YOS9, the Putative Yeast Homolog of a Gene Amplified in Osteosarcomas, Is Involved in the Endoplasmic Reticulum (ER)-Golgi Transport of GPI-anchored Proteins*

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The OS-9 gene maps to a region (q13–15) of chromosome 12 that is highly amplified in human osteosarcomas and encodes a protein of unknown function. Here we have characterized a homolog designated as YOS9 (YDR057w) from Saccharomyces cerevisiae. The yeast protein (Yos9) is a membrane-associated glycoprotein that localizes to the endoplasmic reticulum (ER). YOS9 interacts genetically with genes involved in ER-Golgi transport, particularly SEC34, whose temperature-sensitive mutant is rescued by YOS9 overexpression. Interestingly, Yos9 appears to play a direct role in the transport of glycosylphosphatidylinositol (GPI)-anchored proteins to the Golgi apparatus. Yos9 binds directly to Gas1 and Mkc7 and accelerates Golgi transport and processing in cells overexpressing YOS9. Correspondingly, Gas1 processing is slowed in cells bearing a deletion in YOS9. No effect upon the transport and processing of non-GPI-anchored proteins (e.g. invertase, carboxypeptidase Y) was detected in cells either lacking or overexpressing Yos9. As Yos9 is not a component of the Emp24 complex, it may act as a novel escort factor for GPI-anchored proteins in ER-Golgi transport in yeast and possibly in mammals.

Membrane-bound compartments are in a state of dynamic flux in eukaryotic cells. Transport vesicles emerge from donor compartments and are targeted to specific acceptor compartments where they deliver cargo molecules through membrane fusion (1). Vesicular transport not only permits the trafficking of soluble and membranal components from one compartment to the other but also controls the protein and lipid composition of membranes as well as the size of cellular organelles. Complimentary biochemical and genetic studies in both yeast and mammalian cells have established the basic principles and molecular mechanisms that underlie protein sorting. Initially, proteins are translocated into the ER1 where they undergo folding and N-linked glycosylation. Proteins are then transported to the Golgi before reaching their final destination, such as the cell surface or the lysosome/vacuole. ER-Golgi protein trafficking in the yeast Saccharomyces cerevisiae has been studied intensely, and many of the components involved in the derivation and consumption of Golgi-targeted transport vesicles have been characterized. These include the Sar1 GTPase and COPII coat components that are recruited to the sites of vesicle formation at the ER and that are required for vesicle biogenesis (2). In addition, those components involved in the docking and fusion of ER-derived vesicles at the Golgi, i.e. the SNAREs (e.g. Bet1, Bos1, Sec22, and Sed5) as well as the Ypt1 GTPase and t-SNARE-associated protein, Sly1, have been studied (3–8). Additional factors operate upstream of Ypt1 and the SNAREs to mediate the tethering of COPII vesicles and include Uso1 (9), the Sec34/Sec35 complex (10, 11), and a large multisubunit particle, transport protein particle (TRAPP) (12).

Other proteins are implicated in the maintenance of the ER and Golgi as well as protein transport therein. These include soluble and membrane-bound resident ER proteins such as BiP/KAR2 (13–15), calreticulin and calnexin (16–18), and PDI (19, 20). These proteins function as chaperones in conferring quality control (17, 21). Despite their high concentration, luminal-resident proteins are not secreted and are hardly detectable in the Golgi. Two complementary systems, one that ensures retention in the ER and another that retrieves escapees from the Golgi, confer this highly selective steady-state distribution. ER-resident proteins are retrieved by discrete targeting motifs such as cytoplasmic KKXX and RR signals or luminal H/KDEL sequences (22). Most soluble ER-resident proteins are characterized by the H/KDEL sequence that prevents their secretion (23, 24).

The protein translated from open reading frame (ORF) YDR057w is the last HDEL-containing protein in S. cerevisiae that has not been characterized. ORFs encoding proteins of similar structure are found in the genomes of Schizosaccharomyces pombe, Caenorhabditis elegans, and Homo sapiens, etc. (25). The probable human homolog, hOS-9, is present in all tissues and is overexpressed in osteosarcomas, although no function has been assigned to the protein thus far. Here, we characterize the yeast homolog of the OS-9 family, designated Yos9. We show that Yos9 is an ER-resident protein that is membrane associated and facilitates delivery of the glycosylphosphatidylinositol (GPI)-anchored proteins to the Golgi. Yos9 interacts directly with the Gas1 and Mkc7 GPI-anchored proteins, and the processing of Gas1 is significantly altered in cells either lacking or overexpressing the YOS9 gene. In contrast, processing and secretion of non-GPI-anchored proteins is unaffected. Thus, the yeast OS-9 homolog plays an important role in the maturation and exit from the ER of GPI-anchored proteins.

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This work is dedicated to the memory of Prof. Shmuel Shaltiel who passed away earlier this year.

¶ Recipient of the Henry Kaplan Chair in Cancer Research.

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1 The abbreviations used are: ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; SNARE, soluble NSF attachment protein receptors; NSF, N-ethylmaleimide-sensitive factor; ORF, open reading frame; HA, hemagglutinin; GFF, green fluorescent protein; IP, immunoprecipitation; co-IP, co-immunoprecipitation; endoH, endoglycosidase-H; CPY, carboxypeptidase-Y; WT, wild type; UPR, unfolded protein response; SL, sphingolipid; TCL, total cell lysate; abs, antibodies; PDI, protein disulfide isomerase.

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EXPERIMENTAL PROCEDURES

Media and Genetic Manipulation—Yeast were grown in standard media containing 2% glucose as a carbon source (26). DNA introduction into yeast was performed using standard procedures (26). Homologous recombination was used to disrupt the YOS9 gene. Strains were transformed with linearized pYOS9U (cut with EcoRI to release a 4085-bp fragment). After selection on plates lacking uracil, DNA derived from the subsequent colonies was subjected to Southern analysis to verify the disruption of YOS9. Yeast strains are listed in Table I.

Plasmids—Constructs for YOS9 overexpression: A DNA fragment encoding HA-tagged Yos9 was created by polymerase chain reaction (PCR) using genomic DNA. Primers included: YOS9HA (5'-CATCCCTTTCCTCCCTGGACAGATCGGC-3'), which introduces a PstI site at the 5' end of YOS9, and YOS9AR (5'-CTCCTGAGCTCTTAAAGC-3'), which introduces the HA tag directly upstream of the encoded HDEL sequence and a SacI site 9' to the subsequent stop codon. A multi-copy plasmid, pADH-YOS9HA, that bears the LEU2 selectable marker, was created by subcloning a PCR fragment containing a full-length genomic YOS9 (with flanking regions) was amplified from genomic DNA using (5'-GATCCTCGAGGTAGTAAAGGAGAAGAACTTTT-3') and GPFXHO1R (5'-GATCCTCGAGCTGCTACGTTTGTTACCATGCAATGCTGCAATGCC-3') oligonucleotides. This fragment was inserted into the XhoI site in pADH-YOS9HAX to yield plasmid pADH-YOS9HAGFP.

A multicopy plasmid bearing a URA3 marker and expressing HA-tagged Mek7, pHKM71HA, was kindly provided by R. Fuller (University of Michigan, Ann Arbor). An integrating plasmid expressing Sec7-RFP, Ylplac204-7C-SEC7-DSRED.T4 was kindly provided by B. Glick (University of Chicago). After linearization with Bsu36I, this construct was integrated into the trpl locus of SP1 wild-type yeast to yield SP1-SEC7RFP yeast.

Phenotypic Assays—Yeast strains either overexpressing YOS9 or bearing a disruption in the YOS9 gene were assayed for viability at different temperatures. Cells were patched onto plates for growth at room temperature prior to replica plating to the experimental conditions.

To measure Kar2 secretion on filters, cells were first grown as patches on plates, then replicated onto nitrocellulose filters (Schleicher and Schuell, BA-S85) that were placed yeast-side-up on fresh plates and grown for 36–48 h. Next, the filters were washed three times with phosphate-buffered saline and blocked in phosphate-buffered saline containing 0.1% Tween 20 and 3% non-fat dry milk. Filters were then probed with anti-Kar2 antibodies (1:20000 dilution) (a gift of C. Barlowe, Dartmouth University) and detected by chemiluminescence using secondary antibody and anti-rabbit secondary antibodies (1:10000 dilution) (Amersham Biosciences).

Immunoprecipitation—Yeast were grown to log phase and processed for immunoprecipitation (IP) and co-immunoprecipitation (co-IP) experiments, as described (27), with the exception that 0.5% Nonidet P-40 was substituted for Triton X-100 in the co-IP buffer. For pulse-chase experiments, detergent solubilized cell extracts were incubated with the appropriate antibodies, including anti-Gas1 (gift of H. Riezman, Biozentrum); anti-CPY (gift of Scott Emr, University of California, San Diego) and anti-sec14 antibodies. For pulse-chase experiments, detergent solubilized cell extracts were incubated with the appropriate antibodies, including anti-Gas1 (gift of H. Riezman, Biozentrum); anti-CPY (gift of Scott Emr, University of California, San Diego) and anti-sec14 antibodies. For pulse-chase experiments, detergent solubilized cell extracts were incubated with the appropriate antibodies, including anti-Gas1 (gift of H. Riezman, Biozentrum); anti-CPY (gift of Scott Emr, University of California, San Diego) and anti-sec14 antibodies. For pulse-chase experiments, detergent solubilized cell extracts were incubated with the appropriate antibodies, including anti-Gas1 (gift of H. Riezman, Biozentrum); anti-CPY (gift of Scott Emr, University of California, San Diego) and anti-sec14 antibodies.
from the 10,000 × g pellet were resuspended in 200 μl of lysis buffer (30) and incubated with one of the following reagents: 0.5 mM potassium acetate, pH 5.5; 0.1 M sodium carbonate, pH 11.5; 0.5% Triton X-100; 1 mM NaCl; 0.25 μg glycine, pH 2.5, or buffer alone (control). Samples were incubated on ice for 1 h and then centrifuged at 100,000 × g for 1 h. The supernatants were precipitated with 5% trichloroacetic acid, washed with acetone, suspended in sample buffer, and resolved on 10% SDS-PAGE gels for immunoblot analysis.

Extraction with precondensed Triton X-114 was carried out as described (30). Protein samples (0.5 mg) were added to a solution containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1% Triton X-114 in a final volume of 200 μl. For better phase separation, a 6% (w/v) cushion of sucrose was used. Proteins from the detergent and aqueous phases were concentrated by precipitation with 5% trichloroacetic acid for 30 min (4 °C) before being washed, resuspended in sample buffer, and electrophoresed on SDS-PAGE gels.

Glycoprotein Analysis—Endoglycosidase Treatment: Deglycosylation of yeast extracts was performed using endoglycosidase H (endoH) according to the supplier’s instructions (New England Biolabs). Briefly, 100-μg protein aliquots from cell lysates were incubated with endoH (1,000 units) for 2, 4, and 16 h. Samples were resolved on SDS-PAGE gels and probed in blots using anti-HA or anti-CFP antibodies.

Concanavalin-A (Con-A) chromatography: Total cell lysates prepared using the glass bead method were diluted 10-fold with the column buffer (10 mM Tris HCl, pH 7.5; 0.25 M NaCl; 1 mM CaCl2; 1 mM MnCl2; 0.25 M glycine, pH 2.5, or buffer alone (control). Samples were applied to Con-A-Sepharose 4S beads (Sigma). Cleared lysates were incubated for 3 h at 4 °C with beads that had been prewashed with buffer. Next, the beads were washed extensively, the bound proteins eluted with sample buffer and resolved on 10% SDS-PAGE gels, and detected in immunoblots.

Immunofluorescence Studies—Yeast were fixed and permeabilized for immunofluorescence as described (30). Yos9 was detected using affinity-purified anti-HA monoclonal antibodies and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibodies (Jackson Laboratories). Yos9, Yos9HA, Sso1, and Sso2 conjugated goat anti-mouse antibodies (Jackson Laboratories) for immunofluorescence as described (30). Yos9HA was found to be present in the pre-

RESULTS

OS-9 Is Conserved in Evolution—Comparison of the amino acid sequences translated from full-length human and rat OS-9 cDNAs with the sequences of possible C. elegans (F48E8.4) and S. cerevisiae (YDR057w) homologs (Fig. 1) reveals that OS-9 is highly conserved. This is especially true for the NH2 terminus of the proteins (residues 1–250), which shares 96% identity between rats and humans and 20% identity between mammalian OS-9 and that of ORF YDR057w (12% identity overall with mammalian OS-9). Moreover, six of eight cysteine residues found in the mammalian forms are positionally conserved with yeast OS-9 (Yos9). Thus, Yos9 is a probable OS-9 homolog and can be studied in order to elucidate the function of this conserved family of proteins. Interestingly, we noted that Yos9 contains a HDEL ER retention signal present at the COOH terminus of the protein, suggesting that it is a luminal ER resident protein and may be involved in the transport of proteins through the ER.

Disruption of YOS9 in Yeast—To determine the function of Yos9 in yeast, we checked the effects of a null mutation in YOS9 by disrupting the gene using homologous recombination. A YOS9 disruptant gene cassette containing the URA3 selectable marker was transformed into wild-type cells, and the disruption was verified by Southern analysis. yos9Δ strains were found to grow normally and showed no obvious phenotype. These cells had a normal morphology and were able to grow under different conditions of pH, salt concentration, and temperature (i.e., 15°, 26°, 30°, 35°, and 37°C) as well as the parental SP1 strain, and the cells were not defective for mating (data not shown). Thus, the YOS9 gene does not provide any essential function that can be identified phenotypically upon its disruption.

Yos9 Is a Membrane-associated, ER-localized, Glycoprotein—A HA epitope-tagged form of Yos9 (Yos9HA) was expressed in order to monitor its localization in yeast. The calculated molecular mass of native Yos9 is 61 kDa, while addition of the HA tag should increase the mass to 63 kDa. However, when identified on Western blots using anti-HA antibodies, we found that Yos9HA runs on SDS-PAGE gels at a mass corresponding to 75 kDa (data not shown, and Figs. 2B and 4). Yos9 is predicted to be a luminal ER protein because of its ER retention signal. To help determine its localization in cells, fractionation techniques were used to prepare extracts from yeast expressing Yos9HA. The 10,000 × g soluble (S10) and pellet (P10) fractions as well as the 100,000 × g soluble (S100) and pellet (P100) fractions were separated by centrifugation and then probed for Yos9 using anti-HA antibodies (Fig. 2A). Western blot analysis revealed that Yos9HA is found mostly in the P10 membrane fraction, with some in the P100. This distribution was basically identical to that seen for bona fide membrane proteins such as Sso1 and 2, which are integral membrane proteins functioning as t-SNAREs at the cell surface (32). In contrast, a soluble yeast vacuolar hydrolase, carboxypeptidase Y (CPY) (33), was found in the supernatant fractions only. Thus, Yos9 is associated with the total membrane fraction and not with the cytosol (Fig. 2A).

As YOS9 does not appear to encode a membrane protein, aliquots of the P10 membrane fraction were subjected to various treatments to reveal the nature of the interaction between Yos9 and the membrane. High pH extraction with 0.2 M Na2CO3 (pH 11) and low pH extraction with 0.2 M glycine (pH 2.4) were performed in order to detach proteins associated with membranes, but not integral membrane proteins. Yos9HA was found to be split evenly between the soluble and pellet fractions in both cases, whereas Sso proteins were associated with the pellet fractions, and CPY remained soluble (Fig. 2A). However, upon extraction with high salt (0.5 M NaCl), Yos9HA remained associated with the pellet like the Sso proteins. Thus, although the amino acid sequence of Yos9 does not predict a hydrophobic region (aside perhaps from the NH2-terminal signal peptide), the data suggests that it is tightly associated with membranes. To determine whether Yos9 is an integral membrane protein, we examined its distribution between lipid and aqueous phases using extraction with Triton X-114. Upon such treatment, transmembrane or lipid-anchored proteins separate to the Triton X-114 phase, while membrane-associated and soluble proteins are found in aqueous phase. As seen in Fig. 2A, Yos9HA is found in the aqueous fraction upon phase separation, unlike Sso. Thus, Yos9 is not an integral membrane protein but is tightly membrane associated.

Analysis of the Yos9 protein sequence predicts potential sites for phosphorylation and glycosylation. To check whether Yos9 undergoes phosphorylation, an in vivo metabolic labeling assay was performed with [32P]orthophosphate. After IP with anti-HA antibodies, Yos9HA was found to be present in the precipitates, but no signal was detected by autoradiography (data not shown). Thus, Yos9 is not phosphorylated in vivo under these conditions.

As stated above, Yos9HA runs at a higher molecular mass than expected, possibly because of glycosylation. To determine whether Yos9 is a glycoprotein, we examined whether Yos9 is sensitive to treatment with endoglycosidase H (endoH) or binds to concanavalin A (con A)-conjugated beads. Treatment of cell lysates with endoH resulted in a clear increase in the mobility
of Yos9HA on SDS-PAGE gels (Fig. 2B), the change in molecular mass being on the order of 10 kDa. This treatment also resulted in a change in mobility of CPY, a highly glycosylated protein, which served as a control. Similarly, we found that Yos9HA bound tightly to con A beads, as did CPY (Fig. 2C). However, in contrast Sso could not bind to the resin and eluted in the wash phase. Thus, Yos9 is a glycoprotein.

Although Yos9 bears a COOH-terminal HDEL sequence and pellets in the P10 fraction, we wished to verify its localization to the ER. To do so, we expressed a Yos9-GFP fusion protein in yeast and examined its localization using confocal microscopy (Fig. 2D). We found the GFP fluorescence to label the intracellular aspect of the cell surface and often ring the nucleus, which is typical for ER-localized proteins such as Kar2 (34). Some punctate labeling was also observed at the cell surface and may indicate possible ER exit sites. In contrast, there was no significant co-localization with the Sec7 Golgi marker when expressed as a fusion with red fluorescent protein (RFP) (35). Similar results were also obtained by immunofluorescence, using affinity-purified antibodies to detect Yos9HA and the Golgi-resident protein, MnnI, which were visualized via the fluorescein isothiocyanate (FITC) and rhodamine channels, respectively (data not shown). Thus, Yos9 appears to be a membrane-associated ER-localized glycoprotein.

**FIG. 1.** A comparison of OS-9 and related sequences. The amino acid sequence of human OS-9 (hOS9) was compared with rat OS-9 (rOS9) and to homologous sequences from Caenorhabditis elegans (C. elegans) and Saccharomyces cerevisiae (S. cerevisiae). Identical residues are shaded in black, similar residues in gray. Asterisks indicate conserved cysteine residues.
and Sec7RFP were grown to log phase and visualized by confocal microscopy. A
expressing Yos9HAGFP were grown to log phase and visualized by confocal microscopy.
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processing of the GPI-anchored Protein Gas1 Is Slowed in
yos9 Cells and Accelerated in Cells Overexpressing YOS9—
One way to determine the role of a given protein in intracellular
trafficking is to examine its effect upon the processing of
other proteins as they are transported along the pathway. For
example, immediately after synthesis CPY is glycosylated in
the ER to yield an initial form called p1 CPY. After transport to
the Golgi it is further glycosylated to yield p2 CPY. Finally,
after reaching the vacuole the protein is cleaved, resulting in
the mature lower molecular weight form, mCPY (33). Another
example is Gas1, a GPI-anchored plasma membrane protein
that becomes highly glycosylated as it transits the ER-Golgi,
yielding a protein with a higher molecular weight (mature
Gas1). Invertase, a soluble secreted enzyme, also undergoes a
similar pattern of maturation.

The processing of these proteins was followed in wild-type
(WT) cells, yos9Δ cells, and WT cells overexpressing Yos9HA by
pulse-chase analysis. We found that neither the overexpression
nor deletion of YOS9 had any effect upon the processing rate
and extent of CPY and invertase (data not shown). In contrast,
Gas1 processing was significantly affected in both the yos9Δ
knockout strain as well as when Yos9 was overexpressed (Fig.
3B). We found that Gas1 processing was much faster in yeast
overexpressing YOS9. After 10 min of chase, all of Gas1 was
found in its mature form in the overexpressing cells, while
immature Gas1 was still visible for up to 30 min in both WT
and yos9Δ cells. Correspondingly, a significant fraction (~35%) of
immature Gas1 was present in yos9Δ cells for up to 45 min
after the start of the chase. These results indicate that Yos9
exerts a specific effect upon the intracellular trafficking of a
GPI-anchored protein from the ER to Golgi.

Yos9 Binds to GPI-anchored Proteins—Since overexpression
of YOS9 increases the rate of Gas1 trafficking from the ER to
the Golgi, while its deletion has an opposite effect, we exam-
ined whether Yos9 interacts physically with Gas1. Such an
interaction could suggest that Yos9 is a potential chaperone or
escort protein for the trafficking of certain proteins within the ER.

To examine this possibility, we immunoprecipitated Yos9HA
from cell lysates using anti-HA antibodies and probed for the
presence of Gas1. We found that Gas1 readily co-immunopre-
cipitated with Yos9HA, suggesting that these proteins physical-
ly interact (Fig. 4A). Gas1 that co-immunoprecipitated with
Yos9HA is likely to be in its immature form because of the
smaller mobility shift seen upon endoH treatment versus that
of mature Gas1 from the total cell lysate (data not shown).

To determine whether Yos9 interacts with other GPI-
proteins, we immunoprecipitated Yos9HA, followed by
Western blotting. Blotted samples of the TCL (Input)
and eluted proteins (Eluate) from either control cells or cells expressing Yos9HA (HAOS9) were probed with
abs against the HA epitope, CPY, and Sso proteins. D, Yos9 localizes to the ER by confocal microscopy. Left panel, SP1 wild-type yeast
expressing Yos9HAGFP were grown to log phase and visualized by confocal microscopy. Right panel, SP1-SEC7RFP yeast expressing Yos9HAGFP
and Sec7RFP were grown to log phase and visualized by confocal microscopy.
anchored proteins, we examined whether it could bind a HA-tagged form of Mxc7 (39). To do this we co-expressed a myc-tagged form of Yos9 in the same cells and performed immunoprecipitation. As can be seen (Fig. 4B), Yos9myc also interacts with HA-Mxc7. Thus, Yos9 binds GPI-anchored proteins non-specifically and appears to regulate their transport between the ER and Golgi.

**TABLE II**

| Cell growth at restrictive and semi-restrictive temperatures |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Vector          | YOS9            |                 |                 |                 |
| sec12           | +              | +              | +              | -              | -              |
| sec13           | +              | +              | +              | -              | -              |
| sec14           | +              | +              | +              | -              | -              |
| sec23           | +              | +              | +              | -              | -              |
| sec18           | +              | +              | +              | -              | -              |
| sec22           | +              | +              | +              | -              | -              |
| bss1-1          | +              | +              | +              | -              | -              |
| sde5-1          | +              | +              | +              | -              | -              |
| uso1-1          | +              | +              | +              | -              | -              |
| sec34-1         | +              | +              | +              | -              | -              |
| sec34-2         | +              | +              | +              | -              | -              |
| sec35-1         | +              | +              | +              | +              | +              |
| bet1-1          | +              | +              | +              | -              | -              |
| ypt1-1          | +              | +              | +              | -              | -              |
| sec6-1          | +              | +              | +              | -              | -              |
| sec9-4          | +              | +              | +              | -              | -              |
| sec21           | +              | +              | +              | -              | -              |
| WT              | +              | +              | +              | -              | -              |

Deletion of Emp24 family members in yeast does not have significant effects upon cell growth, but results in the secretion of the Kar2 chaperone as a part of a general unfolded protein response (UPR) (43). To determine whether the deletion of YOS9 results in a UPR we grew yos9Δ and control cells on filters and examined them for the secretion of Kar2 using anti-Kar2 antibodies. We found that Kar2 was highly secreted from emp24Δ control cells but not from yos9Δ or wild-type cells (Fig. 5C). Thus, YOS9 deletion does not result in a UPR.

Finally, we looked at genetic interactions between YOS9 and EMP24. However, we found that neither the overexpression nor deletion of YOS9 could restore Kar2 retention in emp24Δ cells (data not shown). Moreover, no additive growth defects were observed in emp24Δ yos9Δ double mutants.

**Yos9 Is Probably Not Involved in Ceramide and Sphingolipid Synthesis**—Sphingolipid (SL) synthesis has been shown to be important for the trafficking of GPI-anchored proteins (44). Therefore, alterations in ceramide and subsequent SL production could account for the changes in Gas1 trafficking observed in cells lacking or overexpressing YOS9 (Fig. 3B). To determine whether Yos9 could play a role in SL production, we examined SL synthesis by metabolic labeling with [3H]-myo-inositol. After organic phase extraction, SLs were separated by thin-layer chromatography and quantified. We found that cells either lacking or overexpressing YOS9 had no observable changes in the levels of the three known yeast SLs (e.g. inositol-phosphoryl-ceramide (IPC); mannose IPC, and mannose (inositol-phosphoryl)2-ceramide) and were similar in this regard to wild-type cells (data not shown). In contrast, control cells bearing mutations in either the ELO3/VBM1 or ELO2/VBM2 genes showed large changes in the levels of the SLs produced, as expected (29). Since SL levels were not affected by YOS9 deletion or overexpression, it is unlikely that Yos9 is involved in ceramide or sphingolipid production.
Yeast OS-9 in ER-Golgi Transport

**DISCUSSION**

We have characterized a novel yeast ORF (YDR057w; YOS9) and shown that it encodes a membrane-associated ER-resident glycoprotein. Experiments performed with YOS9 reveal genetic interactions with early sec mutants such as sec34, which was originally identified in a novel screen for secretion mutants in the secretory pathway (36, 37). Known genetic interactions between SEC34 and genes (i.e. SEC35) encoding proteins that may be involved in the tethering of ER-derived vesicles to the Golgi (11) suggest that Yos9 could be involved in this process. However, as Yos9 is likely to be a luminal ER protein, whereas Sec34/35 are not, it is unclear how Yos9 could have tethering functions. An alternative and more attractive scenario is that Sec34 has functions that have not been fully described. Indeed, a recent work indicates that Sec34 binds to the Sed5 t-SNARE and mediates intra-Golgi retrograde transport (38). Interestingly, yet another recent study has shown that Sec34/35 play a direct role in the exit of GPI-anchored proteins from the ER and their targeting to the Golgi (45). If so, this finding could also implicate Yos9 in the sorting and, perhaps, exit of GPI-anchored proteins from the ER, a role consistent with many of the results described herein.

Our results show that the trafficking of Gas1 (but not of CPY or invertase) is significantly affected by either the overexpression or deletion of YOS9 (Fig. 3B). These data suggest, therefore, a possible role for YOS9 as an escort factor in the trafficking of GPI-anchored proteins. Whereas Gas1 and Mkc7 interact physically with Yos9 (Fig. 4), this is only the second example of an ER-resident protein that binds to GPI-anchored cargo. The first was Emp24, a p24 family member that is required for the packaging of Gas1 into ER-derived vesicles (41). Emp24 is a type I membrane protein that can be cross-linked to protein complexes that contain Gas1 (41). This suggests that the Emp24 complex (which also contains Erv25, Erp1, and Erp2) may act as a cargo receptor for Gas1 (41, 42) and perhaps other GPI-anchored proteins. The deletion of genes encoding the Erp1 and Erp2 p24 family members also causes defects in the transport of Gas1 (42). Based upon these strong similarities, we expected that Yos9 might be associated with the heteromeric Emp24 complex. However, we found that Yos9 could not co-IP with either Emp24 or Erv25 (Fig. 5, A and B). In addition, the disruption of YOS9 did not result in a UPR (Fig. 5C) and did not alter the processing of invertase, which is typically seen in cells lacking components of the Emp24 complex (43). Moreover, neither the deletion nor overexpression of YOS9 affected sphingolipid metabolism, which is known to be important for the trafficking of GPI-anchored proteins (44). Thus, Yos9 is likely to be a novel factor involved in the specific trafficking of GPI-anchored proteins. Since Yos9 bears a number of conserved cysteine residues (Fig. 1), it could be directly involved in protein folding like PDI or perhaps in the modulation of GPI-anchor attachment. More work is necessary to explore these connections.

The function of mammalian OS-9 proteins is unclear, though recent work shows they interact with N-copine, a membrane-associated protein that associates with secretory vesicles in a calcium-dependent manner (46). In addition, they interact with the cytoplasmic domain of a secreted kinase, meprin.2 Thus,
the cytoplasmic mammalian OS-9 proteins might confer some type of trafficking function in mammalian cells.

Although the disruption of YOS9 has only minor effects upon protein transport in yeast, its conservation in evolution suggests that it fulfills an important function. Interestingly, deletions in genes that encode the conserved p24 family members, which function as cargo receptors, also do not lead to growth defects in yeast (47). Thus, it is not entirely surprising that the deletion of YOS9 is without major effect. Although we do not yet know the precise role of Yos9, our work suggests that this ER-resident glycoprotein has a lectin-like function that is required for the transport of GPI-anchored proteins. As Yos9 is unique in yeast, its continued study will be useful for understanding the role of OS-9 family members in protein trafficking. In addition, this work may shed light on the importance of OS-9 amplification in the onset and growth of osteosarcomas.

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