% glucose. Zymolyase 100-T was obtained from Seikagaku Kogyo. Horse radish peroxidase (HRP)-conjugated goat IgG was from Organon Teknika Corp. Superose-6 was from Pharmacia Fine Chemicals. Other chemicals were from Sigma Chemical Co.

Protein detection: SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli using 7.5% polyacrylamide gel (10). Proteins were visualized by silver staining or Western blotting (11). Anti-Uso1 IgG was used at 1:300 dilution and HRP-conjugated goat anti-rabbit IgG was used at 1:1000 dilution. Filter-bound antibody was detected by DAB substrate kit (Vector Lab, Inc).

Preparation of Uso1 fusion proteins and rabbit immunization: N-terminal 536 amino acids of the Uso1 protein (amino acid 37-572) fused with glutathione S-transferase (GST) and C-terminal 661 amino acids of the Uso1 protein (amino acid 1130-1790) fused with T7 gene 10 protein were produced in *Escherichia coli* (JM109, JM109(DE3)) using pGEX-2T (Pharmacia) and pGEMEX-I (Promega) as expression vectors, respectively (12). Fusion proteins were purified by SDS-PAGE and used to raise antibodies in three rabbits for each antigen (13).

Cell fractionation: Yeast cells were grown to the mid-log phase in YEPD medium at 25°C. Cells (usually 100-200 OD_{599} U) were collected, washed in cold washing solution (1.4 M sorbitol, 10 mM sodium azide), resuspended in 20 ml of a spheroplast medium (1.4 M sorbitol, 50 mM potassium phosphate (pH7.5), 10 mM sodium azide, 40 mM β-mercaptoethanol, 3 mg of Zymolyase-100T) and converted to spheroplasts during 30 min at 30°C. The spheroplasts were pelleted and resuspended in ice-cold lysis buffer (50 mM Tris-HCl pH7.5, 0.8 mM sorbitol, 1 mM EDTA, containing 1 mM PMSF, and 1/1000 vol. of protease inhibitor cocktail (1 mg/ml each of leupeptin, chymostatin, pepstatin, aprotinin and antipain)). The spheroplasts were lysed with glass beads. The lysate was centrifuged at 450 x g for 3 min. The supernatant (S1) was spun at 10,000 x g for 10 min. The supernatant (S2) was spun at 100,000 x g for 1 h to produce a P3 pellet and S3 supernatant (14). The protein concentrations were determined according to Bradford (15).

Sucrose gradient fractionation: For velocity gradient analysis, S3 supernatant (4mg of protein) was layered on a 5-20% (wt/wt) continuous sucrose gradient containing 200 mM Tris-HCl (pH 8.0) and 1/1000 vol. of protease inhibitor cocktail. The gradient was centrifuged at 150,000 x g for 13 h in a RPS-40T rotor (Hitachi). Fractions were collected from the bottom of the tube. Horseradish peroxidase (6S), bovine liver catalase (11.5S), bovine thyroglobulin (19.3S) were used as molecular mass standards (16).

Gel filtration: S3 fraction (7 mg of protein in 0.5 ml of lysis buffer) was applied to Superose-6 column (40 cm x 1.2 cm) and fractionation was performed using 50 mM Tris-HCl (pH7.5), 1 mM EDTA at a flow rate of 0.35 ml/min. Bovine serum albumin (69 kDa), bovine liver catalase (232 kDa), horse spleen ferritin (440 kDa), bovine thyroglobulin (669 kDa) were used as standards (16). Fractions of 1 ml were collected and proteins were analyzed by SDS-PAGE or Western blotting.

Results: The *USO1* DNA sequence predicted an open reading frame of 1,790 amino acids (calculated molecular weight of 206,423) (3). To determine if yeast actually produces a corresponding polypeptide, and to examine the relationship between the primary translation product and native functional protein, polyclonal antibodies directed against a N-terminal fragment and a C-terminal fragment of the Uso1 protein were raised in rabbits (see **MATERIALS AND METHODS**). All antisera detected a band of a common size, 200 kDa in wild-type cell lysate (Fig. 1, lanes 1, 4). The size of this protein was in good agreement with the predicted molecular weight of the *USO1* gene product. This 200 kDa band was not recognized when preimmune serum was used (Fig. 1, lanes 7-9). When *USO1* gene was present on a multicopy plasmid, an approximately 20-30 fold increase in the 200 kDa protein species was observed (Fig.

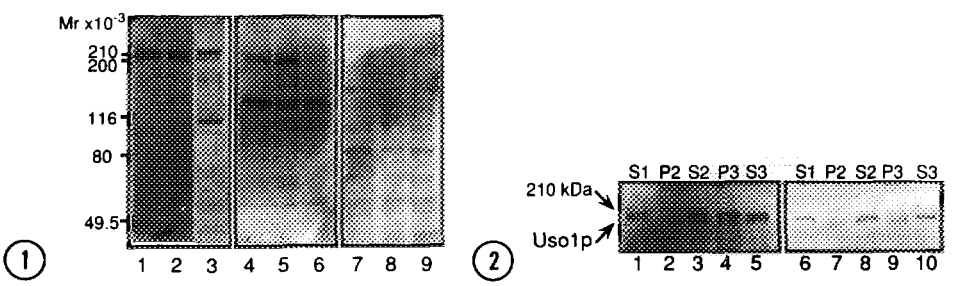

Figure 1. Detection of the Uso1 protein by Western blotting.

Antibodies raised against the GST-N-terminal Uso1 and T7 gene 10-C-terminal Uso1 fusion proteins recognized a 200 kDa protein on Western blots. Spheroplasts of a wild-type strain (X2180-1A, lanes 1, 4 and 7), a strain carrying *USO1* gene on a multicopy plasmid (lanes 2, 5 and 8) and a *uso1-1* mutant strain (lanes 3, 6 and 9) were broken by glass beads. The lysates were immediately centrifuged for 3 min at 450 x g. The supernatant was mixed with Laemmli sample buffer and subjected to electrophoresis. Immunoblotting was done with anti-Uso1 N-terminal antibody (lanes 1-3), anti-Uso1 C-terminal antibody (lanes 4-6) or a pre-immune serum (lanes 7-9).

Figure 2. Localization of Uso1 protein.

Yeast cells (X2180-1A) were grown to early exponential phase at 25°C in YEPD medium, converted to spheroplasts and lysed in 50 mM Tris (pH 7.5), 0.8 M sorbitol, 1 mM EDTA containing protease inhibitor cocktail. The spheroplasts were lysed with glass beads and unbroken cells were removed during a 3 min spin at 450 x g. The supernatant (S1) from this spin was centrifuged for 10 min at 10,000 x g to generate a supernatant (S2) and pellet (P2) fractions. The supernatant (S2) was centrifuged for 1 h at 100,000 x g to generate a supernatant (S3) and pellet (P3) fraction. The 450 x g supernatant (lane 1), 10,000 x g pellet (lane 2), 10,000 x g supernatant (lane 3), 100,000 x g pellet (lane 4) and 100,000 x g supernatant (lane 5) fractions were boiled in SDS and subjected to Western blot analysis using a 1:300 dilution of anti-Uso1 N-terminal antibody (lanes 1-5) and anti-Uso1 C-terminal antibody (lanes 6-10).

1, lanes 2, 5). Several bands other than 200 kDa were detected by Western blots (for example, 210 kDa (upper band in lanes 1-3), 130 kDa (lower band in lanes 4-6)). These bands were not common either between antigens used or among immunized rabbits. They were probably caused by non-specific antibodies rather than modified forms and/or degradation products of the Uso1 protein. Thus we assigned the 200 kDa protein as the wild-type *USO1* gene product. In *uso1-1* mutant lysate, however, 200 kDa protein was not detected by these antisera (Fig. 1, lanes 3, 6) but a 100 kDa protein was detected only by anti-Uso1 N-terminal antiserum (Fig. 1, lane 3).

To determine the localization of the Uso1 protein, the wild type cell lysate was prepared by osmotic lysis of the spheroplasts. This lysate was centrifuged at 750 x g to remove unbroken cells. The supernatant (S1) was spun for 10 min at 10,000 x g to obtain a supernatants (S2) and pellet (P2). The S2 was further centrifuged for 1 h at 100,000 x g to obtain a high speed supernatant (S3) and pellet (P3). Each fraction was analysed by SDS gel electrophoresis and the Uso1 protein was detected by Western blotting. Almost all of the Uso1 protein was localized in S3 indicating the Uso1 protein is a soluble protein (Fig. 2).

We have previously predicted that the carboxyl-terminal 1010 amino acid-long sequence had an α-helical structure characteristic of the coiled-coil rod region (3). If so, the Uso1 protein should interact with itself or with other protein(s). Then it should display a native molecular weight significantly higher than that of the SDS-denatured protein (200 kDa). To test this possibility, the S3 fraction was applied to a Superose-6 gel filtration and eluted by 50 mM Tris (pH7.5), 1mM EDTA. Uso1 protein was detected by Western blotting with anti-Uso1 C-terminal antibody. The Uso1 protein was eluted with a estimated molecular weight of 800-900 kDa (Fig. 3) in comparison with molecular mass standards of globular proteins. These results demonstrated that the Uso1 protein is present in a soluble high molecular mass complex.

If, as predicted by its sequence, the Uso1 protein forms a long coiled-coil rod between amino acid 760 and 1770, it should display a much lower sedimentation coefficient than the expected value for a 800-900 kDa globular protein. The cytosolic fraction of yeast lysate was characterized by velocity sedimentation in sucrose gradient. The sucrose gradient (5-20%) was centrifuged at 150,000 x g for 13 h. Fractions from the gradient were analyzed for Uso1 protein distribution by Western blotting with anti-Uso1 C-terminal antibody. Uso1 protein had a sedimentation coefficient of ca. 6S, which corresponds to a globular protein of 44 kDa (Fig. 4). This data supported our prediction that Uso1 protein is a nonglobular oligomer forming a long rod structure by dimerization or trimerization at the C-terminal halves.

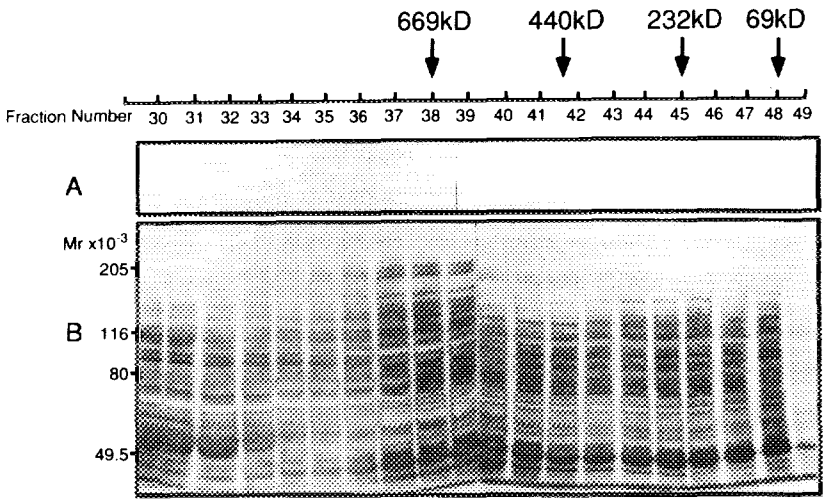

Figure 3. Superose 6 gel filtration of soluble fraction.
Soluble fraction was chromatographed on the Superose 6 column. Eluted fractions were separated by SDS-PAGE and visualized with Western blotting by anti-Uso1 C-terminal antibody (A) or silver staining (B). Molecular size standards for gel filtration were bovine thyroglobulin (669 kDa), horse spleen ferritin (440 kDa), bovine liver catalase (232 kDa) and BSA (69 kDa).

DISCUSSION: A 200 kDa polypepetide was detected by all the antisera raised against partial Uso1 fusion polypeptides but not with preimmune serum. We assigned this band as the *USO1* gene product. Gene dosage effect supported this conclusion. If *USO1* gene was expressed with the aid of *GAL7* promoter, the amount of 200 kDa polypeptide also increased ca. 100-folds (data not shown). The Uso1 proteins was

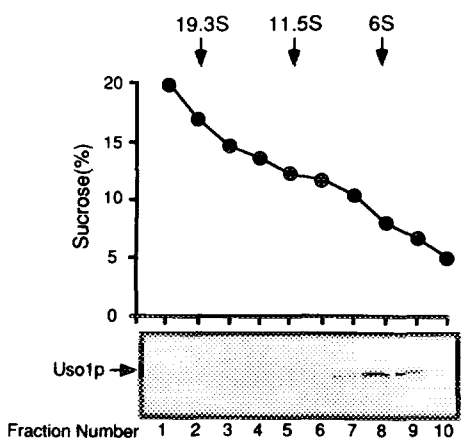

Figure 4. Velocity sedimentation of soluble fraction.
Yeast soluble fraction was sedimented through a sucrose density gradient (5-20%) (see **MATERIALS AND METHODS**). Fractions were collected and Uso1 protein was detected by Western blotting with anti-Uso1 C-terminal antibody.

localized predominantly in the soluble fraction. This localization is in good agreement with the prediction that there are no hydrophobic stretches long enough to function as a signal sequence or a membrane insertion sequence.

Several nonglobular proteins were reported to function in vesicle transport. Mammalian p115 is reported to be in a nonglobular molecular shape and involved in intercisternal protein transport among Golgi apparatus (17). Yeast Sec2 protein which is required at the final stage of the secretory pathway was reported to be in an α-helical coiled-coil conformation (18). We have previously predicted that *USO1* gene encodes 1790 amino acid polypeptide with a C-terminal 1010 amino acid-long coiled-coil α-helical structure like many cytoskeletal proteins (3). In the temperature-sensitive *uso1-1* mutant lysate, however, 200 kDa protein was not detected but a 100 kDa protein was detected only by anti-Uso1 N-terminal antisera (Fig. 1, lane 3). This suggests *uso1-1* mutation might be a nonsense or frameshift mutation as found in the case of *sec2* mutants which truncated the C-terminal region(18). Recently we found that a base substitution in the 951st codon occurred in *uso1-1* which converted a glutamine codon to an amber codon. The *uso1-1* sequence predicted a protein of 950 amino acids and an calculated molecular mass of 108.8 kDa, consistent with the detected 100 kDa band on immunoblots (to be published elsewhere).

Myosin has a long rod like tail in the molecules. The structure of this protein depends on a common structural motif in which two α-helices with a characteristic spacing of hydrophobic residues coil around each other to form a coiled-coil. Myosin consists of six polypeptide chains: two identical heavy chains and two pairs of light chains (MW, 500 kDa). However, sedimentation coefficient of this protein in 0.6 M KCl or 1 M ammonium acetate is 6S because of its filamentous conformation (19, 20). Uso1 protein also displayed a native molecular mass of 800-900 kDa in gel filtration (Fig. 3). But it had a sedimentation coefficient of about 6S (Fig. 4). This discrepancy is quite similar to the case of myosin molecule. These data suggest that Uso1 protein is a nonglobular oligomer forming a long rod structure. Analysis of the molecular function of the Uso1 protein should provide a clue for studies of the nonglobular protein in the protein transport.

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