Membrane Topology of the Yeast Endoplasmic Reticulum-localized Ubiquitin Ligase Doa10 and Comparison with Its Human Ortholog TEB4 (MARCH-VI)*

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Quality control machinery in the endoplasmic reticulum (ER) helps ensure that only properly folded and assembled proteins accumulate in the ER or continue along the secretory pathway. Aberrant proteins are retrotranslocated to the cytosol and degraded by the proteasome, a process called ER-associated degradation. Doa10, a transmembrane protein of the ER/nuclear envelope, is one of the primary ubiquitin ligases (E3s) participating in ER-associated degradation in *Saccharomyces cerevisiae*. Here we report the membrane organization of the 1319-residue Doa10 polypeptide. The topology was determined by fusing a dual-topology reporter after 16 different Doa10 fragments. Our results indicate that Doa10 contains 14 transmembrane helices (TMs). Based on protease digestion of yeast microsomes, both the N-terminal RING-CH domain and the C terminus face the cytosol. Notably, the experimentally derived topology was not predicted correctly by any of the generally available TM prediction algorithms. Bioinformatic analysis and *in silico* mutagenesis guided the topological studies through problematic regions. The conserved TD domain in Doa10 includes three TMs. These TMs might function in cofactor binding or substrate recognition, or they might be part of a retrotranslocation channel. The Derlins were previously proposed to provide such channels, but we show that the two yeast Derlins are not required for degradation of Doa10 membrane substrates, as was found before for the Sec61 translocon. Finally, we provide evidence that the likely human Doa10 ortholog, TEB4 (MARCH-VI), adopts a topology similar to that of Doa10.

In eukaryotes, most proteins that must traverse or insert into a lipid bilayer are first translocated from the cytoplasm into or through the ER membrane, after which they are sorted to various cellular compartments. A resident quality control machinery surveys the ER and eliminates misfolded, unassembled, or aberrantly modified proteins by the ERAD system (for review, see Refs. 1–3). In addition to its role in quality control, ERAD is also employed in the physiologically regulated degradation of normal ER proteins, such as the rate-limiting enzyme for sterol biosynthesis, hydroxymethylglutaryl-coenzyme A reductase (4). Activity of the ERAD system can sometimes also have deleterious effects, as in cystic fibrosis in humans. The most common mutation causing this disease is a single amino acid deletion (ΔF508) in the cystic fibrosis conductance regulator protein, which triggers the rapid proteasome-dependent degradation of the protein at the ER (5).

ERAD substrates are retrotranslocated from the ER lumen or membrane to the cytosol before or concomitant with degradation by the 26 S proteasome (6, 7). Ubiquitination of the substrate is generally required for both efficient retrotranslocation and degradation by the proteasome (3). Ubiquitination involves the conjugation of ubiquitin polymers to a substrate protein (8), which is accomplished through a linked series of enzymatic reactions catalyzed by three classes of enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s), and ubiquitin-protein ligases (E3s). E3s facilitate transfer of ubiquitin from an E2 to a substrate protein, and they are the main determinants of substrate binding and specificity. Within the ERAD pathway, ubiquitination of substrates appears to take place at the cytosolic face of the ER membrane during or prior to retrotranslocation (3).

To date, two ER-localized E3 ligases have been identified in the yeast *Saccharomyces cerevisiae*, Hrd1/Der3 (9, 10) and Doa10 (11). Both are multispanning membrane proteins, and both are members of the RING family of E3s. Ubc6 and Ubc7, the E2s that function with these E3s, are also either embedded in or bound to the ER membrane. Interestingly, Doa10 was initially found as an E3 that functions in the degradation of the soluble Mata2 transcriptional repressor (11). The identification of ER-localized transmembrane substrates of Doa10, such as Ubc6 (11), a mutant form of the ATP-binding cassette transporter Ste6 (12, 13), and a mutant Pma1 plasma membrane H⁺-ATPase (14), directly linked Doa10 to the ERAD pathway. Loss of Doa10 causes a mild induction of the ER unfolded protein response, an effect greatly exacerbated by simultaneous deletion of Hrd1. The *doa10Δ hrd1Δ* double mutant is also extremely sensitive to the heavy metal cadmium, a common defect in ERAD mutants (11). These results indicate that Doa10 is a central component of the yeast ER stress response and ERAD pathways.

Genes encoding presumptive Doa10 orthologs are found in all fully sequenced eukaryotic genomes. These orthologs include the human protein TEB4 (also called MARCH-VI or KIAA0597) (11, 15, 16). All contain a variant zinc-coordinating RING motif at their N termini called a RING-CH domain (11). The well conserved RING-CH domain defines a subfamily of RING-type E3s which encompasses a number of viral proteins and at least eleven different human proteins. Nine of the human proteins were noted previously and called membrane-anchored RING-CH or MARCH proteins (15). Our more recent searches identified two additional human RING-CH proteins, one that is also predicted to be a transmembrane protein (GenBank™ XP_496738) and one that is not (GenBank™ NP_689811). In addition to the more widespread RING-CH motif, all of the presumptive Doa10 orthologs are predicted to have at least 10 transmembrane segments.
(TM)s, and all contain a conserved ~130-residue element dubbed the TD (TEB4-Doa10) domain (11).

Based on cell fractionation studies, Doa10 behaves as an integral membrane protein, but its membrane topology was not examined previously (11). Hydropathy plot analysis and TM prediction algorithms suggest some 12–16 TM s. As a first step in the structure-function analysis of Doa10, we have determined its detailed membrane topology. Our results indicate that the Doa10 ubiquitin ligase contains 14 transmembrane segments with both termini facing the cytoplasm. Interestingly, the actual topology of Doa10 was not predicted correctly by any of the standard computer algorithms currently available. Guided by sequence alignment with the closely related Saccharomyces paradoxus Doa10 ortholog, we tested in silico mutants of the S. cerevisiae protein for changes in predicted topology. Combining TM prediction algorithms with this type of bioinformatic analysis should prove generally useful for the topological dissection of polytopic membrane proteins. Finally, topology mapping with a limited set of TEB4-topological reporter fusions expressed in yeast suggested that the human ortholog of Doa10, TEB4, adopts a topology that is similar or identical to that of Doa10, contrary to a recent report. The conservation of membrane topology across such a large evolutionary distance suggests that Doa10 orthologs in many if not all eukaryotes will share important roles in the ER stress and ERAD pathways.

EXPERIMENTAL PROCEDURES

Yeast and Bacterial Methods—Yeast rich (YPD) and minimal (SD) plates were prepared as described previously, and standard methods were used for genetic manipulation of yeast (18). Standard techniques were used for recombinant DNA work in Escherichia coli.

Construction of Dual-topology Reporter (DTR) Fusion Plasmids—The DTR consists of a triplicated HA epitope tag, a Suc2 moiety, and a His4C protein fragment. The DTR was fused after different N-terminal fragments of either Doa10 or TEB4 by homologous recombination between their corresponding DNA sequences (19). In brief, the 2μ/URA3 plasmid pK90, which carries a gene encoding an OST4-DTR fusion protein under control of the triosephosphate isomerase promoter, was linearized at its unique Smal site just upstream of the OST4-DTR open reading frame (19). Sequences for the different N-terminal Doa10 or TEB4 fragments were PCR-amplified from genomic yeast DNA (strain MHY500 (20)) for DOA10 or a plasmid bearing a full-length TEB4 (KIA00597) cDNA, respectively. Amplification was done with Ex-Taq DNA polymerase (Takara) using a forward primer that included a 5′ 35-nucleotide segment matching the end of the triosephosphate isomerase promoter and a reverse primer that included a 5′ segment matching the beginning of the 3′ HA tag coding sequence (19). The linearized pK90 plasmid was cotransformed with individual PCR fragments into the yeast strain SY50 (21), and uracil prototrophs were selected. By homologous recombination, the OST4 sequence was replaced by the corresponding Doa10 or TEB4 sequences, and a stable circular plasmid was reconstituted. Lysates derived from single colonies were checked by anti-HA immunoblot analysis to verify expression of a DTR fusion protein of the expected size. At least two independent clones of each fusion construct were tested by growth and glycosylation assays (see below). Primer sequences are available upon request.

Growth Assay on Histidinol—Yeast (SY50) cells expressing different Doa10-DTR and TEB4-DTR derivatives were streaked or patched onto minimal media lacking uracil (SD-Ura) or lacking histidine but containing 6 mM histidinol (SD-His+histidinol). Plates were incubated at 30 °C for 2 days and 4 days, respectively.

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Endoglycosidase H (Endo H) Treatment of Yeast Lysates—Endo H treatments were done essentially as described previously (19). In brief, STY50 cells containing a plasmid encoding a particular Doa10-DTR or TEB4-DTR fusion protein were grown in SD—Ura medium to an A600 of 0.8–1.0. 10 A600 equivalents were collected and washed in water, and cell pellets were placed at −20 °C for 1 h. Cells were resuspended in 200 μl of SDS sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.0025% bromphenol blue, 0.5 mM EDTA, and protease inhibitors (2 μg/ml aprotinin, 10 μg/ml aprotinin, 10 μg/ml chymostatin, 10 μg/ml leupeptin, 20 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride)), incubated for 15 min at 60 °C, and centrifuged for 15 min at 13,200 rpm in an Eppendorf microfuge. The supernatant was used for Endo H treatment. Lysates (200 μl) were adjusted to 80 mM potassium acetate, pH 5.6, and subsequently split into two halves. Two μl of Endo H (1 unit/200 μl, Roche Applied Science) was added to one half, and the other half served as control. Samples were incubated for 2 h at 37 °C, and the reactions were analyzed by anti-HA immunoblotting.

Proteinase K Digestion of Yeast Microsomes—Microsomes were prepared by a procedure modified from Ref. 11. Yeast cells expressing high levels of Doa10 tagged at its C terminus with the DTR or a 13× Myc epitope3 were grown in SD—Ura to an A600 of 0.8–1.0. 10–15 A600 equivalents were harvested and resuspended in 1 ml of 10 mM Tris-HCl, pH 9.4, containing 10 mM dithiothreitol and incubated at room temperature for 10 min. Cells were collected by centrifugation and washed with spheroplast buffer (1 M sorbitol, 20 mM sodium phosphate, pH 7.5, 150 mM NaCl, 2 mM dithiothreitol). Cells were centrifuged and resuspended in 1 ml of spheroplast buffer. Cell walls were digested with 140 μg of zymolase 100T (Seikagaku Corporation)/10 A600 equivalents for 20 min at 30 °C. Spheroplasts were pelleted by centrifugation (5 min at 600 × g at 4 °C) and washed in prechilled spheroplast buffer containing 20 μg/ml pepstatin and 1 mM EDTA, pH 8.0. Cells were pelleted again by centrifugation and resuspended in 1 ml of fractionation buffer (200 mM D-mannitol, 20 mM sodium phosphate, pH 7.5, 150 mM NaCl) containing 20 μg/ml pepstatin and 1 mM EDTA, pH 8.0. Lysates were obtained by the addition of 300 μl of acid-washed glass beads (Sigma) and 1 min of vortexing at 4 °C. Unlysod cells and cell debris were pelleted by centrifugation for 5 min at 600 × g at 4 °C. The supernatant was used directly or centrifuged once to yield a crude microsome fraction.

The microsome fraction was split into two halves. One was digested with 5 μg/ml proteinase K (American Bioanalytical) and incubated for 30 min at 4 °C, and the other was incubated without enzyme. For microsomes that were also examined with 1% Triton X-100, the original yeast culture was scaled up to 15 ml, and the final microsome fraction was divided into thirds. Proteinase K and mock digests were stopped by the addition of 10 mM phenylmethylsulfonyl fluoride and further incubation for 10 min at 4 °C. Samples were precipitated with 10% trichloroacetic acid (15 min at 4 °C) followed by a 10-min centrifugation at 13,200 rpm at 4 °C. Pellets were washed once with 2% trichloroacetic acid and resuspended in 35 μl of lysate buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1% sodium deoxycholate, 1% SDS, 8 mM urea) followed by 15 μl of 4× sample buffer (4% SDS, 125 mM Tris-HCl, pH 6.8, 10% glycerol, 10% β-mercaptoethanol, 0.02% bromphenol blue). 5 μl of 1 M Tris was added to neutralize the pH. For proteinase K digestion in the presence of Triton X-100, 3 μl of 10 mM NaOH was also added. Samples were incubated for 10 min at 37 °C before SDS-PAGE.

Immunoblot Analysis—Proteins separated by SDS-PAGE were electroblotted onto polyvinylidene difluoride membranes (Amersham Biosciences) and incubated with the appropriate primary antibody followed

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by horseradish peroxidase-conjugated secondary antibodies. Immuno-
reactive species were visualized using the ECL system (Amersham Bio-
sciences). The following antibodies were used: anti-HA mouse mono-
clonal antibody 16B12 (Covance); anti-Myc mouse monoclonal
antibody 9E10 (Covance); and an affinity-purified anti-Doa10 anti-
serum from rabbit. The rabbit antiserum was raised against a His6-
tagged N-terminal 128-residue fragment of Doa10 and was affinity puri-
ified by binding to a maltose-binding protein-Doa10(1–128) fusion
protein immobilized on polyvinylidene difluoride membrane as
described previously (22). Pulse-chase analysis of Ste6–166(HA) was
done as detailed previously (13).

Computational Methods—Using their respective Web servers, the
following algorithms were used for predicting the topology of Doa10:
TMHMM version 2.0 (23), Polyphobius (24), TMMOD (25), PHDhtm
(26), DAS (27), TMpred (28), SOSUI (29), and TopPred 2.0 (30).

RESULTS

Membrane Topology Predictions for S. cerevisiae Doa10—To deter-
mine whether particular loops of Doa10 were on the cytosolic or lumi-
nal face of the ER, an initial provisional model locating the TMs was
needed. We therefore subjected the Doa10 sequence to eight common
topological prediction programs (TMHMM2.0, PHD, TMpred, Poly-
phobius, TMMOD, DAS, SOSUI, and TopPred 2.0). The various algo-
rithms yielded different topologies for Doa10, with 12–16 TMs, but, as
will be discussed below, none matched the experimentally determined
topology. Because it has been one of the more successful prediction
algorithms when tested against other programs (31), the TMHMM2.0
program (www.cbs.dtu.dk/services/TMHMM/) was chosen for cre-
ating a starting topological model for Doa10 (Fig. 1A).

To gauge the robustness of the predictions from this program, we
compared the predicted S. cerevisiae Doa10 topology with that pre-
dicted for Doa10 orthologs from other Saccharomyces species whose
genomes have been sequenced (www.yeastgenome.org/). Unexpect-
edly, even the Doa10 protein from the most closely related of these yeast
species, S. paradoxus, yielded a significantly different topology despite
sharing 94% amino acid identity with S. cerevisiae Doa10 (Fig. 1B). Like
the ScDoa10 prediction, the predicted topology for SpDoa10 consisted
of 14 TMs with both termini facing the cytosol. However, the position
and orientation of the TMs in the C-terminal ~40% of the proteins
differed. For example, no TM was predicted for the hydrophobic stretch
of ScDoa10 at ~780–800 by TMHMM2.0 (Fig. 1A), whereas a TM in
this region was predicted for the S. paradoxus homolog, which differs
in three residues over this stretch (I779V, T785A, and T789I; residue iden-

ty and position from S. cerevisiae are listed first). The presence of a TM
at 780–800 would lead to a cytosolic orientation of the following large
loop, whereas the loop would be luminal (and even larger) in the absence
of that TM (Fig. 1, A and B).

Interestingly, in silico replacement of the two Thr residues (Thr785
and Thr789) of ScDoa10 with the respective residues from SpDoa10
resulted in the prediction of a TM at ~777–794 by TMHMM2.0 (Fig. 1C).
Moreover, the presence of this TM affected the topological predictions
for downstream sequences in ScDoa10. First, the orientation of the
next predicted TM at 965–987 was reversed and shifted slightly (to 969–
991). Second, the originally predicted TM at 989–1008 was lost (com-
pare Fig. 1, A and C). Downstream of this position, the TMHMM2.0
predictions for both wild-type Doa10 and the in silico mutant converged
again and were identical for the last five TMs. The value of this bioin-
formatic extension on standard topological prediction was testable, and,
as will be shown below, the topology predicted for the in silico mutant

FIGURE 1. Membrane topology predictions for Doa10 proteins. A, topology of S. cer-
evisiae Doa10 predicted by TMHMM2.0. The 14 TM segments are depicted as small gray
boxes near the top; lines connected to the bottom edge of these boxes represent cytosolic
loops, and those connected to the top edges depict luminal loops. Arrowheads with
residue numbers indicate the different sites within Doa10 at which the DTR was fused. B,
topology predicted for the S. paradoxus Doa10 ortholog. C, topology predicted for the in
silico mutant Doa10-T785A/T789I. The two amino acid changes are to the residues found
in S. paradoxus Doa10. Arrowheads correspond to those in A.

turned out to match the experimentally determined topology of wild-
type S. cerevisiae Doa10.

Membrane Topology of Doa10 Determined with DTR Fusions—The
Suc2-His4C DTR (32) was fused C-terminally to a set of N-terminal
fragments of Doa10. When fused to the C terminus of a membrane
protein, the DTR allows one to assay the disposition of the C terminus
relative to the ER membrane (cytosolic versus luminal). Fusion sites in
Doa10 were chosen within loop regions between the TMs predicted
computationally as described above, including sites that would distin-
guish the TMHMM2.0 predictions for normal Doa10 and the in silico
Doa10 mutant. The 126-kDa DTR bears a fragment of Suc2 (yeast
invertase) and a His4C fragment, His4C, that codes for histidinol dehy-
drogenase. A triplicated HA epitope tag is present upstream of the Suc2
sequence. When located in the cytosol, the His4C moiety allows growth
of a his4C yeast mutant on SD–His plates supplemented with 6 mM
histidinol through its ability to convert histidinol to histidine. Con-
versely, a luminal orientation of the reporter results in glycosylation of
the Suc2 moiety on its eight N-glycosylation sites. This causes an
increase in the molecular mass of the fusion protein that is sensitive
to Endo H treatment and can be followed by SDS-PAGE.

A total of 16 Doa10–DTR fusion proteins were expressed in the his4C
mutant strain STY50 and tested both for growth on histidinol plates and
Endo H sensitivity (Fig. 2). The alleles encoding the different proteins
were generated in a high copy plasmid that also carried the URA3
marker (see “Experimental Procedures”). Eight of the 16 strains grew on
histidinol (Fig. 2A, right panel), indicating a cytosolic disposition of the
DTR in these fusions. The glycosylation status of each DTR fusion was determined by Endo H digestion and immunoblot analysis. As shown in Fig. 2A, the eight DTR fusion proteins that failed to support growth on histidinol were heavily glycosylated, as indicated by an increase in mobility after Endo H treatment. The apparent sizes of the Endo H-treated proteins matched the predicted molecular masses of the unglycosylated forms. The lack of growth on histidinol combined with the glycosylation of the DTR in these eight strains indicated a luminal disposition of the DTR in each case. The eight Doa10-DTR fusions that supported growth on histidinol did not get glycosylated. Hence, the two assays gave fully self-consistent results, yielding the topology shown in Fig. 2A (also see Fig. 7). It appears that Doa10 itself is not normally glycosylated because the untagged protein migrated on SDS gels at ~150 kDa, its predicted protein mass, and this is unaffected by Endo H treatment (supplemental Fig. S1). Moreover, neither the full-length Doa10-DTR fusion nor any of the shorter fusions in which the DTR was cytosolic showed a detectable size reduction upon Endo H treatment (Fig. 2B).

We note that for several of the DTR fusions, either a small fraction appeared to have the opposite membrane orientation from the bulk of the protein, as measured by the gel mobility assay, or a portion of the protein was apparently cleaved proteolytically. Presumably these effects derived from context-specific interference by the DTR with protein folding or membrane insertion, although it is conceivable that dynamic changes in membrane insertion might normally occur in some regions of the protein. The most complex protein pattern was seen for the Doa10T785A/T789I-DTR fusion (Fig. 2B). Most of this protein is glycosylated, but a large fraction of the glycosylated form is proteolytically clipped (lowest band) for unknown reasons. Specifically, three protein bands were observed in the untreated lane. A small amount of full-length protein was detected (top band), and this was glycosylated (Endo H-sensitive).

The second band from the top was Endo H-resistant, implying that this derived from a fraction of Doa10T785A-DTR in which the DTR was still in the cytosol; this fraction was not sufficient to support growth on histidinol (Fig. 2A). The largest fraction of the Doa10T785A-DTR protein (lowest band) appeared to have been clipped after translocation of the DTR into the ER lumen because the migration of this species was Endo H-sensitive but much faster than predicted for the full-length unglycosylated protein.

In summary, the results of the Doa10-DTR mapping studies support the TMHMM2.0 topology model for the in silico Doa10-T785A/T789I mutant (Fig. 1C) rather than any computational model made for wild-type Doa10.

Mapping the Orientation of the Doa10 Termini by Protease Protection—The DTR mapping studies demonstrated a cytosolic orientation of the C terminus of Doa10. The disposition of the N terminus could not be tested directly by this method, although the prediction of a TM at 130–150 and the luminal orientation of the DTR in Doa10T785A-DTR strongly suggested that the N terminus would be cytosolic. Moreover, the hydrophilic Doa10 N-terminal segment includes the RING-CH domain, which is believed to interact directly with one or both of its cognate E2s in the cytosol. To determine directly the orientation of the Doa10 N terminus as well as that of the C terminus, we employed protease protection assays on intact yeast microsomes. In this method, freshly prepared yeast microsomal membrane fractions are treated with protease K, and the digested microsomal proteins are subjected to immunoblot analysis to determine whether the antigenic region of the target protein is protected from proteolysis.

We first tested whether our method of microscopic preparation yielded intact, right-side-out membrane vesicles by carrying out protease protection assays on microsomes from cells expressing the Doa10T785A-DTR or Doa10T789I-DTR fusion proteins. Based on the analysis in Fig. 2, the DTR (which includes a 3×HA epitope) in Doa10T785A-DTR should be in the ER lumen and therefore resistant to protease cleavage. Only the N-terminal RING-CH domain should be facing the outside of the vesicle, where it would be proteolysed. Indeed, the large glycosylated fusion protein was only slightly reduced in size after protease K digestion of Doa10T785A-DTR-containing microsomes, consistent with removal of just the N-terminal cytosolically disposed domain, which...
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FIGURE 3. Both termini of S. cerevisiae Doa10 face the cytosol. A, protease protection assays with yeast microsomal fractions from cells expressing the Doa10<sub>168</sub>-DTR and Doa10<sub>244</sub>-DTR fusions. Microsomes were digested with proteinase K (+) or were mock digested (−); samples were separated by SDS-PAGE (6–15% gradient gel) followed by anti-HA immunoblotting against the 3×HA tag in the DTR. 1% Triton X-100 (TX) was added in the reaction in lane 3. All detectable Doa10<sub>168</sub>-DTR was glycosylated, as expected, and a small fraction of Doa10<sub>244</sub>-DTR was as well (upper arrowhead). The DTR in this latter fraction was protected from proteinase K (prot. K) digestion (lower arrowhead), so only the N-terminal domain of Doa10 was cleaved. However, the majority of the DTR in Doa10<sub>244</sub>-DTR was digested. An asterisk marks a cross-reacting protease-insensitive protein, which serves as a loading control. B, protease protection assays of microsomes from yeast expressing a C-terminally 13×Myc-tagged Doa10 protein. Immunoblotting was performed with an antiserum specific for the Doa10 N terminus (left panel) or an anti-Myc antibody (right panel). Arrowhead, full-length Doa10-Myc. The lower bands seen in the anti-Doa10 blot are observed in doa10Δ extracts (not shown); therefore, these species are cross-reacting yeast proteins.

should be no more than 14.6 kDa in size (Fig. 3A, lanes 1 and 2). If the microsomal membranes were solubilized with the detergent Triton X-100 to allow protease access to the luminal domain of Doa10<sub>168</sub>-DTR, anti-HA reactivity was lost (lane 3). In contrast, the cytosolically exposed HA-tagged DTR domain in the Doa10<sub>244</sub>-DTR fusion protein was cleaved in the absence of added detergent (lanes 4 and 5). As was also seen in Fig. 2B, a small fraction (<5%) of Doa10<sub>244</sub>-DTR was glycosylated, reflecting a low frequency of translocation of the DTR into the ER lumen. Consistent with this, proteinase K did not eliminate this species but reduced it in size, as predicted if just the N-terminal domain of the glycosylated protein were cytosolically exposed.

These data indicate that the microsomal vesicles in our preparations were largely intact and right side out. Furthermore, the small reduction in the size of Doa10<sub>168</sub>-DTR (and the glycosylated Doa10<sub>244</sub>-DTR) in protease-treated microsomes is precisely as predicted for a cytosolic exposure of the Doa10 N terminus.

To assay the orientation of the N and C termini of a full-length Doa10 protein that did not carry the large DTR extension, microsomes were isolated from yeast cells expressing a fully functional C-terminally 13×Myc epitope-tagged Doa10 protein (Doa10-Myc). The microsomes were digested with proteinase K and analyzed by immunoblotting with either an antibody to the Myc epitope or an antiserum raised against a soluble N-terminal 128-residue fragment of Doa10 (Fig. 3B). No protected immunoreactive protein fragments were detected in either case. If the N terminus had been luminal, a protected fragment of at least 17 kDa should have been observed. Similarly, if the C terminus were in the ER lumen, a protected fragment of at least 17 kDa should have been seen.

We conclude from these biochemical experiments that Doa10 has an N<sub>cytosolic</sub>/C<sub>cytosolic</sub> topology, in agreement with the DTR mapping results.

Derlins Are Dispensable for the Doa10 Pathway—The large number of TMs in Doa10 is surprising if these serve only to anchor the protein in the ER membrane and help bind cofactors such as E2s. For comparison, the other integral E3 of the yeast ER, Hrd1, has only six TMs (10). One potential function for a subset of the TMs in Doa10 would be that they are part of a channel that functions in the retrotranslocation and degradation of membrane protein substrates of Doa10. However, other ER proteins have already been proposed as ER retrotranslocation channels. The first was the Sec61 translocon (3), but degradation of two well-studied Doa10 membrane protein substrates, Ubc6 and Ste6−166, is independent of the Sec61 complex (33, 13). The transmembrane proteins known as Derlins were recently proposed to be retrotranslocons as well (34). We therefore tested whether the two yeast Derlins, Der1 and Dfm1 (35), were necessary for the degradation of single pass and polytopic membrane substrates of Doa10 (Fig. 4). Simultaneous deletion of
both Derlin genes had no detectable effect on the degradation rate of the Ste6–166 or Ubc6 substrates, in contrast to the effect of deleting Doa10. We note that degradation of these two substrates was shown previously to be unaffected by loss of Der1 alone (12, 33). Our results imply that neither Derlin is essential for the extraction of Doa10 substrates from the ER membrane and leave open the possibility that a retrotranslocation channel is formed by Doa10, although other proteins might have this role.

Topological Analysis of TEB4, the Human Ortholog of Doa10—We previously identified the human TEB4 (MARCH-VI) protein as the likely human ortholog of Doa10 (11). The 910-residue protein is most similar to Doa10 in the N-terminal RING-CH domain (36% identity) and an internal ~130-residue block, the TEB4-Doa10 (TD) domain (30% identity). A function for TEB4 as an ERAD E3 is supported by recent findings demonstrating ubiquitin ligase activity of the TEB4 RING-CH domain in vitro and ER localization of full-length TEB4 in transfected HeLa cells4 (16).

To strengthen the hypothesis that TEB4 and Doa10 are functional counterparts, we determined whether the topology of TEB4 and Doa10 was likely to be the same. Intriguingly, the TMHMM2.0 program predicted 14 TMs for TEB4 with similar relative positions and the same orientations as the experimentally mapped TMs in Doa10 (Fig. 5, A and B, 14-TM model). Recently, however, a different topological model for TEB4 was proposed based mainly on hydrophathy analysis and protease protection assays of the TEB4 protein termini in isolated microsomes (16). This TEB4 model had 13 TMs, with the N terminus facing the cytosol and the C terminus luminally disposed (Fig. 5B, 13-TM model). The major difference between the 13-TM and 14-TM models is that in the latter, a TM at residues 520–540 is predicted, whereas this is missing from the 13-TM model. All 13 predicted TMs in the 13-TM model are also predicted as TMs in the 14-TM model, but insertion of the TM at residues 520–540 results in an inversion of the orientation of all the predicted TMs C-terminal to this segment.

To find out which of the two models best predicts the actual topology of TEB4, we analyzed a limited set of TEB4-DTR fusion proteins. The DTR described above was fused to three different sites in TEB4, and the resulting fusion proteins were expressed in yeast. The orientation of the DTR for each fusion protein was determined by both the histidinol growth and Endo H sensitivity assays. The three TEB4 fusion sites, after residues 598, 844, and 910, were chosen because the two competing topology models make opposite predictions about which side of the ER membrane these positions face (Fig. 5B). In particular, fusion after residue 598 was chosen to determine whether the TMHMM2.0-predicted TM helix at ~520–540 was likely to be present. DTR fusion to TEB4 residue 910, the last residue of the protein, directly tested the disposition of the C terminus, and the fusion to residue 844 tested a loop that should be on the opposite side of the ER membrane from the C terminus.

Fusion of the DTR to residue 598 or 910 conferred growth on histidinol plates, whereas fusion to residue 844 did not (Fig. 6A). Conversely, of the three fusions, only TEB4844-DTR was glycosylated as determined by Endo H treatment (Fig. 6B). In addition, protease protection assays of microsomes from yeast expressing a C-terminally Myc epitope-tagged
Membrane Topology of the Doa10 Ubiquitin Ligase

FIGURE 7. Experimentally derived topology model for S. cerevisiae Doa10. Doa10 contains 14 TMs, and both termini face the cytosol. The (predicted) borders of each TM are indicated. The RING-CH at the N terminus is depicted as a black box. The conserved TD domain (~residues 625–759) is highlighted by gray shading. A previously predicted WW-motif (residues 775–807) is also highlighted but can be seen to span the membrane.

TEB4 protein revealed complete loss of reactivity to anti-Myc antibody upon proteinase K treatment (data not shown). These findings are consistent with the 14-TM model for TEB4 and not the 13-TM version. Therefore, we infer that TEB4 adopts a topology similar to that of Doa10, with both termini facing the cytosol, and a total of 14 TMs. Notably, the cytosolic Doa10 C-terminal segment is important for Doa10 function (supplemental Fig. S2). Assuming that the function of TEB4 and Doa10 is conserved, this would imply a cytosolic disposition of the TEB4 C terminus as well.

DISCUSSION

In the present work, we have used a combination of standard TM prediction algorithms, sequence alignment between proteins from closely related species, and in silico mutagenesis to generate a detailed prediction for the complex topology of the S. cerevisiae Doa10 ubiquitin ligase. The model was tested extensively by fusions of a topological reporter to at least one position within every predicted loop as well as to the Doa10 C terminus. The 151-kDa Doa10 protein was found to have 14 transmembrane helices and to expose the bulk of its sequence, including the N and C termini, to the cytosolic side of the ER membrane (Fig. 7). A cytosolic orientation of both Doa10 termini was confirmed by protease protection assays with yeast microsomes. These data inform other aspects of Doa10 structure and function, as will be discussed below. Finally we have compared the topology of Doa10 with that of its likely human ortholog, TEB4. Despite extensive sequence divergence, TEB4 appears to have a topology identical to Doa10, supporting the idea that the yeast and human proteins are functionally conserved.

Computational Analysis of Membrane Topology—Interestingly, our experimental TM mapping data matches the TMHM2.0 topology model for the in silico Doa10-T785A/T789I mutant rather than the topology model for wild-type Doa10 predicted by TMHM2.0 or any of the other algorithms we employed. In fact, the experiments included specific tests of the Doa10-T785A/T789I-based model, which greatly aided elucidation of the actual Doa10 topology. Because the exact features of membrane proteins that dictate how they insert into membranes are not fully understood, the available prediction algorithms can only serve as heuristic guides for the actual topology. The TMHM2.0 program considers multiple protein properties, including hydrophobicity, charge bias, helix length, and helix orientation, in deriving its TM probabilities (23). The increase in hydrophobicity caused by the in silico mutations is the likely reason that the TM probability threshold was reached for residues 777–794 in the mutant Doa10; a TM at this position will affect the orientation of downstream segments and therefore their TM probabilities. Supplementing standard computational models with comparative sequence analyses and exploratory in silico mutations, as we have done here, should be useful for analyzing other polytopic membrane proteins as well.

It is striking that orthologs with sequences as divergent as yeast Doa10 and human TEB4 (MARCH-VI) apparently adopt an identical topology. Other Doa10 orthologs need not necessarily have the same number of TMs, although all of them appear to have at least 12. The presumptive counterpart of Doa10 from the fission yeast Schizosaccharomyces pombe, for instance, is predicted by TMHMM2.0 to have 16 TMs, with two additional TMs inserted upstream of the TD domain. Nevertheless, we expect that all Doa10/TEB4 orthologs will have an even number of TMs. This inference is based on the fact that 1) the conserved N-terminal RING-CH is essential for ubiquitin ligase activity (11) and is cytosolic (Fig. 3); and 2) the C-terminal hydrophilic segment of Doa10 is sensitive to mutational inactivation (supplemental Fig. S2) and is also cytosolic. The implication is that Doa10 function requires that both its termini be on the cytosolic side of the ER membrane, perhaps because both interact with cytosolic (or cytosolically exposed) factors. If one assumes evolutionary conservation of function, this should be true of Doa10 orthologs as well.

Human TEB4 Topology—The TMHM2.0 topology model for human TEB4 predicts 14 TMs in similar relative positions and the same orientations as those in yeast Doa10. Our analysis of TEB4 protein expressed in yeast is fully consistent with this model, supporting the hypothesis that Doa10 and TEB4 have similar biochemical functions (although we have not observed genetic complementation of doa10Δ by heterologously expressed TEB4). Another group (16) recently reported data that are in concordance with ours on the cytosolic exposure of the N-terminal RING-CH domain of TEB4, but they suggested that TEB4 has a luminally exposed C terminus and only 13 TMs.

A reexamination of the Hassink et al. (16) data suggests, however, that their results are actually compatible with ours. Hassink and colleagues limited their analysis to a determination of the orientation of the two termini of TEB4. Microsomes treated with low concentrations of proteinase K yielded a C-terminal ~34-kDa protein fragment, although this was no longer observed at higher protease concentration. Incomplete digestion at the lowest protease concentration was also reflected by the presence of uncleaved full-length TEB4. The partial resistance was interpreted to result from a luminal disposition of the TEB4 C terminus, but it is unclear why degradation would be observed at the higher protease concentration if this were true. Moreover, the 34-kDa fragment includes 3–4 TMs based on our topological predictions as well as those of Hassink et al.; therefore, the partial digestion must be because of a general relative protease resistance of all of the cytoplasmic loops distal to the cleavage site that generated the 34-kDa species.

Functional Implications of Doa10 Membrane Organization—The experimental localization of the 14 TMs in Doa10 has significant implications for other structure-function aspects of this ubiquitin ligase. In our original report on Doa10, we noted a match to the minimal consensus sequence for a WW motif at residues 775–807. Class I WW motifs fold into a three-stranded β sheet and bind PPxY motifs in their binding partners (36). Ubc6, a cognate E2 for Doa10, includes a PPxY peptide. Our topological mapping data, however, now show that the bulk of residues 775–807 is part of a transmembrane helix (Fig. 7). Therefore, unless membrane insertion in this region is highly dynamic, a WW β sheet will not form.

Notably, the topological analysis of Doa10 reveals that the well conserved TD domain (11) includes three TMs, and the most conserved
stretch in the TD domain is a loop that faces the cytosol (Fig. 7). The conservation of sequence in these three TMαs contrasts with the other TMαs in the Doa10/TEB4 protein family, which share a general hydrophobicity but are not readily aligned. Among the conserved residues within the TMαs of the TD domain are several Gly, Pro, and charged amino acids, which are relatively uncommon in TMαs. Such conservation of sequence implies a shared core function of the TD domain within the ER/nuclear envelope membrane.

The Doa10 ubiquitin ligase has an unusually broad range of substrates that includes soluble cytoplasmic and nuclear proteins as well as various transmembrane proteins of the ER and nuclear envelope (37). It must be noted that with both soluble and transmembrane cofactors (3). The proteasome-mediated degradation of membrane protein substrates of Doa10 requires their retrotranslocation out of the ER membrane as well. It is useful to consider these various Doa10-linked functions in the context of the experimentally derived topology of the protein.

The 14 TMαs in Doa10 are arranged such that the majority of the protein is exposed to the cytosol (~63%), and only a small portion is facing the ER lumen (~14%). The cytoplasmic loops are between ~21 and ~250 residues in length, whereas the luminal loops are all relatively short, ranging from ~3 to ~48 residues (Fig. 7). The cytosolic orientation of most of the Doa10 sequence suggests that many key Doa10 interactions with other proteins are likely to take place at the cytosolic face of the ER membrane. This is presumed to be the case for the binding of soluble Doa10 substrates (and cofactors), but it might also be true for at least a subset of its membrane substrates. The best studied membrane substrate of Doa10 is Ste6–166, a mutant form of the pheromone transporter that is missing the last 42 residues of its cytosolically disposed C-terminal domain (13, 12). Vashist and Ng (12) demonstrated that this truncated C-terminal domain is sufficient for recognition by the Doa10 pathway. On the other hand, Ub6c is also ubiquitinated and degraded by the Doa10 pathway, and its C-terminal membrane anchor has been suggested to be sufficient for E3 recognition because its translocation to another E2 protein causes the resulting fusion to be degraded (33).

The large number of TMαs in Doa10 greatly exceeds what is required for localization to the ER membrane. Presumably some of the structural complexity of Doa10 is necessary for its ability to bind diverse soluble and membrane cofactors and substrates. However, another possibility, first raised in our original description of Doa10 (11), is that at least some of the TMαs may contribute to a protein exit channel. Although several other ER proteins have been proposed as retrotranslocons, these particular proteins have been shown to be nonessential for the degradation of Doa10 membrane substrates (Fig. 4; Refs. 12, 13, 33). Retrotranslocation of certain ERAD substrates can initiate prior to substrate ubiquitination, but ubiquitin conjugation is required for complete transport and degradation (38). Initiation of transport of a transmembrane substrate through a specific retrotranslocon domain in an E3 such as Doa10 might couple further transport to its ubiquitin ligase activity. A substrate polypeptide could begin to thread back into the cytoplasm and en route expose a lysine(s) susceptible to ubiquitin ligation by a membrane-juxtaposed E2–E3 complex. If Doa10 has such a retrotranslocation function, it should be phylogenetically conserved. An obvious candidate for a region involved in this would be the transmembrane TD domain.

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