DAD1 Is Required for the Function and the Structural Integrity of the Oligosaccharyltransferase Complex*

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Asparagine-linked glycosylation is a highly conserved protein modification reaction that occurs in all eukaryotic organisms. The oligosaccharyltransferase (OST), which has its active site exposed on the luminal face of the endoplasmic reticulum (ER), catalyzes the transfer of preassembled high mannose oligosaccharides onto certain asparagine residues of nascent polypeptides. The mammalian OST complex was initially thought to be composed of three transmembrane proteins, ribophorin I (RI), ribophorin II (RII), and OST48. Most recently, a small integral membrane protein of 12 kDa called DAD1 has been identified as an additional member of the mammalian OST complex. A point mutation in the DAD1 gene is responsible for the temperature-sensitive phenotype of a baby hamster kidney-derived cell line (tsBN7) that undergoes apoptosis at the non-permissive temperature. Furthermore, the mutant protein DAD1 is not detectable in tsBN7 cells 6 h after shifting the cells to the non-permissive temperature. This temperature-sensitive cell line offered unique opportunities to study the effects caused by the loss of one OST subunit on the other three subunits and also on N-linked glycosylation. Western blot analysis of cell lysates showed that after 6 h at the non-permissive temperature, steady-state levels of the ribophorins were reduced by about 50%, and OST48 was barely detectable. On the other hand, steady-state levels of other components of the rough ER, such as the α-subunits of the TRAP (translocon-associated membrane protein) and the Sec61 complex, which are components of the translocation apparatus, are not affected by the instability of the OST subunits. Furthermore, N-glycosylation of the ribophorins was seriously affected 6 h after shifting the cells to the non-permissive temperature, and after 12 h they were synthesized only in the non-glycosylated form. As may be expected, this defect in the OST complex at the non-permissive temperature caused also the underglycosylation of a secretory glycoprotein. We concluded that degradation of DAD1 at the non-permissive temperature not only affects the stability of OST48 and the ribophorins but also results in the functional inactivation of the OST complex.

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1 The abbreviations used are: OST, oligosaccharyltransferase; ER, endoplasmic reticulum; BiP, heavy chain binding protein; endo H, endoglycosidase H; BHK, baby hamster kidney; RI and RII, ribophorin I and II, respectively; SEAP, soluble form of the bovine alkaline phosphatase; PAGE, polyacrylamide gel electrophoresis; SSRα, signal sequence receptor α.
DAD1 was initially identified as a negative regulator of apoptosis in a temperature-sensitive mutant hamster cell line (tsBN7) (20). The mutant DAD1 protein contains a single amino acid substitution, which results in its rapid degradation at the non-permissive temperature, such that the protein can no longer be detected after 6 h. This degradation of DAD1 precedes the onset of apoptosis, suggesting that it may be the event that triggers programmed cell death. The tsBN7 cells could be rescued at the non-permissive temperature upon transfection with the DAD1 wild-type gene (20). Recently, it has been shown that this membrane protein assumes a U-loop disposition in the ER membrane with both the N and C termini exposed at the cytoplasmic surface. Furthermore, at the non-permissive temperature N-glycosylation was suppressed (22). We became interested in the tsBN7 cell line as it offered an opportunity to analyze the effects of the deletion of one of the OST subunits on the stability of other subunits and also on the enzymatic activity of the whole complex. The results of this study demonstrate that DAD1 is an essential component of the OST complex and that its loss causes the rapid degradation of OST48 and, also to a lesser extent, of the ribophorins. As might be expected, the degradation of the OST subunits is accompanied by a complete loss of the N-glycosylation activity.

MATERIALS AND METHODS

Cell Culture—BHK-21 and tsBN7 cells were obtained from Dr. C. Basilico (New York University Medical Center, New York). Cells were cultured in 10% fetal bovine serum (Hyclone) with penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were grown at 33 °C (compare lanes k and l) or as controls (compare lanes a and b) tsBN7 cells were grown on coverslips at 33 °C (a) or at 39.5 °C (b and c) for 24 h (b) or 48 h (c). Control BHK cells were also grown for 48 h at 39.5 °C (d). Cells were fixed with 2% paraformaldehyde and permeabilized with 0.2% Triton X-100, and the nuclei were stained with the Hoechst 33258 stain.

Antibodies—Antibodies directed against RI and RII are those previously described (23). The antibody against the signal sequence receptor (SSR), was raised against an amino-terminal peptide (20 amino acids) of the canine protein (24). Antibodies directed against the soluble form of the bovine alkaline phosphatase (SEAP, metabolic labeling, and immunoprecipitations were done as described before (25).

RESULTS

**tsBN7 Cells Undergo Apoptosis at the Non-permissive Temperature**—It has been previously shown by DNA fragmentation analysis that tsBN7 cells, which harbor a point mutation in the DAD1 gene, undergo apoptosis at the non-permissive temperature (20). To demonstrate that cells used for the experiments described below indeed shifted into an apoptotic state when grown at the non-permissive temperatures, we used the Hoechst staining procedure to visualize the nuclei by fluorescence microscopy. Compared with wild-type BHK-21 cells (Fig. 1D), the mutant tsBN7 cells grown at the non-permissive temperature for 48 h (C) displayed the typical morphology of apoptotic nuclei, as indicated by their condensed or fragmented appearance (Fig. 1, compare panels C and D). This effect was more profound when cells were kept for longer times at the non-permissive temperature.

**Effect of Temperature Shift on the Steady-state Levels of the OST Subunits**—The mammalian OST is a heterooligomer composed of four non-identical subunits. It contains the two ribophorins, OST48 and a recently identified small hydrophobic protein DAD1 (4, 22). Based on results obtained from Western blot analysis of cell lysates, Nakashima et al. (20) have previously shown that the DAD1 protein is no longer detectable by Western blot analysis 6 h after incubation of the temperature-sensitive tsBN7 cell line at the non-permissive temperature. To test whether the loss of the DAD1 protein affects the steady-state levels of other subunits of the OST complex, tsBN7 cells (ts in Fig. 2) or as controls (C in Fig. 2), BHK cells their wild-type counterpart were grown at the non-permissive temperature (39.5 °C) for up to 48 h. Equal amounts of protein from lysates of tsBN7 and BHK-21 cells were electrophoresed and blotted onto nitrocellulose sheets, followed by immunostaining of RI, RII, and OST48 with the respective antibodies. In contrast to DAD1, which disappears within 6 h after the temperature shift (20), a band corresponding to RI can be recognized even after prolonged incubation at the non-permissive temperature (Fig. 2A). After 48 h, protein levels decreased to about 50% of those observed in lysates of wild-type BHK cells grown for the same time period at 39.5 °C (compare lanes k and l). At

![Fig. 1. tsBN7 cells undergo apoptosis at the non-permissive temperature](image-url)
Indicated by the similar intensities of the RI bands immuno-
viable because the rate of RI synthesis is not affected, as
seen in tsBN7 cells after growth of the non-permissive temper-
ature (Fig. 3, lanes e–h). This indicates that ribophorin II is a rather stable protein. The
ratio of the glycosylated (RII) to the non-glycosylated form
of RII also did not change. As expected, endo H treat-
ment shifted the upper band to the position of the non-glyco-
sylated form (lanes b and d). On the other hand, when the
cells were grown for 24 h at the non-permissive temperature
and analyzed immediately after the pulse (lanes e and f),
only the non-glycosylated form of RII was made. Furthermore,
after a 6-h chase period at 39.5 °C (lanes g and h), the
only non-glycosylated form of RII was made. This confirms our presumption that degradation of DAD1 at the
non-permissive temperature results in the destabilization of
the whole OST complex, which is accompanied by a loss in
N-glycosylation activity.

To determine whether the inhibition of N-glycosylation in
tsBN7 cells grown at the non-permissive temperature is a
generalized effect and is not limited to the RI and RII subunits
of the OST complex, we studied the N-glycosylation pattern of
a secretory glycoprotein, the secreted form of alkaline phospha-
tase (SEAP) (25, 28), which contains two N-linked oligosacca-
rides. SEAP is a truncation mutant in which the 24 most
COOH-terminal amino acids of this normally membrane-anchored plasma membrane protein are deleted (28). This protein
is normally not expressed in tsBN7 cells; as expected, immu-
noprecipitates obtained from non-transfected cells did not show

At the Non-permissive Temperature, N-Glycosylation Is In-
hibited in tsBN7 Cells—To study the effects of degradation of
DAD1 and OST48 on the N-glycosylation of RI, tsBN7 cells
were grown at the non-permissive temperature for 0, 6, 12, and
24 h, and they were then labeled for 1 h with [35S]methionine.
Cell lysates were immunoprecipitated with anti-RI antibodies,
and aliquots of the immunoprecipitates were left untreated
(Fig. 3, lanes a, c, e, and g) or treated with endo H (lanes b, d,
f, and h). Samples were subjected to SDS-PAGE, followed by
autoradiography. As shown in Fig. 3, two closely spaced bands
were immunoprecipitated by the anti-RI antibody 6 h after
culturing tsBN7 cells at the non-permissive temperature (lane
c). The band of lower mobility disappears upon endo H treat-
ment (lane d), indicating that at this time point both the glyco-
sylated and the non-glycosylated forms of RI were made. 12 h
after the temperature shift, only the non-glycosylated form of
RI was made (lane e), which was, as expected, insensitive to
endo H (lane f). It is important to note that even after 24 h at
the non-permissive temperature (lanes g and h), the cells are
viable because the rate of RI synthesis is not affected, as
indicated by the similar intensities of the RI bands immuno-
precipitated after labeling cells 6, 12, or 24 h after the temper-

ature shift. Therefore, the decrease in the steady-state level of
RI at the non-permissive temperature, as determined by West-
ern blot analysis (Fig. 2A), must be attributed to higher rates of degradation and not to a lower rate of synthesis.

Fig. 4 illustrates experiments in which the glycosylation patterns of RII and its stability were compared in tsBN7 cells
grown at the permissive (lanes a–d) or at the non-permissive temperature (lanes e–h). Cells were grown for 24 h at 33 °C
(lanes a–d) or 39.5 °C (lanes e–h) and labeled for 1 h with
[35S]methionine. Cells were harvested after 0, 6, 12, 24, 36,
and 48 h. Cell lysates (50 μg of protein each) were electrophoresed and analyzed by immunoblotting
with an antibody directed against RI (A) or OST48 (B).

Fig. 2. tsBN7 cells grown at the non-permissive temperature
show decreased steady-state levels of ribophorin I. tsBN7 (ts)
and BHK-21 (C) cells were shifted to the non-permissive temperature, and
lanes g
the non-permissive temperature (
lanes a–d) or at the non-permissive

temperature (lanes e–h). Cells were grown for 24 h at 33 °C
(lanes a–d) or 39.5 °C (lanes e–h) and labeled for 1 h with
[35S]methionine. Cells were harvested immediately after the
pulse (0 h, lanes a, b, e, and f), after a chase period of 6 h at
33 °C (lanes c and d) or after a 6-h chase at 39.5 °C (lanes g and
h). Samples were kept untreated (lanes a, c, e, and g) or were
were grown for 24 h at the non-permissive temperature for 0, 6, 12, and 24 h, and
cells were labeled with [35S]methionine for 1 h. 50-μg protein aliquots
of cell lysates were prepared and immunoprecipitated with anti-RI anti-

bodies. The immunoprecipitates were kept untreated (lanes a, c, e, g) or treated with endo H (lanes b, d, f, and h) to remove high mannos
oligosaccharides. Samples were subjected to SDS-PAGE followed by
autoradiography.
bands corresponding to SEAP (Fig. 5, lanes a and b). When tsBN7 cells were transfected with cDNA encoding SEAP and were then metabolically labeled at the permissive temperature, a band of about 64 kDa was immunoprecipitated (lane c) that corresponds to SEAP carrying two N-linked oligosaccharides (arrow). A second band of slightly lower electrophoretic mobility (in lanes c and d) represents the N-glycosylated form of SEAP, which has exited from the ER and has received Golgi modifications of the N-linked oligosaccharides, rendering them insensitive to the treatment with endo H (compare lanes c and d). This form is also found in the medium as a secretory product (not shown). Treatment of the ER form of SEAP with endo H results in its deglycosylation, which is accompanied by a shift to a band of increased electrophoretic mobility (lane d). SEAP species immunoprecipitated from cells labeled at the non-permissive temperature (lanes e and f) are underglycosylated and carry only one (*) or no (**) N-linked oligosaccharides (lane e). As expected, these forms of SEAP are sensitive to endo H treatment (lane f). It is interesting to note that the underglycosylated form of SEAP (*) and probably also the non-glycosylated form (**) generated at the non-permissive temperature (lane e) do not exit from the ER, as indicated by the absence of a band of lower electrophoretic mobility, which is seen in cells kept at the permissive temperature (lanes c and d). Accordingly, at the non-permissive temperature, SEAP is not found in the medium (not shown). These results demonstrate that at the non-permissive temperature, inhibition of N-glycosylation affects not only the subunits of the OST complex but also secretory glycoproteins such as SEAP.

Degradation of OST Subunits at the Non-permissive Temperature Does Not Affect the Stability of Other ER Proteins—The finding that degradation of the mutant DAD1 protein at the non-permissive temperature affected the stability of the other components of the OST complex was especially significant because other ER membrane proteins remained completely unaffected. In fact, we observed an increase in the steady-state level of BiP, a soluble protein of the ER lumen, which is a member of the heat shock protein family (29) (Fig. 6). Under normal growth conditions, BiP is synthesized constitutively. Its synthesis can be induced, however, by the accumulation of unfolded proteins or by a variety of stress conditions, including glucose starvation or treatment with tunicamycin (30). When tsBN7 cells were grown at the non-permissive temperature, the steady-state levels of BiP had more than doubled after 24 h, whereas OST48 and the ribophorins became destabilized and were degraded (see Figs. 2 and 4). On the other hand, steady-state levels of the α-subunit of TRAP, also called SSRα, an abundant ER protein located in close proximity to the translocation apparatus (24, 31), are not affected, even when tsBN7 cells are cultured for prolonged periods of time at the non-permissive temperature (Fig. 6). The same is true for Sec61p, a multispanning membrane protein that serves as a receptor for ribosomes (32, 33) and is part of the translocation pore (32). These results demonstrate that degradation of DAD1, which is accompanied by the destabilization of the entire OST complex, does not affect the stability of other components of the ER.

DISCUSSION

The major finding obtained from this study is that DAD1, the recently identified fourth subunit of the mammalian OST complex (4, 22), is required for the functional and structural integrity of the oligosaccharyltransferase. We have shown that when tsBN7 cells, which harbor a temperature-sensitive mutation in the DAD1 gene, are grown at the non-permissive temperature, steady-state levels of RI and RIH are significantly reduced, and OST48 is reduced to barely detectable levels 6 h after the temperature shift. As the mutation, rendering DAD1 unstable at the non-permissive temperature, has a comparable effect on the stability of OST48, one may suspect that OST48 interacts physically with DAD1. Alternatively, the rapid degradation may be a reflection of the intrinsic susceptibility of the unassembled subunit toward degradative mechanisms. The former conclusion is supported by experiments where interactions
among the OST subunits were identified using the yeast two-hybrid assay (21). It was shown that DAD1 is directly linked via its amino-terminal domain to the short cytoplasmic segment of OST48. On the other hand, the luminal domains of both RI and RII interact with that of OST48 in such a way that the ribophorins are only indirectly connected to the DAD1 protein. Similar conclusions were reached from chemical cross-linking experiments on the isolated mammalian OST complex (4), although specific interacting domains could not be determined in this way. Support for interactions between DAD1 and OST48 was also obtained from genetic tests and chemical cross-linking experiments affecting the yeast homologues (16, 19). Studies performed on the yeast OST, which contains apparently seven subunits (8), have demonstrated that the OST2 gene, which is homologous to DAD1, is a suppressor of a mutation in the WBP1 gene (OST48 in mammals) and that overexpression of Ost2p increases the stability of Wbp1p as well as that of the RII homolog Swp1p (19). Accordingly, overexpression of SWP1 results in the suppression of the temperature-sensitive phenotype of WBP1 (16).

Although about 30% of the tsBN7 cells cultured for 24 h at 39.5 °C are at a late state of apoptosis (Fig. 1), there is no change in the rate of synthesis of RI, suggesting that basic metabolic functions are not yet impaired and that the decrease in the steady-state levels of the OST subunits is a consequence of their accelerated rate of degradation at the non-permissive temperature. After culturing tsBN7 cells for 12 h at 39.5 °C, N-glycosylation of RI was completely suppressed, despite the fact that the steady-state levels of both ribophorins were still about 50% compared with cells grown at 33 °C. This indicates that OST48 and DAM1, subunits of the OST that have almost 50% compared with cells grown at 33 °C. This indicates that the steady-state levels of both ribophorins were still about 50% compared with cells grown at 33 °C. This indicates that OST48 and DAM1, subunits of the OST that have almost completely disappeared after 6 h, are needed for the functioning of the oligosaccharyltransferase. This is in agreement with the finding that the yeast OST subunits Wbp1p and Ost2p, which are homologous to OST48 and DAM1, respectively, are both essential gene products, and their deletion affects N-glycosylation (15, 19). In yeast, the genes encoding the OST subunits Ost1p and Swp1p are also essential (17, 18); therefore, it may be expected that the same is true for RI and RII in the mammalian OST complex. According to our yeast two-hybrid assays, where we tested for interactions among the OST subunits, it was found that the two ribophorins do not interact directly with each other, but instead RI and RII interact with OST48 via their luminal domains. Degradation of OST48 is therefore expected to have deleterious effects on the structure and stability of the ribophorins and, therefore, on their function.

Because the active site of the OST complex is located at the luminal side of the ER (2), inhibition of N-glycosylation in tsBN7 cells at the non-permissive temperature is not expected to interfere with the insertional into, and the translocation across the ER of soluble or membrane proteins. In fact, it has been shown that purified Sec61p, which represents apparently the core of the translocation apparatus, can accomplish polypeptide translocation in the absence of N-glycosylation when reconstituted into lipid vesicles (for review, see Ref. 34). Our experiments have demonstrated that despite the biochemical evidence for a tight interaction between the OST complex and Sec61p (32), destabilization of the subunits of the OST complex at the non-permissive temperature does not affect the steady-state concentration of the alpha subunit of the Sec61 complex. This is apparently a reflection of the fact that the two complexes represent distinct oligomers that can be purified as such (3, 4, 32). Inhibition of N-glycosylation may, however, result in the misfolding of proteins (35), which causes an induction of BiP in the ER lumen of tsBN7 cells.

The DAD1 protein was initially characterized as an integral membrane protein that suppresses apoptotic cell death, as a specific point mutation in the DAD1 gene triggered programmed cell death at the non-permissive temperature (Fig. 1; see also Ref. 20). The time lag between complete degradation of the DAD1 protein (6 h) and onset of apoptosis (24 h) suggested an indirect effect on apoptosis. It is plausible that the accumulation of underglycosylated and malformed proteins made in the absence of DAD1 (and OST48) causes a stress response from the ER (35), leading to the activation of certain genes that ultimately result in programmed cell death. In fact, we have shown earlier (36) that levels of CHOP, a transcription factor that is activated by agents that adversely affect the folding of proteins in the endoplasmic reticulum, are elevated in tsBN7 cells at the non-permissive temperature. It was found that CHOP levels peak at 6 h and taper off after 24 h (36). That inhibition of N-glycosylation may indeed lead to apoptosis, albeit by an indirect mechanism, is also supported by experiments where HL-60 cells from a human promyelocytic cell line were treated with tunicamycin, a drug that interferes with the synthesis of the dolichol pyrophosphate oligosaccharide (37).

In this case, the substrate for the oligosaccharyltransferase, dolichol pyrophosphate oligosaccharide, is not made, resulting in the suppression of N-glycosylation of newly synthesized polypeptides. The latter effect is obtained in tsBN7 cells grown at the non-permissive temperature due to degradation of the subunits of the oligosaccharyltransferase complex. Loss of N-glycosylation activity may result in the accumulation of under- and non-glycosylated proteins that may not fold properly and, therefore, do not exit from the ER (35). We have in fact demonstrated that the underglycosylated form of the secreted form of alkaline phosphatase (SEAP), which was used as a reporter protein for the N-glycosylation activity of the OST complex, does not exit from the ER. It seems, therefore, either that a specific N-glycosylated protein is required to suppress apoptosis or that a nonspecific accumulation of improperly folded proteins in the ER initiates the apoptotic response.

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