Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 4994–4999, May 1997
Biochemistry

DAD1, the defender against apoptotic cell death, is a subunit of the mammalian oligosaccharyltransferase

DANIEL J. KELLEHER AND REID GILMORE*

Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, MA 01655-0103

Communicated by William J. Lennarz, State University of New York, Stony Brook, NY, February 11, 1997 (received for review September 26, 1996)

ABSTRACT **DAD1, the *d*efender against *a*poptotic cell *d*eath, was initially identified as a negative regulator of programmed cell death in the BHK21-derived tsBN7 cell line. Of interest, the 12.5-kDa DAD1 protein is 40% identical in sequence to Ost2p, the 16-kDa subunit of the yeast oligosaccharyltransferase (OST). Although the latter observation suggests that DAD1 may be a mammalian OST subunit, biochemical evidence to support this hypothesis has not been reported. Previously, we showed that canine OST activity is associated with an oligomeric complex of ribophorin I, ribophorin II, and OST48. Here, we demonstrate that DAD1 is a tightly associated subunit of the OST both in the intact membrane and in the purified enzyme. Sedimentation velocity analyses of detergent-solubilized WI38 cells and canine rough microsomes show that DAD1 cosediments precisely with OST activity and with the ribophorins and OST48. Radioiodination of the purified OST reveals that DAD1 is present in roughly equimolar amounts relative to the other subunits. DAD1 can be crosslinked to OST48 in intact microsomes with dithio*bis*(succinimidylpropionate). Crosslinked ribophorin II–OST48 heterodimers, DAD1–ribophorin II–OST48 heterotrimers and DAD1–ribophorin I–ribophorin II–OST48 heterotetramers also were detected. The demonstration that DAD1 is a subunit of the OST suggests that induction of a cell death pathway upon loss of DAD1 in the tsBN7 cell line reflects the essential nature of N-linked glycosylation in eukaryotes.**

In multicellular organisms, programmed cell death or apoptosis is an obligatory, exquisitely regulated event during normal cell differentiation, development, and tissue homeostasis of the mature organism (1). During the past several years, it has been established that regulatory proteins (e.g., *ced9* and *bcl-2*) and enzymatic activities [e.g., *ced3* and interleukin 1-β-converting enzyme (ICE)-like proteases] that control commitment to, and mediate progression of, the programmed cell death pathway are conserved between invertebrates and vertebrates (2, 3). The DAD1 protein, the *d*efender against *a*poptotic cell *d*eath, was identified as a mammalian cell death suppressor that may act downstream of the *bcl-2* protein (4, 5). A human cDNA encoding a novel 113-residue hydrophobic protein designated as DAD1 was cloned by complementation of the restrictive phenotype of tsBN7 cells, a temperature-sensitive derivative of BHK21 cells, which undergo apoptosis at 37°C (4). A point mutation in the DAD1 gene introduces a charged residue within the first hydrophobic segment of the DAD1 protein. Rapid loss of the DAD1 protein from tsBN7 cells after shift to the restrictive temperature initiates apoptosis either because DAD1 acts as a cell death suppressor (4) or because DAD1 performs an essential cellular function, the loss of which leads to programmed cell death. The isolation of a *Xenopus laevis* DAD1 cDNA that predicted a protein that was 91% identical in sequence to human DAD1 revealed substantial evolutionary conservation (4), an observation that has since been extended by the isolation of DAD1 cDNAs from *Caenorhabditis elegans* (5) and *Arabidopsis thaliana* (GenBank accession no. X95585). The *C. elegans* and *A. thaliana* DAD1 proteins are, respectively, 61% and 47% identical in amino acid sequence to the human DAD1 protein. A role for *C. elegans dad1* as a cell death suppressor was suggested by studies of transgenic nematodes bearing human or *C. elegans* DAD1 genes under control of a heat shock promoter (5). Transient overexpression of the human or *C. elegans dad1* transgene during development led to the survival of a subset of cells normally programmed for death (5).

A protein sequence comparison revealed that human DAD1 is 40% identical to the yeast Ost2 protein (6). Ost2p is the 16-kDa subunit of the yeast oligosaccharyltransferase (OST) (7). OST catalyzes the transfer of high mannose oligosaccharides onto asparagine residues in nascent polypeptides in the lumen of the rough endoplasmic reticulum (RER). Temperature-sensitive *ost2* mutants are defective in N-linked glycosylation of proteins *in vivo* and in glycosylation of synthetic peptide substrates *in vitro*. Point mutations in the *OST2* gene that cause growth defects alter amino acid residues that are highly conserved between Ost2p and the vertebrate, invertebrate, and plant DAD1 proteins (6). Like the Gly37Arg mutation responsible for the lability of DAD1 in tsBN7 cells, point mutations in several *ost2* alleles introduce charged residues within the predicted membrane spanning segments of Ost2p (6).

The significance of the homology between Ost2p and DAD1 is subject to several interpretations. One conjecture is that DAD1 is a subunit of the vertebrate OST. However, biochemical evidence supporting this hypothesis is lacking. The OST isolated from canine pancreas, hen oviduct, and human liver appears to be a heterotrimer with subunit molecular masses of 66 kDa (ribophorin I), 63/64 kDa (ribophorin II), and 48–50 kDa (OST48) (8–10). An alternative interpretation would be that DAD1 and Ost2p are structurally related proteins that nonetheless perform very dissimilar functions in unicellular and multicellular organisms. To discriminate between these two models, we conducted biochemical studies to determine whether DAD1 in vertebrate cells and tissues is a subunit of the OST.

MATERIALS AND METHODS

Preparation of Puromycin–High Salt-Extracted Rough Microsomes (PK-RM) and Detergent-Permeabilized, High Salt-

Abbreviations: ICE, interleukin 1-β-converting enzyme; DAD1, defender against apoptotic cell death; OST, oligosaccharyltransferase; RER; rough endoplasmic reticulum; PK-RM, puromycin–high salt-stripped microsomes; DK-RM, detergent-permeabilized, high salt-stripped microsomes; DSP, dithio*bis*(succinimidylpropionate); DTSSP, 3,3′-dithio*bis*(sulfosuccinimidylpropionate); TEA-Oac, triethanolamine-acetate, pH 7.5; ICE, interleukin 1-β converting enzyme.

*To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

0027-8424/97/944994-6$2.00/0
PNAS is available online at **http://www.pnas.org**.

Extracted Rough Microsomes (DK-RM). Rough microsomes were isolated from canine pancreas as described (11). To prepare the PK-RM, 14 ml of rough microsomes [1 eq/μl (equivalents as defined in ref. 11)] was adjusted to 50 mM triethanolamine–acetate (TEA-Oac) (pH 7.5), 500 mM KOac, 12 mM $Mg(Oac)_2$, 0.2 mM GTP, 1 mM puromycin, 0.8 mM dithiothreitol, and 200 mM sucrose in a total volume of 17 ml. After a 10-min incubation at 25°C followed by 30 min at 4°C, the solution was adjusted to 1 mM $CaCl_2$ and 16 units/ml micrococcal nuclease and incubated for 10 min at 25°C. After adjustment to 2 mM EGTA, the membranes were transferred into Beckman Ti50.2 centrifuge tubes, underlaid with a cushion of 1.3 M sucrose, 50 mM TEA-Oac, 500 mM KOac, 12 mM $Mg(Oac)_2$, and 1 mM EGTA, and centrifuged for 2.5 h at 150,000 $\times$ *g*. The PK-RM were recovered at the cushion interface, diluted to 25 ml with 50 mM TEA-Oac, 600 mM KOac, 12 mM $Mg(Oac)_2$, and 1 mM EGTA, and centrifuged for 90 min at 150,000 $\times$ *g*. After repeating the preceding centrifugation, the membranes were resuspended in 50 mM TEA-Oac and 250 mM sucrose and stored at -80°C. DK-RM were prepared from rough microsomes by successive extraction with high salt (0.5 M NaCl) and with detergent (0.1% Nikkol) as described (10).

Glycerol Gradient Centrifugation of WI38 Fibroblast Homogenates. WI38 cells were grown to confluence in T-150 flasks in DMEM supplemented with 10% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and nonessential amino acids. The cells were washed three times with 25 ml of 20 mM Tris·HCl (pH 7.4), 150 mM NaCl, and 1 mM $MgCl_2$ supplemented with a protease inhibitor cocktail as described (10). The cells were scraped from the flask and homogenized by 10 strokes with a dounce homogenizer in 1 ml of ice-cold 20 mM Tris·HCl (pH 7.4), 500 mM NaCl, 1.5% digitonin, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, a protease inhibitor cocktail, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The cell homogenate was centrifuged for 15 min at 122,000 $\times$ *g* in a Beckman Type 50 rotor to obtain a detergent extract that was applied to an 11.5-ml 8–30% glycerol gradient in 20 mM Tris·HCl (pH 7.4), 50 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 0.125% digitonin, 25 μg/ml egg yolk phosphatidylcholine, a protease inhibitor cocktail, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. After centrifugation for 14 h at 37,000 rpm in a Beckman SW40 rotor, the gradient was resolved into 14 0.9-ml fractions.

OST Assay and OST Purification. Digitonin-solubilized OST was assayed as described (10) except that the digitonin concentration was reduced to 0.007%. The iodinated tripeptide acceptor (Nα-Ac-Asn-[^{125}I]Tyr-Thr-NH_2; 15,000 cpm/pmol) and the lipid-linked oligosaccharide donor for the OST assay were prepared as described (10). Canine OST was purified from digitonin-solubilized DK-RM by glycerol gradient centrifugation and two successive Mono Q ion exchange columns as described (10) except that the chromatography buffer for the final Mono Q column was altered by (*i*) elimination of the dithiothreitol, (*ii*) elimination of the protease inhibitor cocktail, and (*iii*) replacement of the 20 mM Tris·HC (pH 7.4) buffer with 20 mM Tea-Oac. These modifications were designed to obtain purified OST that was compatible with the protein crosslinking and radioiodination experiments.

Radioiodination of the OST. Purified canine OST (15 pmol in 50 μl of 20 mM TEA-Oac/0.125% digitonin/34 μM egg yolk phosphatidylcholine/1 mM $MgCl_2$/1 mM $MnCl_2$/240 mM NaCl) was denatured by adjustment to 0.2% SDS and incubated for 3 min at 22°C with 100 μCi (1 Ci = 37 GBq) of $Na^{125}I$ (15.9 mCi/μg) in the presence of 72 nmol of chloramine T. The reaction was terminated by the addition of 20 μl of 15 mM $Na_2S_2O_5$ in 20 mM TEA-Oac. The iodinated proteins were precipitated as potassium dodecyl sulfate complexes by the addition of 14 μl of ice-cold 1 M KOac, collected by centrifugation in a microfuge after incubation for 30 min at 0°C, and prepared for SDS/PAGE.

Protein Electrophoresis and Protein Immunoblots. Proteins were resolved by gel electrophoresis in SDS on 7.5–17% polyacrylamide gradient gels except as noted. The gels were either stained with Coomassie blue or were electrophoretically transferred to 0.2-μm Bio-Rad Transblot membranes. The membranes were probed with rabbit antisera or mouse mAb as described (10). Peroxidase-labeled secondary antibodies were detected by enhanced chemiluminescence using kits from Pierce (Supersignal) or Kirkegaard & Perry Laboratories (Lumi-Glo).

The RIC6 antibody that recognizes the cytoplasmic domain of ribophorin I and the two mAb that are specific for the lumenal domains of ribophorins I and II have been characterized (12). The polyclonal antibody specific for the cytoplasmic domain of OST48 was raised against a synthetic peptide (CysHisMetLysGluLysGluLysSerAsp). The OST48 peptide was coupled to thyroglobulin with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester and used to immunize rabbits. The antisera raised against human DAD1 has been described (4). The antisera that recognizes the lumenal domain of OST48 was provided by Gert Kreibich.

Crosslinking of the OST. The homobifunctional amine-reactive crosslinking reagents used in this study were 3,3′-dithio*bis* (sulfosuccinimidylpropionate) (DTSSP) and dithio*bis* (succinimidylpropionate) (DSP). Freshly prepared DTSSP or DSP in dimethyl sulfoxide was added to 8 eq of PK-RM in 50 mM TEA-Oac, 40 mM sucrose, 1 mM $MgCl_2$, and 0.8 mM $MnCl_2$ to obtain a crosslinker concentration of 300 μM and a dimethyl sulfoxide concentration of 1%. Freshly prepared DTSSP or DSP in dimethyl sulfoxide was added to 3 pmol of purified canine OST in 50 mM TEA-Oac, 40 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.125% digitonin, and 4 μg/ml egg yolk phosphatidylcholine to obtain a crosslinker concentration of 75 μM. After 90 min at 0°C, unreacted crosslinker was quenched by the addition of one–fourth volume of 1 M Tris·HCl (pH 7.4). DSP- and DTSSP-treated samples were prepared for SDS/PAGE without exposure to reducing agents and were resolved on 6.5–15% polyacrylamide gradient gels.

Proteolysis of PK-RM. Aliquots of PK-RM (8 eq, $\approx$20 μg of protein) were adjusted to 40 mM TEA-Oac, 100 mM KOac, 0.8 mM $MgCl_2$, 0.8 mM $MnCl_2$, and 100 mM sucrose in a total volume of 25 μl. Some samples were adjusted to 1% Triton X-100 to solubilize the membranes. Trypsin or chymotrypsin was added to obtain a concentration of 60 μg/ml. After 3.75 h of incubation at 37°C, the proteases were inactivated by adjustment of the samples to 40 mM phenylmethylsulfonyl fluoride.

RESULTS

Copurification of DAD1 with the OST. To determine whether DAD1 is a subunit of the canine OST, we examined samples obtained during an OST purification for the presence of DAD1. Detection of DAD1 was achieved using an antibody raised against a hydrophilic peptide that separates the second and third hydrophobic segments of DAD1 (4). Peripheral membrane proteins were removed from canine pancreatic microsomes by extraction with high salt followed by permeabilization of the microsomes with a low concentration of a nonionic detergent to remove soluble lumenal content proteins (10). The integral membrane proteins, including the OST, were solubilized with digitonin plus 0.5 M NaCl and resolved from the majority of RER membrane proteins by glycerol density gradient centrifugation (10). OST activity was recovered as a symmetric peak centered in fraction 10 of the glycerol gradient (Fig. 1*A*). The antibody to DAD1 recognized an 8- to 10-kDa protein that cosedimented precisely with OST activity

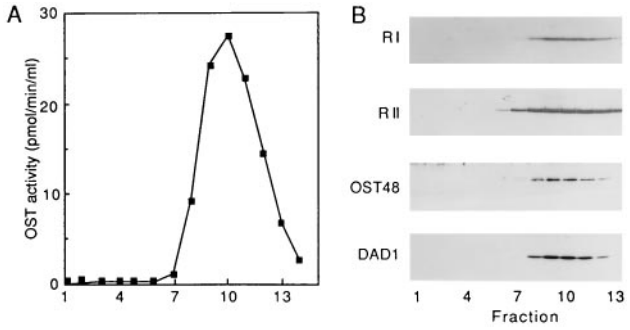

FIG. 1. Cosedimentation of DAD1 with OST activity, ribophorins I and II, and OST48. (*A*) A digitonin–high salt extract prepared from DK-RM was resolved by preparative glycerol density gradient centrifugation. Fraction 1 is the top of the gradient. Duplicate aliquots from each of 14 gradient fractions were assayed for OST activity. (*B*) Aliquots from each gradient fraction were analyzed on protein immunoblots using antibodies to ribophorin I (R I), ribophorin II (R II), OST48, and DAD1. The 64-kDa-glycosylated and 63-kDa-nonglycosylated forms of ribophorin II migrate as a doublet (13).

and with ribophorins I and II and OST48, strongly suggesting that DAD1 is associated with the OST (Fig. 1*B*).

Cell fractionation experiments and N-linked carbohydrate analyses have established that ribophorins I and II are integral membrane glycoproteins restricted to the RER (13–15). To address the intracellular location of DAD1, a total membrane fraction prepared from a porcine pancreas postnuclear supernatant was resolved by sucrose density gradient centrifugation. Protein immunoblots revealed that DAD1 cosedimented with ribophorin I and Sec61α, two well characterized RER markers (14, 16). Markers diagnostic for mitochondria, the Golgi apparatus, and the plasma membrane were recovered in fractions of lower density (data not shown). We next asked whether human tissue culture cells contain a single population of DAD1 that is associated with ribophorins I and II. Human WI38 fibroblasts were homogenized in digitonin and separated into a soluble detergent extract and an insoluble residue by centrifugation. As shown in Fig. 2, DAD1 and the ribophorins were recovered in the detergent extract. Total cellular DAD1 cosedimented with ribophorins I and II on a glycerol density gradient. Low molecular mass monomeric proteins, including two abundant cytosolic proteins of 14 and 16 kDa that react with the DAD1 antisera (Fig. 2, lanes 2–4), sedimented far less

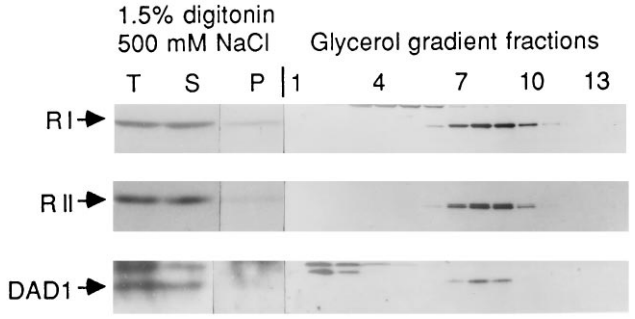

FIG. 2. Total cellular DAD1 cosediments with the OST subunits. Intact WI38 fibroblasts were solubilized in digitonin–high salt solution as described. The total homogenate (T) was centrifuged for 15 min at 160,000 × *g* to prepare a detergent-solubilized supernatant (S) and a detergent-insoluble pellet (P). The digitonin–high salt supernatant (S) was resolved by glycerol density gradient centrifugation into 14 fractions. Fraction 1 is the top of the gradient. Samples corresponding to 3% of the total homogenate, 3% of the supernatant, and 6% of the pellet were resolved on one polyacrylamide gel, and 18% of each gradient fraction was resolved on a second gel. Protein immunoblots used antibodies specific for ribophorins I (R I) and II (R II) and DAD1.

rapidly. Taken together, these results provide evidence that human DAD1 is exclusively associated with the ribophorins and is therefore localized to the RER.

The canine OST was purified from digitonin-solubilized DK-RM by glycerol gradient centrifugation followed by two sequential Mono Q anion exchange columns as described (10). The OST subunits resolve into several activity peaks on the first Mono Q ion exchange column. The majority of each OST subunit (≈70–80%) is recovered in an activity peak that we will refer to as the OST core enzyme. The remainder of each subunit is recovered in several additional activity peaks, two of which have higher apparent turnover numbers (data not shown). The three large subunits (ribophorins I and II and OST48) of the OST are the major proteins detected by staining with Coomassie blue when 10 μg of the OST core enzyme is resolved by SDS/PAGE (Fig. 3*A*, lane a). DAD1 is not detected when the protein gel is stained with Coomassie blue (Fig. 3*A*, lane a). However, protein immunoblot experiments showed that DAD1 copurified with the OST core enzyme during each step of the purification (Fig. 3*B*). Note that the relative stoichiometry among the four polypeptides is not noticeably altered during purification, suggesting that the interaction between DAD1 and the other subunits is not labile. We conclude that DAD1 is a core subunit of the OST and hypothesize that DAD1 was not detected in previous studies because of the poor staining properties of the protein. We also note that the presence or absence of DAD1 cannot account for the difference in activity between the high turnover and core forms of the OST because the high turnover forms of the OST have a similar DAD1 content (data not shown).

Despite the copurification of DAD1 with the OST core enzyme, the absolute stoichiometry of DAD1 relative to the three larger polypeptides was uncertain. To address the question of stoichiometry, the purified OST was denatured in SDS and radiolabeled with ^{125}I using a chloramine T oxidation procedure. Radiolabeled ribophorins I and II and OST48 are readily detected (Fig. 3*A*, lanes b and c). Radioiodination revealed the presence of several additional polypeptides, including an 8- to 10-kDa polypeptide that comigrates with the

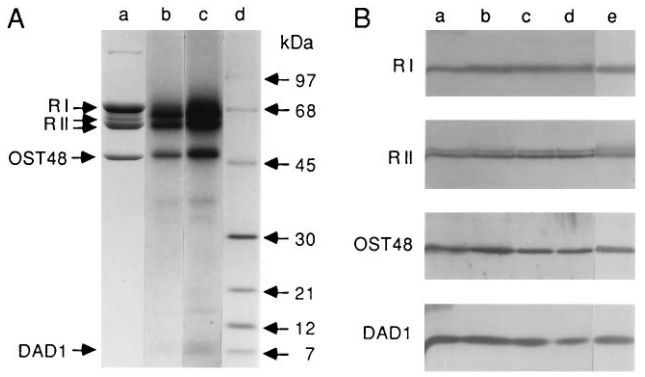

FIG. 3. Copurification of DAD1 with the OST core enzyme. The OST was purified from digitonin–high salt extracts of canine rough microsomes (10). (*A*) Purified OST (lane a, 10 μg) and protein molecular mass standards (not shown) were resolved by SDS/PAGE and stained with Coomassie blue. ^{125}I-labeled OST (lane b, 0.5 pmol; lane c, 1.0 pmol) and ^{14}C-labeled protein molecular mass standards (lane d) were resolved by SDS/PAGE. The photograph of the autoradiogram (lanes b-d) was aligned with the photograph of the stained gel to show identical mobilities for the molecular mass standards. (*B*) Selected fractions from the OST purification were analyzed on protein immunoblots using antibodies specific for ribophorins I or II, OST48, or DAD1. The gel lanes correspond to the following fractions: a, rough microsomes (4 eq); b, digitonin–high salt extract of DK-RM (4 eq); c, pooled active fractions from the glycerol gradient (3 eq); d, active pool after the first Mono Q column (4.1 eq); and e, active pool after the second Mono Q column (5.2 eq). For *A* and *B*, R I, ribophorin I; R II, ribophorin II.

DAD1 antigen. The less intense 15-kDa polypeptide (Fig. 3*A*, lanes b and c) was not recognized by the antibody to DAD1. If the core OST complex contains equimolar amounts of ribophorin I, ribophorin II, OST48, and DAD1, the incorporation of the radiolabel should be proportional to the tyrosine content of the four subunits provided that the radioiodination efficiency of individual tyrosines is comparable in the denatured protein subunits (Table 1). The experimentally determined stoichiometry of radiolabeling supports the conclusion that DAD1 is present in roughly equimolar amounts relative to the other three subunits of the OST core enzyme. The radiolabeled OST preparation does contain a polypeptide of 36 kDa that we have detected as a minor component with conventional protein-staining methods. The 36-kDa canine polypeptide may be the canine equivalent of the 40-kDa subunit of the porcine OST complex (17) or it may be a proteolytic fragment derived from OST48 (see Fig. 5*A*).

Orientation of DAD1 in the RER Membrane. We next asked whether the hydrophilic segments of DAD1 are located on the cytoplasmic face of the membrane. The 113-residue DAD1 protein consists of a 28-residue N-terminal hydrophilic segment followed by three hydrophobic segments that could function as transmembrane spans (Fig. 4*A*). The peptide used to elicit antibody to DAD1 separates the second and third hydrophobic segments (4). Because of the lack of a cleavable signal sequence and the presence of several polar residues in the third hydrophobic segment, neither the orientation of the N terminus nor the number of membrane-spanning segments could be deduced based upon the amino acid sequence alone. Proteolytic digestion experiments of intact yeast microsomes indicated that the amino-terminal hydrophilic segment of Ost2p faces the cytosol (6). If DAD1, the mammalian homologue of Ost2p, is oriented in an identical manner in rough microsomes, both the N-terminal hydrophilic segment and the epitope recognized by the antisera to DAD1 should be oriented toward the cytosol if the topology model depicted in Fig. 4*B* is valid.

To address the topology of DAD1 in the RER membrane, we digested intact microsomes with trypsin or chymotrypsin because the peptide used for antibody production contains cleavage sites for both proteases (Fig. 4*A*). Samples of the proteolyzed membranes were subjected to protein immunoblot analysis using antibodies to DAD1, OST48, and ribophorin I (Fig. 4*C*). Proteolysis of intact membranes with trypsin or chymotrypsin at 37°C resulted in a substantial decrease in DAD1 immunoreactivity. Proteolytic fragments of DAD1 were not detected, indicating that the peptide epitope was destroyed or was instead liberated as a peptide of <2–3 kDa. OST48 and ribophorin I served as control membrane proteins of known topology (Fig. 4*B*). As reported previously (19), the cytoplasmic domain of ribophorin I is digested by trypsin and chymotrypsin; a limit digestion product of 50 kDa was detected with antibodies specific for the lumenal domain of ribophorin I (Fig. 4*C*). Proteolysis with trypsin, but not chymotrypsin, caused a slight increase in the gel mobility of OST48 (Fig. 4*C*),

Table 1. Quantification of radiolabeled OST subunits

Subunit	^{125}I incorporation, units*	Tyrosines†	Ratio
Ribophorin I	16.5	29	1.0
Ribophorin II	13.6	18	1.33
OST48	6.7	17	0.69
DAD1	2.0	4	0.86

*Incorporation of ^{125}I into OST subunits was determined by PhosphorImager quantification of lanes b and c of Fig. 3*A* and is expressed in arbitrary units.

†The tyrosine content of the OST subunits is based upon the mature sequences of human ribophorins I and II, human DAD1, and canine OST48.

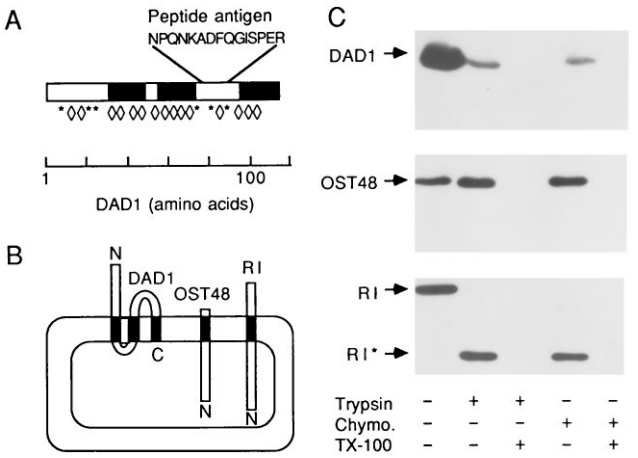

FIG. 4. Membrane topology of DAD1. (*A*) The predicted membrane spans of DAD1 are shown as solid bars. The location of the peptide (AsnProGlnAsnLysAlaAspPheGlnGlyIleSerProGluArg) used for antibody production is shown using the one letter code for amino acids. Potential trypsin sites (Lys and Arg) in DAD1 are indicated by asterisks, and potential chymotrypsin sites (Tyr, Phe, and Trp) are indicated by diamonds. (*B*) A model for the membrane topology of DAD1 locates both the N terminus and the antigenic peptide on the cytoplasmic face of the membrane. OST48 (18) and ribophorin I (R I) (19, 20) are integral membrane proteins with lumenal N termini (N). (*C*) Aliquots of PK-RM were incubated at 37°C for 3.75 h with either trypsin (60 μg/ml) or chymotrypsin (Chymo.) (60 μg/ml) with or without Triton X-100. Each sample was divided for subsequent SDS/PAGE using a 15% polyacrylamide gel for the DAD1 immunoblot or 9% polyacrylamide gels for the OST48 and ribophorin I (R I) immunoblots. The blots were probed with antibodies specific for the lumenal domains of ribophorin I and OST48. Proteolysis of ribophorin I yields a 50-kDa fragment (R I*) in the absence of detergent.

consistent with the predicted exposure of 10 amino acid residues on the cytoplasmic face of the membrane (Fig. 4*B*). A trypsin-dependent reduction in OST48 immunoreactivity was not observed when the immunoblots were probed with the lumenal-specific antibody to OST48 (Fig. 4*C*), but it was observed when the immunoblots were probed with an antibody to the cytoplasmic domain of OST48 (data not shown). The resistance of the lumenal domains of OST48 and ribophorin I to proteolysis by trypsin and chymotrypsin provided strong evidence that the vesicles remained intact unless the membrane bilayer was disrupted with the detergent Triton X-100. We conclude that the DAD1 epitope is exposed on the cytoplasmic face of the RER, consistent with the topology predicted in Fig. 4*B*.

Crosslinking of DAD1 to Other OST Subunits. Protein crosslinking was selected as a method to obtain evidence for direct contact between DAD1 and the three larger OST subunits in microsomal membranes and in the purified OST complex. The purified OST was incubated with either DSP or DTSSP. These two homobifunctional, amine-reactive, chemical crosslinking reagents were tested for the ability to link DAD1 to the other OST subunits using a wide range of crosslinker concentrations (4–600 μM). Here, we present data from a typical experiment using a single concentration of crosslinker. Protein immunoblots using antibodies to DAD1 revealed the presence of a 58-kDa DAD1-immunoreactive product (CL-1) that was produced when the purified OST was incubated with either DSP or DTSSP (Fig. 5*A*). The CL-1 product also was recognized by antibodies raised against OST48. The yield of the CL-1 product was greater with DSP than with the water soluble crosslinker DTSSP.

To demonstrate that the association between DAD1 and the OST subunits exists before detergent solubilization of the membrane, we determined whether CL-1 was produced when

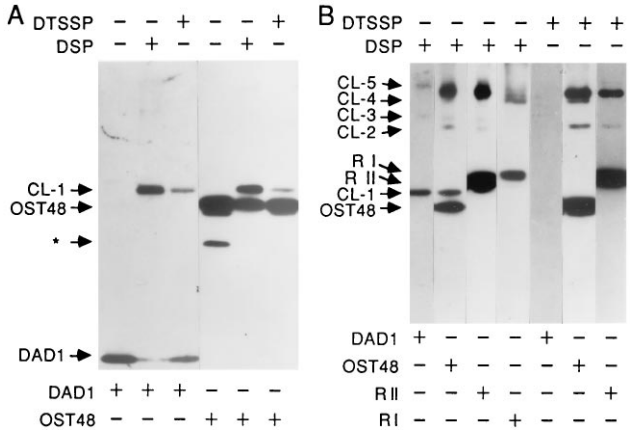

FIG. 5. Crosslinking of DAD1 to OST subunits. The purified OST was incubated with 75 μM DSP or 75 μM DTSSP (*A*), and PK-RM were incubated with 300 μM DSP or 300 μM DTSSP (*B*) as described. The OST subunits and the crosslinked products (CL-1–CL-5) were detected on protein immunoblots using antibodies specific for DAD1, ribophorins I (R I) and II (R II), and the lumenal domain of OST48. After primary and secondary antibodies were stripped from blots by exposure to acid pH, the blots were reprobed with antibodies to the other subunit to demonstrate the precise comigration of a given crosslinked product. The asterisk in *A* designates a proteolytic fragment of OST48 that was detected in the purified OST complex.

microsomal vesicles were incubated with DSP or DTSSP. PK-RM, which lack peripheral membrane components, including ribosomes, were incubated with the crosslinkers before protein immunoblot analysis (Fig. 5*B*). Control PK-RM samples that were not treated with DSP or DTSSP were probed with the panel of OST-specific antisera in parallel to establish the crosslinker dependence of each immunoreactive product, as in Fig. 5*A* (data not shown). A series of DSP-dependent products labeled CL-1–CL-5 were detected using antibodies to OST48. The CL-5 band consisted of two or more incompletely resolved products. The antibodies to DAD1 recognized the DSP-dependent products labeled CL-1 and CL-3 and the upper portion of the CL-5 band (Fig. 5B) in addition to DAD1 (not shown). Antibodies to ribophorin II recognized CL-2, CL-3, and the entire CL-5 region. A subset of the crosslinked products (CL-2, CL-4, and the faster migrating portion of CL-5) were obtained when PK-RM were incubated with the membrane-impermeable crosslinker DTSSP (Fig. 5*B*). Note that the DSP-dependent, crosslinked products that contained DAD1 (CL-1, CL-3, and the upper portion of CL-5) are absent. Thus, DAD1 was not crosslinked to OST48 with DTSSP in intact membranes, despite the observation that the α-amino and ε-amino groups in DAD1 were located on the cytoplasmic face of the membrane. As noted above, formation of crosslinks between DAD1 and OST48 in the purified enzyme was also less efficient with DTSSP than with DSP. The simplest interpretation of our results is that CL-2 was produced by crosslinking ribophorin II to OST48, and CL-3 was a heterotrimer of DAD1, OST48, and ribophorin II. The antibody to ribophorin I recognized the CL-4 and CL-5 products. These results suggest that the CL-5 region consists of crosslinked heterotrimers (ribophorins I and II and OST48) and heterotetramers (ribophorins I and II, OST48, and DAD1). The results of these crosslinking experiments provide evidence that DAD1 is directly associated with the previously identified subunits of the OST in intact membranes. The two antigens we detected in CL-4 were ribophorin I and OST48. However, this product migrates anomalously slowly relative to the CL-2 heterodimer and the CL-5 heterotrimer, raising the possibility that the CL-4 product may contain additional proteins that are not recognized by our panel of antibodies.

DISCUSSION

We have addressed the question of whether the DAD1 protein is an authentic homologue of the yeast Ost2 protein. The results presented here strongly support the conclusion that the vertebrate DAD1 protein is an equimolar and integral subunit of the OST. In WI38 cells, we were not able to detect a separate pool of DAD1; hence, we conclude that the DAD1 protein, like ribophorins I and II, is restricted to the RER. Based on these conclusions, we propose that our previous observations concerning the phenotype of yeast *ost2* mutants are of direct relevance to the apoptotic phenotype of the tsBN7 cell line. At the restrictive temperature, *ost2* mutants show a reduced stability of the Wbp1p and Swp1p subunits of the OST complex and a substantial reduction in the OST activity (6). Several features of the restrictive phenotype of the tsBN7 cell line are expected for cells with a defect in N-linked glycosylation. Immunoreactive DAD1 protein disappears from tsBN7 cells within 6 h after shift to the restrictive temperature. However, cell death and DNA fragmentation do not occur for an additional 24 h, suggesting that the loss of DAD1 does not result in an immediate initiation of apoptosis but instead that the cellular lesion induced by loss of DAD1 requires an extended incubation at the restrictive temperature. Indeed, recent evidence indicates that hypoglycosylated proteins are synthesized by tsBN7 cells at the restrictive temperature (Takeharu Nishimoto, personal communication). A gradual loss in viability also occurs when yeast *ost2* mutants are grown at the restrictive temperature (6). Unlike apoptosis induced by a variety of other stimuli, apoptosis of tsBN7 cells is not rescued by overexpression of *bcl-2* (4). Of interest, inhibition of protein synthesis by cycloheximide prevents, or at least delays, the DNA fragmentation and cell death that occurs when tsBN7 cells are cultured at the restrictive temperature (4), perhaps because cycloheximide prevents the synthesis of the hypoglycosylated proteins. The recent report that tunicamycin, an inhibitor of dolichol-linked oligosaccharide assembly, induces DNA fragmentation and apoptosis of HL-60 cells (21) is consistent with the hypothesis that a disruption of N-linked glycosylation can lead to apoptotic cell death.

A role for DAD1 as a subunit of the OST does not provide an adequate explanation for the more recent observation that transient overexpression of human DAD1 in *C. elegans* permits survival of a subset of cells that normally undergo programmed cell death (5). As noted by Sugimoto *et al.* (5), overexpression of human or *C. elegans* DAD1 does not cause the robust enhancement in cell survival that has been observed upon transient overexpression of human *bcl-2* (22) or *C. elegans ced-9* (2). Could the DAD1 protein be an important target for destruction by ICE-like proteases in cells that are programmed for death? Although our protein topology experiment indicates that the hydrophilic domains of DAD1 are located on the cytoplasmic side of the RER membrane, we believe that a compelling hypothesis for how the DAD1 subunit of the OST might perform a pivotal role in programmed cell death during development is currently lacking. Instead, we feel that transient overexpression of DAD1 in *C. elegans* may indirectly influence commitment to cell death perhaps by sequestration of a positive regulatory component.

Proteolysis of intact membranes with either chymotrypsin or trypsin destroyed the DAD1 epitope without producing smaller immunoreactive fragments that could be detected on high percentage polyacrylamide gels. These results confirm and extend our previous analysis of the membrane topology of Ost2p. Taken together, these two studies show that both hydrophilic regions of DAD1/Ost2 protein face the cytoplasm and suggest that the protein contains three membrane-spanning segments. A comparison of the DAD1/OST2 sequences from vertebrates, invertebrates, plants, and fungi reveals that the amino-terminal region preceding the first

membrane-spanning segment is the least conserved region of the protein both with respect to length and amino acid sequence identity. The most highly conserved region of DAD1/Ost2p consists of the second and third membrane-spanning segments plus the cytoplasmic loop. These regions of the protein are probably the most crucial for DAD1/Ost2p function. As noted previously (6), point mutations that interfere with DAD1/Ost2p stability or function occur at the site of invariant or highly conserved amino acid residues that are located within the membrane-spanning segments or at the predicted boundaries of the membrane-spanning segments.

Previous observations concerning the conservation and expression of the DAD1 protein are consistent with a role for DAD1 in N-linked glycosylation. The human DAD1 protein, like the ribophorins (13), is expressed in all tissues that have been examined (4). DAD1 homologues have been identified in vertebrates, invertebrates, plants, and fungi consistent with the ubiquitous N-linked glycosylation of proteins in eukaryotes. The demonstration that DAD1 is an OST subunit provides further evidence for the structural conservation of this enzyme between yeast and vertebrates. The yeast enzyme was initially purified as a complex of six polypeptides (7) that are designated Ost1p, Wbp1p, Ost3p, Swp1p, Ost2p, and Ost5p. Protein sequence comparisons have disclosed the following relationships: Ost1p is homologous to ribophorin I; Swp1p is homologous to ribophorin II; and Wbp1p is homologous to OST48 (as reviewed in ref. 23). The Ost3 protein is a nonessential 34-kDa subunit of the yeast OST complex (24), and the yeast *OST5* gene encodes a nonessential 9-kDa subunit (25). Genetic screens have identified two additional yeast loci (*STT3* and *OST4*) that are essential for OST activity *in vitro* and *in vivo* (26, 27). We hypothesize that the less abundant, yet more active, forms of the canine OST contain one or more additional polypeptides that are homologues of Stt3p, Ost3p, Ost4p, and Ost5p. Current efforts in our laboratory are directed toward the isolation and characterization of the more active forms of the canine OST complex.

The crosslinking studies confirmed that the DAD1 protein was adjacent to the previously identified OST subunits in the microsomal membrane. Specifically, we observed that DAD1 can be crosslinked to OST48 via lysine residues that are located on the cytoplasmic face of the membrane. Crosslinked heterodimers of OST48–ribophorin II and crosslinked heterotrimers of OST48, ribophorin II, and DAD1 also were detected. Previously, direct contact between the yeast homologues of these protein subunits (Wbp1p, Ost2p, and Swp1p) was surmised based upon the observation that the *SWP1* and *OST2* genes are allele-specific, high copy suppressors of the conditional *wbp1–2* mutant (6, 28). Gene product depletion experiments have disclosed a reciprocal relationship between the stability of Swp1p and Wbp1p; reduced expression of one subunit leads to a reduced membrane content of the other subunit (29). Together, these studies suggest that ribophorin II, OST48, and DAD1 constitute a structural unit within the OST core enzyme. The results we present regarding the topological properties of DAD1 and the nearest neighbor relationships between DAD1 and the other OST subunits will serve to focus future work on the vital role of DAD1 in the OST complex.

We thank Dr. Kennan Kellaris (Georgetown University) for the preparation of antibodies to the cytoplasmic domain of OST48. We thank Dr. Takeharu Nishimoto (Kyushu University) for providing the antibody to DAD1 and for communicating results concerning OST activity in tsBN7 cells before publication. We thank Dr. Gert Kreibich (New York University) for providing antibodies to ribophorins I and II and to OST48. This work was supported by Public Health Services Grant GM 43768.

1. Vaux, D. L. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 786–799.
2. Hengartner, M. O. & Horvitz, H. R. (1994) *Cell* **76,** 665–676.
3. Chinnaiyan, A. M., Orth, K. K. O. R., Duan, H., Poirier, G. G. & Dixit, V. M. (1996) *J. Biol. Chem.* **271,** 4573–4576.
4. Nakashima, T., Sekiguchi, T., Kuraoka, A., Fukushima, K., Shibata, Y., Komiyama, S. & Nishimoto, T. (1993) *Mol. Cell. Biol.* **13,** 6367–6374.
5. Sugimoto, A., Hozak, R. R., Nakashima, T., Nishimoto, T. & Rothman, J. H. (1995) *EMBO J.* **14,** 4434–4441.
6. Silberstein, S., Collins, P. G., Kelleher, D. J. & Gilmore, R. (1995) *J. Cell Biol.* **131,** 371–383.
7. Kelleher, D. J. & Gilmore, R. (1994) *J. Biol. Chem.* **269,** 12908–12917.
8. Kumar, V., Heinemann, S. & Ozols, J. (1994) *J. Biol. Chem.* **269,** 13451–13457.
9. Kumar, V., Korza, G., Heinemann, F. S. & Ozols, J. (1995) *Arch. Biochem. Biophys.* **320,** 217–223.
10. Kelleher, D. J., Kreibich, G. & Gilmore, R. (1992) *Cell* **69,** 55–65.
11. Walter, P. & Blobel, G. (1983) *Methods Enzymol.* **96,** 84–93.
12. Yu, Y., Sabatini, D. D. & Kreibich, G. (1990) *J. Cell Biol.* **111,** 1335–1342.
13. Marcantonio, E. E., Grebenau, R. C., Sabatini, D. D. & Kreibich, G. (1982) *Eur. J. Biochem.* **124,** 217–222.
14. Marcantonio, E. E., Amar-Costesec, A. & Kreibich, G. (1984) *J. Cell. Biol.* **99,** 2254–2259.
15. Rosenfeld, M. G., Marcantonio, E. E., Hakimi, J., Ort, V. M., Atkinson, P. H., Sabatini, D. D. & Kreibich, G. (1984) *J. Cell Biol.* **99,** 1076–1082.
16. Görlich, D., Prehn, S., Hartmann, E., Kalies, K.-U. & Rapoport, T. A. (1992) *Cell* **71,** 489–503.
17. Breuer, W. & Bause, E. (1995) *Eur. J. Biochem.* **228,** 689–696.
18. Silberstein, S., Kelleher, D. J. & Gilmore, R. (1992) *J. Biol. Chem.* **267,** 23658–23663.
19. Crimaudo, C., Hortsch, M., Gausepohl, H. & Meyer, D. I. (1987) *EMBO J.* **6,** 75–82.
20. Harnik-Ort, V., Prakash, K., Marcantonio, E., Colman, D. R., Rosenfeld, M. G., Adesnik, M., Sabatini, D. D. & Kreibich, G. (1987) *J. Cell Biol.* **104,** 855–863.
21. Pérez-Salva, D. & Mollinedo, F. (1995) *J. Cell. Physiol.* **163,** 523–531.
22. Vaux, D. L., Weissman, I. L. & Kim, S. K. (1992) *Science* **258,** 1955–1957.
23. Silberstein, S. & Gilmore, R. (1996) *FASEB J.* **10,** 849–858.
24. Karaoglu, D., Kelleher, D. J. & Gilmore, R. (1995) *J. Cell Biol.* **130,** 567–577.
25. Reiss, G., te Heesen, S., Gilmore, R., Zufferay, R. & Aebi, M. *EMBO J.* **16,** 1164–1172.
26. Chi, J. H., Roos, J. & Dean, N. (1996) *J. Biol. Chem.* **271,** 3132–3140.
27. Zufferay, R., Knauer, R., Burda, P., Stagljar, I., te Heesen, S., Lehle, L. & Aebi, M. (1995) *EMBO J.* **14,** 4949–4960.
28. te Heesen, S., Knauer, R., Lehle, L. & Aebi, M. (1993) *EMBO J.* **12,** 279–284.
29. Knauer, R. & Lehle, L. (1994) *FEBS Lett.* **344,** 83–86.