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Specific Membrane Recruitment of Uso1 Protein, the Essential Endoplasmic Reticulum-to-Golgi Tethering Factor in Yeast Vesicular Transport

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Abstract Uso1 is a yeast essential protein that functions to tether vesicles in the ER-to-Golgi transport. Its recruitment to the ER-derived vesicles has been demonstrated in in vitro membrane transport systems using semi-intact cells. Here we report that the binding of Uso1 to specific membranes can be detected through simple sucrose density block centrifugation. The purified Uso1 protein binds to slowly sedimenting membranes generated from rapidly sedimenting P10 membranes. These membranes were produced dependent on ATP hydrolysis, contained COPII vesicle components, but had neither of the coat subunits or ER proteins, which indicates that they were representative of the uncoated ER-derived COPII vesicles. The slowly sedimenting membranes of different origins were physically linked when they were mixed in the presence of Uso1. The C-terminal acidic region was not required in membrane binding. The presence of membranes to which Uso1 could bind in the yeast cell lysate was detected using the current method. J. Cell. Biochem. 101: 686–694, 2007. © 2006 Wiley-Liss, Inc.

Key words: endoplasmic reticulum; Golgi; membrane vesicle tethering; *Saccharomyces cerevisiae*; Uso1 protein

In vesicle traffic within the eukaryotic cell, membrane fusion is mediated by SNARE {SNAP [soluble NSF (N-ethylmaleimide-sensitive fusion protein) attachment protein] receptor} proteins [Bonifacino and Glick, 2004]. Fusion occurs when a particular combination of vesicular (v)- and target (t)-SNAREs are located on the opposite membranes, meaning that SNAREs mainly determine the specificity of membrane fusion. However, other components, many of which are peripheral membrane proteins, also play important roles in achieving this membrane fusion [Gillingham and Munro, 2003; Whyte and Munro, 2002]. They seem to link two membranes physically before their SNAREs actually interact, therefore, they are called tethering factors. Tethering factors are classified into two major groups; the multisubunit complexes and long coiled-coil proteins [Lupashin and Sztul, 2005].

Saccharomyces cerevisiae Uso1 protein was discovered during the study of a temperature-sensitive secretion mutation *uso1-1* [Nakajima et al., 1991]. It is a hydrophilic 1,790 amino-acid protein which forms a dimer with two globular heads and a long coiled-coil tail of longer than 1,000 amino acids [Yamakawa et al., 1996]. Most of the tail was lost in the *uso1-1* mutant by an amber codon at the 951st residue. Its mammalian ortholog TAP/p115 also participates in the vesicle trafficking and Golgi organization [Nakamura et al., 1997; Nelson et al., 1998; Lesa et al., 2000; Linstedt et al., 2000; Puthenveedu and Linstedt, 2001, 2004]. Requirement of Uso1 in vesicular transport was demonstrated in an in vitro system using semi-intact cells [Barlowe, 1997]. Uso1 tethers COPII vesicles budded from the ER to the Golgi compartments in the semi-intact cell, thus preventing their free dispersion before the membranes fuse [Cao et al., 1998]. Interestingly, Uso1 was also reported to participate

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in the sorting of GPI-anchored proteins at the exit from the ER [Morsomme and Riezman, 2002]. Tethering of the ER-derived vesicles without membrane fusion was demonstrated in vitro [Morsomme and Riezman, 2002]. Although the requirement and interaction with rab GTPase Ypt1 were indicated [Barlowe, 1997], the molecular nature of the membrane receptor for tethering by Uso1 still remains unclear.

To investigate the mechanisms of vesicle tethering by Uso1 in more detail, we improved the cell-free membrane-binding assay. We found that Uso1 bound to slowly sedimenting membranes using simple sucrose density block centrifugation. Immunological examination and their productive conditions indicated that Uso1 preferentially bound to the uncoated COPII transport vesicles. Although previous methods required coincidental production of COPII vesicles to show the membrane binding of Uso1, the current method could detect the Uso1-binding membranes present in the yeast cell lysate.

MATERIALS AND METHODS

Yeast Strains and Media

The *Saccharomyces cerevisiae* strains used in this study are listed in Table I. CJY146 was created from KA31a by replacing the chromosomal *ERV41* gene with an *ERV41-3HA* construct using pCJ20 (*ERV41-3HA* in pRS303) [Cho et al., 2001]. YNY197 was constructed by transforming CJY146 with pRS416-3HA-Sec71 (a kind gift from Dr. K. Sato and Dr. A. Nakano, Riken Institute, Japan) and was routinely used in this study. The 3′-region of the chromosomal *USO1* gene of KA31a was replaced with the *USO1-5myc-Streptag* (YTY21) or *USO1-3HA-Streptag* (YTY50) construct by homologous recombination using *URA3* as the selection marker. For overproduction of the tagged protein, the *USO1* promoter was replaced with the glyceraldehyde 3-phosphate dehydrogenase promoter (pGAPDH) by homologous recombination using *LEU2* as the selection marker to construct the strain YTY25 or YTY51. The viable *uso1* truncation mutants were similarly created by replacing the chromosomal *USO1* gene with the constructs shown in Figure 6. Myc-tagged Uso1C protein was produced using pYN106 in the wild-type yeast. Yeast cells were grown in YEPD (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% glucose) or SD (0.17% Bacto-yeast nitrogen base w/o amino acids and ammonium sulfate, 0.5% ammonium sulfate, 2% glucose and appropriate supplements) medium at 30°C.

Plasmid Construction

The plasmid pYN275 which produces N-terminally 3HA-tagged Yif1 (3HA-Yif1) was made by PCR amplification of the sequence encoding the ORF and its downstream sequence from the genomic DNA using primers (5′-CCCTCGAGATGTAAATTAAATCTATC-3′ and 5′-CGGGATCCATGTCTTATAATCCGTAC-3′) and ligation into the plasmid pYN339 (a moderate expression vector having the *YPT1* promoter to produce 3HA-tagged protein in pRS416). The nucleotide sequence of each amplified fragment was verified.

Antibodies

Anti-Sed5 antibody was described previously [Kosodo et al., 2002]. Antibodies against Ypt1, Bet1, and Scs2 were generous gifts from Dr. D. Gallwitz (Max Planck Institute, Germany),

TABLE I. Yeast Strains Used in This Study

Strain	*Genotype*/plasmid	Reference
KA31a	*MATa*, Δ*his3* Δ*leu2* Δ*trp1* Δ*ura3*	Laboratory strain
CJY146	KA31a, *ERV41-3HA HIS3*	Cho et al., 2001
CJY119	KA31a, *6myc-SED5 URA3*	Cho et al., 2001
YNY197	CJY146/pRS316-*3HA-SEC71*	This study
YNY204	KA31a/pYN275 (pYN339-*3HA-YIF1*)	This study
YTY21	KA31a, *USO1-5myc-Streptag URA3*	This study
YTY50	KA31a, *USO1-3HA-Streptag URA3*	This study
YTY25	YTY21, pGAPDH-*USO1-5myc-Streptag LEU2*	This study
YTY51	YTY50, pGAPDH-*USO1-3HA-Streptag LEU2*	This study
YNY117	KA31a, *uso1-1-6myc LEU2*	This study
YNY158	KA31a, *USO1-6myc URA3*	This study
CJY105	KA31a, *USO1*Δ*a-6myc URA3*	This study
YNY362	KA31a/pYN346 (pYN106-*6myc-uso1C*)	This study

Dr. S. Ferro-Novick (Yale University, CT) and Dr. S. Kagiwada (Nara Women's University, Japan), respectively. The anti-myc mouse (9E10, Berkeley Antibody) and anti-HA rat (3F10, Roche Diagnostics) monoclonal antibodies (mAb) were purchased.

Purification of Tagged Uso1 Proteins

The cells of YTY25 or YTY51 were grown in YEPD to $OD_{600\ nm}$ 1.0–2.0, collected and converted to spheroplasts by lyticase. The spheroplasts were suspended in B88 buffer (20 mM HEPES, pH 6.8, 150 mM potassium acetate, 5 mM magnesium acetate, 200 mM sorbitol) containing protease inhibitors (1 μg/ml each of chymostatin, aprotinin, leupeptin, pepstatin A and antipain, and 1 mM phenylmethylsulfonylfluoride) and disrupted using a Teflon-glass homogenizer (Wheaton). The homogenate was centrifuged at 1,000*g* for 5 min to collect the supernatant, and then it was centrifuged at 100,000*g* for 60 min. The supernatant obtained from 1 L of the culture was concentrated to 1 ml using Centricon YM-100 (Millipore). One hundred micrograms of avidin/ml was added to the supernatant and kept on ice for 15 min. Then, StrepTactin-Sepharose beads (Novagen) were added and the suspension was kept rotating overnight. After washing the beads with B88 buffer, the tagged protein was eluted using 2.5 mM desthiobiotin. The purified protein was checked by SDS/PAGE and stored at −20°C with 50% glycerol (v/v) until use.

In Vitro Tethering Assay

YNY197 was grown in 1 L of SD medium to $OD_{600\ nm}$ 0.8–1.0. Cells were collected, resuspended in B88 buffer containing protease inhibitors, and disrupted by vortexing with acid-washed glass beads. Unbroken cells were removed by centrifugation at 1,000*g* for 5 min at 4°C to prepare S1 fraction. To prepare the cytosol, S1 was centrifuged at 100,000*g* for 60 min. The supernatant (approximately 10 ml) was aliquoted, quickly frozen in liquid nitrogen, and stored at −80°C. To prepare the P10 membranes, S1 was centrifuged at 10,000*g* for 10 min. The resulting pellet was resuspended in B88 containing protease inhibitors and centrifuged again to yield P10. The first supernatant was further centrifuged at 100,000*g* for 60 min and the pellet was used as P100. To prepare the total membrane, S1 was directly centrifuged at 100,000*g* for 60 min. The protein concentration of membrane fractions were determined by measuring $OD_{280\ nm}$ of a 1:100 dilution in 2% SDS [Shimoni and Schekman, 2002]. The membranes were used at a final concentration of approximately 1.2 mg/ml.

Four hundred microliters of the cytosol and the membranes (720 μg) were homogenized using a Teflon-glass homogenizer. To 360 μl of this mixture, 0.5 μg purified Uso1 protein, ATP (final concentration 1 mM), GTP (0.2 mM), phosphocreatine (40 mM), and creatine kinase (0.2 mg/ml) were added in a final 600 μl reaction volume. The reaction mix was incubated for 30 min at 25°C or on ice as a control. Then, most of the rapidly sedimenting membranes were removed by centrifugation at 10,000*g* for 10 min, and 550 μl was layered over sucrose density blocks consisting of 250 μl of 65% and 400 μl of 25% sucrose in B88 (Fig. 1A). After centrifugation at 100,000*g* (Beckman Coulter, TLS55, 45,000 rpm) for 60 min, 150 μl fractions were collected from the top (Fraction 1) to bottom (Fraction 8). They were mixed with 50 μl of 4 × SDS sample buffer (0.5 M Tris-HCl, pH6.8, 4% SDS, 40% glycerol, 20% 2-mercaptoethanol) and boiled for 1 min. Twenty microliters of each sample was loaded on a gel.

In the experiment of Figure 4, the rapidly sedimenting membranes were not removed after the incubation, and 550 μl of the reaction mix was directly applied on the sucrose blocks. In the experiment of Figure 6, the cytosol containing various mutant forms of Uso1 were used instead of adding the purified tagged Uso1.

Immunoisolation of Uso1-Bound Membranes and of Membranes With 6Myc-Tagged Sed5 in Mixed Membrane Experiments

After the sucrose density block centrifugation, Fraction 6 was recovered and subjected to immunoisolation using anti-myc monoclonal antibody 9E10, as described previously [Inadome et al., 2005]. After washing, membrane proteins were solubilized from Protein A-Sepharose beads with 1% Triton X-100 and analyzed by Western blotting. In the experiments using 6myc-Sed5 (Fig. 5), Uso1-3HA-Streptag protein purified from YTY51 was used instead of Uso1-5myc-Streptag.

RESULTS

Binding of Uso1 Protein to Slowly Sedimenting Membranes Detectable by Sucrose Density Block Centrifugation

It has been reported that Uso1 binds to the ER-derived COPII vesicles produced from the permeabilized semi-intact cells in vitro [Cao et al., 1998; Morsomme and Riezman, 2002]. To facilitate further molecular analysis of this binding, we sought to develop a more concise and improved assay system. Uso1 protein with 5myc or 3HA and Strep tags at the C-terminus (Uso1-5myc-Streptag in YTY21 or Uso1-3HA-Streptag in YTY50) was fully functional, because the haploid yeast having the chromosomal single-copy modified gene with its own *USO1* promoter grew as well as the wild-type yeast, while the *uso1*Δ null mutant was lethal. We purified the tagged protein from the cytosol of its overproducer (YTY25 or YTY51) and used it as the functional protein in our in vitro studies. For convenience, we refer the tagged protein as Uso1 unless otherwise indicated.

Uso1 was mixed with the P10 membranes in the presence of cytosol, ATP-regeneration system and GTP, and incubated for 30 min at 25°C or on ice as a control. Then, the reaction mix was centrifuged at 10,000*g* for 10 min to remove most of the P10 membranes. The supernatant was subjected to a centrifugation at 100,000*g* for 60 min on two sucrose density blocks and fractionated as shown in Figure 1A. The soluble materials remained in the sample layer (Fractions 1–4) and most of the membranes sedimented on the 65% sucrose cushion (Fraction 7). As shown in the Western blots (Fig. 1B, 25°C), a significant amount of Uso1 was found in Fractions 6 and 7 when incubation was done at 25°C. Fraction 6 reproducibly had more Uso1 than Fraction 7. When incubation was done on ice, Uso1 remained in the sample layer (Fractions 1–4) and practically no Uso1 was found in the sucrose blocks (Fig. 1B, on ice). When P100 membranes were used instead of P10, Uso1 remained in the sample layer, and did not sediment into sucrose blocks (Fig. 1B, bottom panel).

Proteins in each fraction were analyzed by Western blotting using antibodies to the membrane marker proteins (Fig. 1B). A COPII-vesicle vSNARE Bet1 [Newman et al., 1992], an early Golgi tSNARE Sed5 [Inadome et al., 2005] and an ER-resident protein Scs2 [Kagiwada et al., 1998] were found in Fractions 6 and 7 of the 25°C sample. None of them were found in the sample layer. In the case of incubation on ice, only a small amount of Scs2 was found in Fraction 7, but no Bet1 or Sed5 were found in Fractions 3–7. Considering that Uso1 tethers COPII vesicles [Cao et al., 1998; Morsomme and Riezman, 2002], the simultaneous recovery of Uso1 and Bet1 in Fraction 6 suggests that Uso1 bound to the ER-derived COPII vesicles which were generated by 25°C incubation [Otte and Barlowe, 2002].

Characterization of the Slowly Sedimenting Membranes to Which Uso1 Bound

We further characterized the membranes to which Uso1 bound by immunoisolation. Uso1

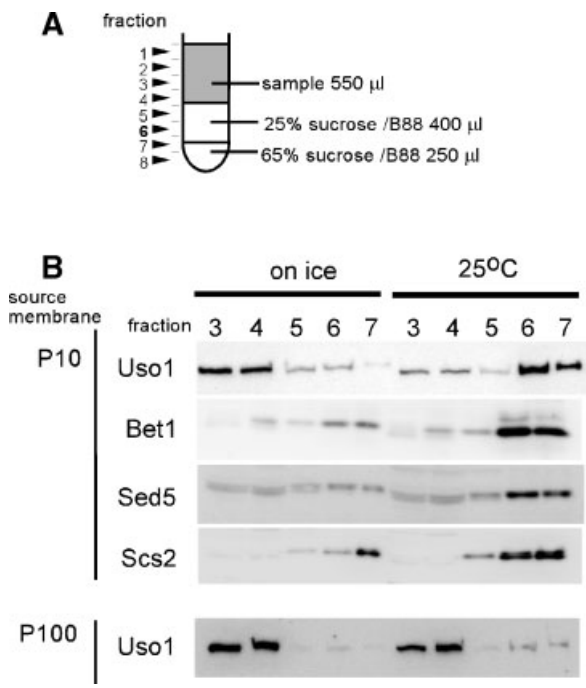

Fig. 1. Binding of Uso1 to slowly sedimenting membranes generated from rapidly sedimenting P10 membranes in the cell-free system. **A**: Correspondence of the composition of sucrose density blocks and fractions after centrifugation. **B**: Western blot analysis of the proteins in the fractions. The cytosol, P10 membranes and purified Uso1 were mixed and incubated as described in Materials and Methods section. The reaction mix was centrifuged to remove most P10, and the sample was overlaid on the sucrose density blocks as shown in subpart A. After centrifugation at 100,000*g* for 60 min, the fractions were collected and analyzed using antisera against Uso1, Bet1, Sed5, and Scs2. As the fractions from 1 to 3 were indistinguishable and Fraction 8 had no signal, Fractions 3–7 are shown.

and its associated materials were collected on Protein A-Sepharose beads from Fraction 6, using an anti-myc monoclonal antibody. The beads were treated with 1% Triton X-100, and the solubilized proteins were analyzed by Western blotting. As shown in Figure 2, the components of the COPII vesicles, Bet1, Erv41, and Yif1 [Otte and Barlowe, 2002], were clearly detected in the bound materials in the presence of Uso1, which further supported that Uso1 bound to the COPII vesicles. Sed5 and rab-GTPase Ypt1 were also found, which might suggest that the early Golgi compartments interacted with Uso1 directly or indirectly. An intra-Golgi vSNARE Sft1 was present in Fraction 6, but it was not found in the bound materials, which suggested that Uso1 did not bind non-specifically to the Golgi membranes. A COPII coat GTPase Sar1 and a COPI coat subunit Ret2 were not detected (data not shown). Those experiments showed that Uso1 binds specifically to the membranes of COPII vesicles and/or early Golgi compartments.

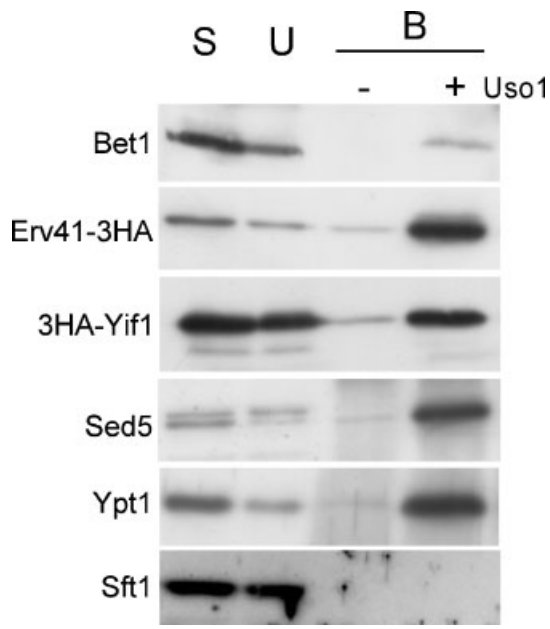

Fig. 2. Proteins in the Uso1-binding membranes. Membranes to which myc-tagged Uso1 bound in Fraction 6 were immunoisolated using anti-myc monoclonal antibody 9E10. The proteins indicated at the left were detected by Western blotting after elution with 1% Triton X-100 (B, bound). Reactions were done with (+) or without (−) adding purified Uso1 protein. A 1/25 portion of the total proteins in Fraction 6 before (S, start) and after (U, unbound) the immunoisolation was also examined.

Conditions to Generate the Slowly Sedimenting Membranes to Which Uso1 Binds

The membranes to which Uso1 binds were produced by a temperature-dependent reaction. We examined the role of nucleotide triphosphates added in the reaction mix. It is well known that GTP hydrolysis is required for uncoating of the COPII vesicles. Replacement of GTP with a non-hydrolyzable analog GMPPNP slightly reduced the amounts of Uso1 and Bet1 migrated in the sucrose blocks (Fig. 3). On the other hand, replacement of ATP with non-hydrolyzable AMPPNP resulted in a significant decrease of the amount of Uso1 and Bet1 in Fraction 6 reproducibly. Therefore, ATP hydrolysis plays an important role in the generation of the Bet1-containing, slowly sedimenting membranes to which Uso1 binds in the current in vitro system.

To shed light on the role of ATP hydrolysis, we tested several inhibitors. *N*-ethylmaleimide, a potent inhibitor of Sec18 (NSF) SNARE-dissociating ATPase, and K-252a, a broad-specificity kinase inhibitor, had no effect when used at 1 mM (data not shown). H89, an inhibitor of serine/threonine and A kinases, blocks the COPII assembly in mammalian cell-free assays [Aridor and Balch, 2000]. The amount of Uso1 and Bet1 in Fraction 6 significantly reduced by adding H89 at concentrations higher than 100 μM (Fig. 4). This effective concentration of H89 was similar to that reported in the mammalian system. Therefore, H89 might also inhibit the COPII vesicle formation in *S. cerevisiae*.

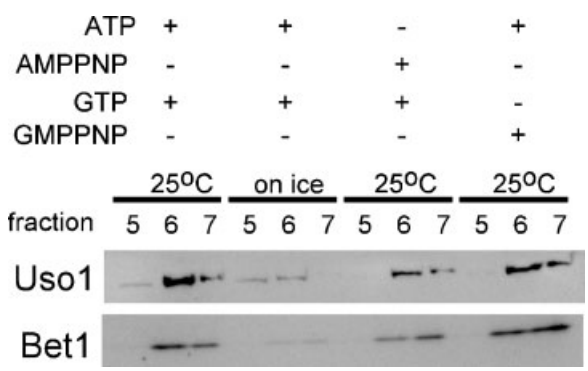

Fig. 3. Nucleotide requirements for the production of the Uso1-binding membranes in Fraction 6. ATP or GTP was replaced with their nonhydrolizable analogs AMPPNP or GMPPNP at the same concentration. Incubation on ice was done as a negative control. Uso1 and Bet1 were detected by Western blotting and significant Fractions 5–7 are shown.

Ability of Uso1 to Bind to Multiple Membranes

Next, we examined whether Uso1 binds only to a single vesicle or piece of membrane or to multiple membranes. We prepared two independent reaction mixtures having different epitope-tagged membrane proteins; one had 6myc-Sed5 and the other had 3HA-Yif1, 3HA-Erv41, and 3HA-Sec71. Uso1-3HA-Streptag protein was used as Uso1. After 25°C incubation for 30 min and removal of rapidly sedimenting membranes, two preparations were independently subjected to centrifugation on the sucrose density blocks and fractionation. Then two Fraction 6s from different blocks were mixed, and kept on ice for 30 min. As the anti-myc immunoisolation of vesicles with 6myc-Sed5 was highly specific [Inadome et al., 2005], we collected 6myc-Sed5-tagged membranes from the mixture. The membrane proteins were eluted from the beads with 1% Triton X-100 and analyzed by Western blotting. The P10 membranes without 6myc-Sed5 were used in a negative control reaction to monitor the non-specific signals of immunoisolation. The addition of Uso1 had no effect on the non-specific binding of membrane proteins to Protein A-Sepharose (Fig. 5, Vector). If one Fraction 6 had 6myc-Sed5 (Fig. 5, 6myc-Sed5), significantly larger amounts of HA-tagged Yif1 and Erv41 were detected by the addition of Uso1 in comparison with the no addition control which had nearly the basal signals as in the Vector control. These HA-tagged proteins were present in the Fraction 6 membranes which were independently prepared from such membranes containing 6myc-Sed5. Absence of 3HA-Sec71 and Sft1 in the immunoisolates gave a support to that the binding of Yif1 and Erv41 in the presence of Uso1 was specific. These results indicated that the different membranes in two Fraction 6s were physically linked during the on ice incubation in the presence of Uso1. Therefore, Uso1 is a multivalent membrane-binding protein, which will be important for tethering of vesicles.

The Acidic C-Terminal Domain of Uso1 Is Dispensable

Uso1 forms a dimer with two N-terminal globular heads and a long rod-shape tail of coiled-coil α-helices (Fig. 6A). The temperature-sensitive *uso1-1* encodes a truncated protein which has only 20% of the tail. Uso1 also has a characteristic acidic stretch of aspartic and glutamic acids at its C-terminal end, which has been reported to play an important role in interaction with Golgi proteins in the case of mammalian ortholog TAP/p115 [Nakamura et al., 1997; Lesa et al., 2000; Linstedt et al., 2000]. We examined the role of these domains in the current membranes binding assay. As shown in Figure 6B, a little but detectable amount of the Uso1-1 mutant protein was in Fraction 6, which is consistent with that it can limitedly support cell growth. The C-terminal coiled-coil domain (Uso1C) was not detected in the Fraction 6. This indicates that the N-terminal globular domain is required in the membrane binding. The C-terminal acidic region could be removed without any defective phenotype in yeast, and it was completely dispensable for membrane binding and the cell growth.

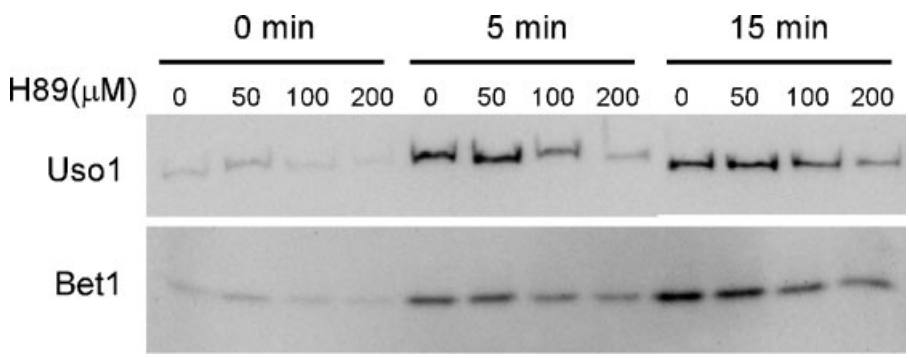

Fig. 4. Effect of the kinase inhibitor H89 on the production of the Uso1-binding membranes. H89 was added to the reaction at the indicated concentration and incubated at 25°C for 0, 5 or 15 min. Reaction mixes were separated by centrifugation as in Figure 1 and proteins in Fraction 6 were subjected to SDS–PAGE and Uso1-5myc and Bet1 were detected by immunoblotting.

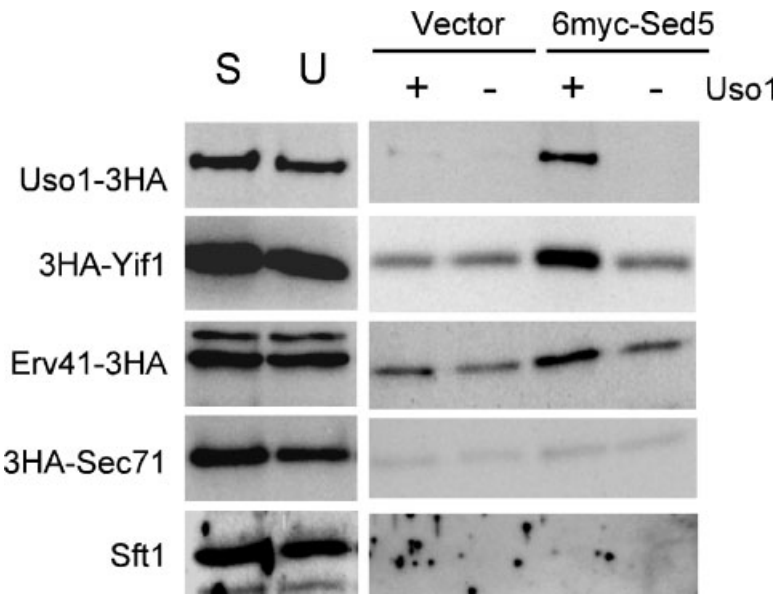

Fig. 5. Physical interaction of independently produced membranes in Fraction 6 in the presence of Uso1. Immunoisolation of 6myc-Sed5-containing membranes was done after mixing two independently prepared Fraction 6s on ice for 30 min; one Fraction 6 contained 6myc-Sed5 and the other Fraction 6 contained 3HA-Yif1, Erv41-3HA and 3HA-Sec71. Preparation of Fraction 6 was done with (+) or without (−) adding purified Uso1-3HA-Streptag. As a negative reference of immunoisolation, a yeast membrane prepared from vector control instead of 6myc-Sed5-producing plasmid was examined (Vector). A 1/30 portion of the total proteins in the mixture of Fraction 6s before (S, start) and after (U, unbound) the immunoisolation was also analyzed. Presence of Sft1 was examined for nonspecific membranes.

Presence of Uso1-Accessible Membranes in the Growing Yeast Cell

We did similar membrane binding experiments using the total membranes instead of P10. Uso1 was found in Fractions 6 and 7, whether incubation was performed at 25°C or on ice (Fig. 6). Therefore, the binding of Uso1 to membranes occurred even on ice if the total membranes were directly used. It was reproducibly observed that a larger amount of Uso1 was found in Fraction 6 than in Fraction 7, while larger amounts of Bet1, Sed5, and Scs2 were found in Fraction 7 than in Fraction 6. The different distribution of these membrane proteins and Uso1 are contrary to a possibility that the binding of Uso1 to membranes occurred nonspecifically. The membranes which bind to Uso1 and migrate in Fraction 6 are likely to present the ER-derived COPII vesicles in growing yeast cell, and the binding capacity remain active during the preparation of the total membrane fraction.

DISCUSSION

Cao et al. [1998] first reported that Uso1 tethers diffusible ER-derived COPII vesicles to the acceptor early Golgi membranes in the semi-intact cell in vitro system. Morsomme and Riezman [2002] showed that vesicles containing radiolabeled Gap1 were immunoprecipitated with vesicles containing unlabelled Gap1-HA by anti-HA antibody when they were mixed in the presence of Uso1. So, it is likely that Uso1 tethers both heterologous and homologous membrane vesicles. As described in the Results section, using the simple sucrose density block centrifugation, binding of Uso1 to specific membranes was reproducibly detected in Fraction 6 (Figs. 1 and 4). Immunological characterization showed that the Uso1-binding membranes in Fraction 6 were the COPII vesicles as described in the above reports [Cao et al., 1998; Morsomme and Riezman, 2002].

Our present data indicate that binding of Uso1 to membranes occurs on ice (Figs. 5 and 7), although generation of Uso1-binding membranes from P10 requires incubation at a biological temperature (Figs. 1B and 3), and is facilitated by ATP hydrolysis (Figs. 3 and 4). The Uso1-binding membranes were likely to be generated from the ER, because they were produced from P10 but not from P100. Ypt1 and Sed5 were found in the Uso1-binding

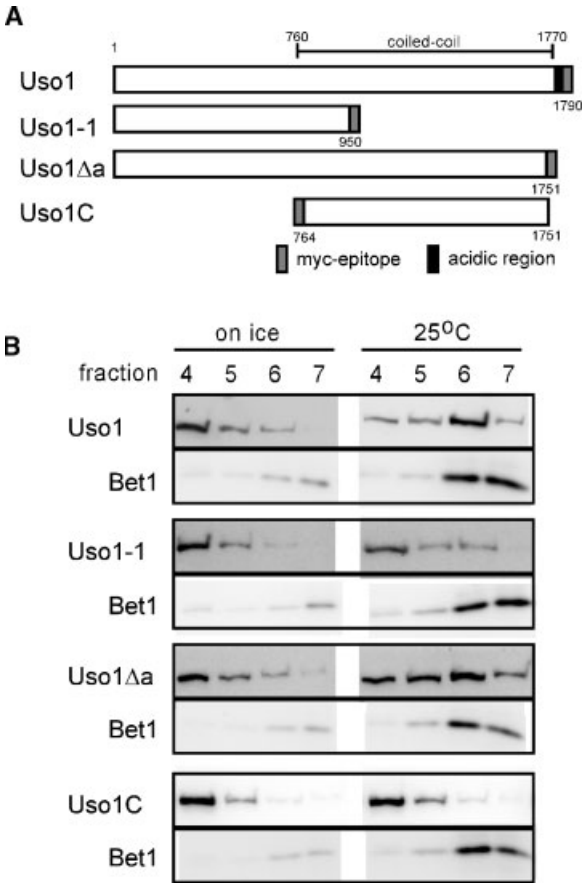

Fig. 6. Domains of Uso1 required for membrane binding. **A**: Structures of Uso1 and its derivatives examined; Uso1-1, a temperature-sensitive mutant protein without most of the tail; Uso1Δa, a truncated protein without the acidic stretch; and Uso1C, the coiled-coil α-helix region. **B**: Binding assays were performed as in Figure 1, using the yeast cytosol containing indicated Uso1 derivatives. After fractionation, Uso1 and the COPII vesicle marker Bet1 were detected by Western blotting. Incubation on ice was performed for negative reference. The significant Fractions 4–7 are shown.

membranes in Fraction 6 (Fig. 2). The presence of Ypt1 may suggest a part of membranes were derived from the early Golgi, but the localization of rab GTPases including Ypt1 are not strongly restricted to the place where they function, in contrast to their activator GEF proteins [Inadome et al., 2005]. Sed5 is an early Golgi tSNARE and has not been included in the reported constituents of COPII vesicles [Otte and Barlowe, 2002]. However, immunological analysis detected Sed5 in the ER-derived COPII vesicle as a specific cargo protein [Miller et al., 2003]. Considering all these points together, we concluded that the membranes in Fraction 6 to which Uso1 efficiently binds are the uncoated COPII vesicles. As Uso1 binds to multiple membranes (Fig. 5), it is likely to help the homotypic fusion of uncoated COPII vesicles.

It was shown that the total membranes of logarithmically growing yeast contained Uso1-binding membranes for the first time using the current assay method (Fig. 7). It is curious that these Uso1-binding membranes were lost by separating the P10 and P100 (Fig. 1B), while they were present among the total (P10 + P100) membranes (Fig. 7). Washing of P10 might have removed them from the samples and/or packing of the P100 pellets without other membranes might have damaged them. Generation of the membranes which settle to Fraction 6 is not dependent on Uso1 binding, because a similar amount of membranes containing the Bet1 protein was generated at 25°C and migrated to this fraction in the case of Uso1-1 protein which showed a reduced binding activity (Fig. 6).

Although the requirement and interaction with rab GTPase Ypt1 were shown [Barlowe, 1997], the membrane receptor for Uso1 remains unclear. Our approaches to determine the membrane proteins that specifically bind to Uso1 have not got reproducible results so far. It has not yet been discussed when and how the tethering factors leave the bound membranes. Absence of the Golgi Sft1 protein as well as the ER proteins suggests that the binding is specific for the membranes after budding and before fusion. We are not sure whether some membranes actually fused following the tethering in the current assay.

Uso1 has a cluster of acidic amino acids at the C terminus as does the mammalian ortholog

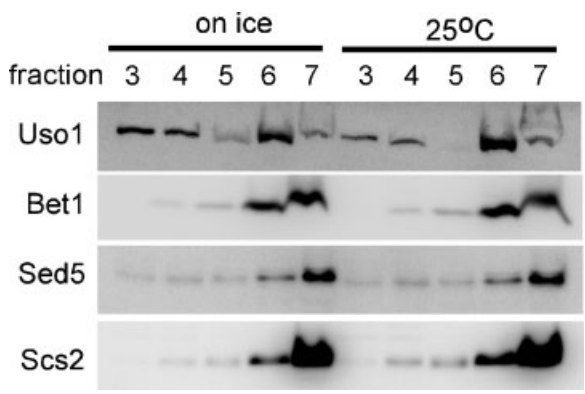

Fig. 7. Binding of Uso1 to membranes present in the total lysate of logarithmically growing cell. Experiments were done as in Figure 1, except that P10 membranes were replaced with unfractionated total membranes. The significant Fractions 3–7 are shown.

TAP/p115. This region was previously reported to function in the tethering of p115 to the Golgi via GM130 and giantin [Nakamura et al., 1997; Lesa et al., 2000; Linstedt et al., 2000], but recent studies have suggested it was not required for either localization or the functioning of the Golgi [Nelson et al., 1998; Puthenveedu and Linstedt, 2001, 2004]. It was not required for tethering of vesicles or growth characteristics of the yeast.

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