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# Localization of ribophorin II to the endoplasmic reticulum involves both its transmembrane and cytoplasmic domains

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**Proteins that are concentrated in specific compartments of the endomembrane system in order to exert their organelle-specific function must possess specific localization signals that prevent their transport to distal regions of the exocytic pathway. Some resident proteins of the endoplasmic reticulum (ER) that are known to escape with low efficiency from this organelle to a post ER compartment are recognized by a recycling receptor and brought back to their site of residence. Other ER proteins, however, appear to be retained in the ER by mechanisms that operate in the organelle itself. The mammalian oligosaccharyltransferase (OST) is a protein complex that effects the cotranslational N-glycosylation of newly synthesized polypeptides, and is composed of at least four rough ER-specific membrane proteins: ribophorins I and II (RI and RII), OST48, and Dad1. The mechanism(s) by which the subunits of this complex are retained in the ER are not well understood. In an effort to identify the domains within RII responsible for its ER localization we have studied the fate of chimeric proteins in which one or more RII domains were replaced by the corresponding ones of the Tac antigen, the latter being a well characterized plasma membrane protein that lacks intrinsic ER retention signals and serves to provide a neutral framework for the identification of retention signals in other proteins. We found that the luminal domain of RII by itself does not contain retention information, while the cytoplasmic and transmembrane domains contain independent ER localization signals. We also show that the retention**

**function of the transmembrane domain is strengthened by the presence of a flanking luminal region consisting of 15 amino acids.**

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*Abbreviations.* BiP Immunoglobulin heavy chain binding protein. – Dad1 Defender against apoptotic death. – ER Endoplasmic reticulum. – OST Oligosaccharyltransferase. – PDI Disulfide isomerase. – RI and RII Ribophorin I and II. – RER Rough endoplasmic reticulum. – RM Rough microsomes. – SDS-PAGE Sodiumdodecylsulfate-polyacrylamide gel electrophoresis. – SSR signal sequence receptor.

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## Introduction

After insertion of polypeptides into, or translocation across the membrane of the ER, some polypeptides remain in that organelle as resident proteins, while others are transported to distal compartments of the endomembrane system. If they reach the plasma membrane and lack a membrane anchor they are released into the extracellular space by exocytosis. The maintenance of the characteristic protein composition of the organelles that comprise the secretory pathway requires that their proteins possess localization signals that determine their site of accumulation. The concentration in the ER of certain proteins that tend to escape from this organelle is assured by mechanisms that involve their retrieval from post-ER compartments, while other ER constituents are actually prevented from leaving the organelle (Sabatini and Adesnik, 1995; Teasdale and Jackson, 1996). The retrieval from a post ER compartment of escaped luminal ER proteins carrying a C-terminal KDEL-type motif is effected by a well characterized receptor, which resides in the Golgi apparatus when not carrying a ligand (Munro and Pelham, 1987; Lewis and Pelham, 1992). Similarly, some type I membrane proteins that may escape the ER have two critical lysine residues in positions -3 and -4 or -5 relative to their carboxyl termini

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(Jackson et al., 1990, 1993) that constitute a signal recognized by coat proteins that function in the formation of vesicles that mediate retrograde transport from the Golgi apparatus to the ER (Letourneur et al., 1994; Fiedler et al., 1996; Cosson et al., 1996; Cosson and Letourneur, 1997). Some ER type II transmembrane proteins contain a cytoplasmically exposed N-terminal double-arginine motif that also serves as a retrieval signal (Schutze et al., 1994). Several mechanisms can lead to retention of proteins that do not have identifiable retrieval signals. Misfolded or incompletely folded proteins in the ER lumen may interact with chaperones, such as BiP, calnexin or PDI, which themselves have retrieval signals (Teasdale and Jackson, 1996; Leitzgen and Haas, 1998; Degen and Williams, 1991; Bergeron et al., 1994). Retention of ER membrane proteins may also be achieved by functional interactions of their luminal domains with an organized matrix of luminal ER proteins, or by interaction of their cytoplasmic domains with cytoskeletal elements, such as microtubules (Dahllöf et al., 1991). Another mechanism that may lead to retention of membrane proteins would entail their incorporation into oligomeric complexes or even larger assemblies that cannot be included in carrier vesicles that leave the organelle. Such macromolecular assemblies have been described and they involve components of the translocation apparatus in the ER, including the oligosaccharyltransferase (OST) complex that catalyzes the N-glycosylation of nascent polypeptides (Sabatini and Adesnik, 1995; Kreibich et al., 1978a,b; Hortsch and Meyer, 1985; Wiedemann et al., 1989; Sitia and Meldolesi, 1992; Görlich et al., 1992). Similar macromolecular assemblies have been implicated in the retention of membrane proteins in individual Golgi stacks (Swift and Machamer, 1991; Machamer, 1991; Munro, 1991; Nilsson et al., 1991; Nilsson and Warren, 1994).

The mammalian oligosaccharyltransferase is a resident ER oligomeric protein complex composed of four subunits: ribophorins I and II, OST48 and Dad1 (Kelleher and Gilmore, 1997). The ribophorins are transmembrane glycoproteins that were originally identified as the first markers for the rough portions of ER membranes (Kreibich et al., 1978a,b). They were later shown to be subunits of the OST complex responsible for transferring a preassembled oligosaccharide from a membrane bound lipid donor to an acceptor asparagine residue within a nascent polypeptide (Kelleher et al., 1992). RI and OST48, and probably also RII, have a type I transmembrane disposition, whereas Dad1 apparently has a U-loop disposition (Makishima et al., 1997). We have recently shown that the luminal domain of OST48 interacts with those of RI and RII and that the cytoplasmic domain of OST48 has affinity for the cytoplasmically exposed N-terminal tail of Dad1 (Fu et al., 1997). Furthermore, the OST complex interacts directly with Sec61p, the core component of the protein translocation apparatus that constitutes the translocation channel (Görlich et al., 1992) and also provides sites for ribosome binding (Kalies et al., 1994).

Results obtained over many years have demonstrated that components of the translocation apparatus, including those involved in the cotranslational modification of newly synthesized polypeptides, are part of a proteinaceous network that confines these membrane proteins to the rough domain of the ER (Kreibich et al., 1978a,b; Hortsch and Meyer, 1985; Marcantonio et al., 1984; Amar-Costesec et al., 1984). Incorporation of proteins into this network was suggested to be an effective means of preventing their exit from the ER towards

the Golgi apparatus (Sabatini and Adesnik, 1995; Kreibich et al., 1978a,b; Hortsch and Meyer, 1985; Sitia and Meldolesi, 1992). Because newly synthesized components forming such a putative network do not become immediately incorporated into the oligomeric complex, and yet do not escape from the ER (Tsao et al., 1992; Ivessa et al., 1992) we hypothesize that individual subunits themselves carry ER localization motifs that guarantee their retention until they are assembled into the network. While OST48 contains a typical dilysine retrieval motif at the C-terminal -3 and -5 positions, no retrieval motifs can be recognized in RI, RII and Dad1. In this study, we searched for ER localization domains in RII and found that retention information is contained independently in the cytoplasmic domain and in a region encompassing the transmembrane domain together with a short luminal flanking segment.

## Materials and methods

### Plasmid construction

The rat RII cDNA was cloned in this laboratory (Pirozzi et al., 1991). To insert RII, II•••, II•T•T, II•II•T, T/II•II•T into pMT-2 (Kaufman, 1990), or T•T•T, T•II•T, and T•T•II into pExp (gift of Dr. Herbert Samuels, New York University Medical Center, New York, NY), standard PCR or PCR for gene splicing by the overlap extension method previously described (Horton et al., 1989) was performed, by using standard molecular cloning methods. The primers used are described in Table IA and B. A schematic representation of the structure of all constructs is shown in Figure 1.

### Proteolytic digestion of dog pancreas rough microsomes

Rough microsomes were isolated from canine pancreas and stripped of ribosomes by KOAc treatment as described by Walter and Blobel (1983). The resulting stripped membranes (EKRM) were adjusted to a final concentration of 1 mg protein per ml in 50 mM Hepes-KOH pH 7.5. Proteinase K was added at a final concentration of 0.2 mg/ml, while duplicate samples contained, in addition, 0.5% Triton X-100 (Eastman Kodak Co., Rochester, NY). The samples were incubated on ice for 4 h, after which PMSF was added to 10 mM and protein was recovered by precipitation with trichloroacetic acid. Samples were analyzed by SDS-PAGE and Western blotting with an antibody against the RII luminal domain, using the ECL kit (Boehringer Mannheim, Indianapolis) for visualization. The blots were stripped of antibody by incubation at 50°C for 30 min with 0.1M 2-mercaptoethanol and 2% SDS, and were reprobed with an antibody against BiP.

### Antibodies

For the detection of RII, the polyclonal antibody RIIL<sub>3</sub> directed against RII was used. RIIL<sub>3</sub> is a polyclonal rabbit antiserum raised against a synthetic peptide corresponding to the N-terminal 22 residues of rat ribophorin II (Yu et al., 1990). To prepare the antibody that detects the Tac antigen the mouse myeloma cell line 7G7B6 was propagated in Balbc 3T3 mice (Rubin et al., 1985) and the IgG fraction was purified from the ascites fluid by passing the fluid over a protein A-Sepharose column. The purified IgG fraction used directly for immunoprecipitation experiments was coupled to protein A-Sepharose beads by the submeridate method described previously (Cosson et al., 1991). The antibody directed against BiP was generated by injecting a synthetic peptide corresponding to the 10 most C-terminal amino acids of the rat protein into rabbits. The mouse monoclonal antibody against human Lamp-2 (H4B4) was a gift from Dr. Thomas August (Johns Hopkins University, Baltimore).

**Tab. IA.** Sequences of oligonucleotides used for constructing chimeras containing domains of RII and the Tac antigen.

number	Oligo Sequences (5'-3')
1	CCC <b>GAATTC</b> ATG GCG CCG CCG GGT TCA AGT
2	CCC <b>GAA TTC</b> CTA ATG TGC TGT TCT CTT AAC
3	CCC <b>GAA TTC</b> GGG GGG CCT CTT CTC AGG
4	CCC <b>GAA TTC</b> ATG GAT TCA TAC CTG CTG ATG TGG GGA
5	CCC <b>GAA TTC</b> CTA GAT TGT TCT TCT ACT CTT CCT CTG TCT CCG
6	CCC <b>AAG CTT#</b> ATG GAT TCA TAC CTG CTG ATG TGG GGA
7	CCC <b>GGT ACC</b> CTA GAT TGT TCT TCT ACT CTT CCT CTG TCT CCG
8	<u>ACA GCC GGC CAC TGC TAC GGG GGG CCT CTT CTC AGG CTC</u>
9	<u>CCT GAG AAG AGG CCC CCC GTA GCA GTG GCC GGC TGT GTT</u>
10	<u>TGT ATT GGA CAC CAC AGT CTG GTA CTC TGT TGT AAA TAT GGA</u>
11	<u>TTT ACA ACA GAG TAC CAG ACT GTG GTG TCC AAT ACA TTC</u>
12	<u>ACT CTT CCT CTG TCT CCG CTG CCA GGT GAT CCA CAG TGC AAA GAG CAG</u>
13	<u>CAG GTG CTG AAT TTC CTG CTG CTA CTC TGT TGT AAA</u>
14	<u>TTT ACA ACA GAG TAC CAG CAG GAA ATT CAG CAC CTG</u>
15	CCC <b>GGT ACC*</b> CTA ATG TGC TGT TCT CTT AAC TGC CTG CTG GGC

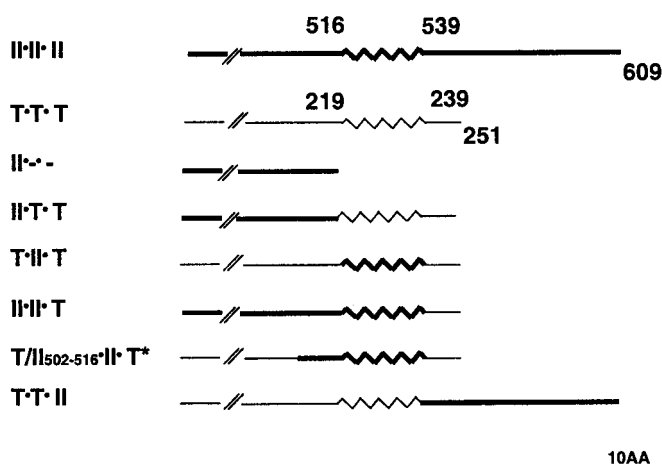
Note: Nucleotides typed in bold letters encode the EcoRI site, except when marked by “#” or “\*”, which represent HindIII and KpnI sites, respectively. Underlined parts of oligonucleotides correspond to various parts of the Tac antigen coding sequence.

**Tab. IB.** Oligonucleotides used for making the constructs.

Abbreviation of constructs*	Oligo numbers**
II●II●II	1, 2
II●●●	1, 3
T●T●T	4, 5
II●T●T	1, 8, 9, 7
T●II●T	4, 10, 11, 12, 5
II●II●T	1, 12, 7
T/II <sub>502-516</sub> ●II●T	4, 13, 14, 7
T●T●II	4, 12, 15

For the structure of these constructs see Figure 1.

\*\* These oligonucleotide numbers are those listed in Table IA.



**Fig. 1.** Schematic representation of chimera containing domains of RII and the Tac antigen. RII (II●II●II) and the Tac antigen (T●T●T) are represented as type I membrane proteins. The native proteins and the chimera are abbreviated such that the symbols II or T corresponding to the luminal, transmembrane and cytoplasmic domains are separated by dots. RII domains are represented by thick lines while the domains of the Tac antigen are represented by thin lines. The zigzag lines indicate the transmembrane domains. The numbers next to the representation of the native RII (II●II●II) or the Tac antigen (T●T●T) correspond to amino acid numbers at the C-terminal ends of the luminal domains, at the N-terminal ends of the cytoplasmic domains and the C-termini, thus defining the boundaries of the constructs. \*, T/II<sub>502-516</sub>●II●T means that the luminal domain of this construct is composed of the full luminal domain of the Tac antigen and the luminal 15 amino acids of RII adjacent to the transmembrane domain.

## Transfection of HeLa cells

Ten µg of plasmid DNA was mixed with 20 µl of lipofectamine in 4 ml of opti-medium (Life Technologies, Gaithersburg, MD). The mixture was incubated at room temperature for 20 min and then added to 10-cm dishes in which the original culture medium was just removed. Next day, the medium was removed and then 10 ml of fresh DMEM + 8% FCS + antibiotics was added. For 60-mm dishes, half of the plasmid DNA and reagents were used.

## Cell fixation and immunostaining

Transfected cells were grown on coverslips to 50–70% confluence (about 48 h after transfection), then rinsed three times with 1× cold PBS and fixed with 3% paraformaldehyde for 20 min, followed by the same rinsing steps. For permeabilization, fixed cells were treated with 0.2% Triton-X-100 for 10 min. Permeabilized or non-permeabilized cells were then incubated with 10 mM glycine for 15 min. After incubation with blocking solution (5% dried milk in 1×PBS) for 30 min, cells were incubated at 37°C for 1 h with the primary antibody diluted in blocking solution. The polyclonal antibody against RII was used at 1:200 dilution. The antibody against the Tac antigen, 7G7B6, was used at 1:100 dilution. The cells were then washed three times with blocking solution and incubated at 37°C for 1 h with either FITC-conjugated donkey anti-rabbit IgG antibody or Texas-red-conjugated donkey anti-mouse IgG antibody diluted 1:100 in blocking solution, followed by 3 washing steps as described before. The coverslips were then mounted with citiflour (Ted Pella Inc., Redding, CA). If not stated otherwise, fluorescently labeled proteins were detected using an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with 63× and 100× Plan-neofluar objective lenses. For double immunofluorescence experiments, cells were first incubated with a mixture of primary antibodies (rabbit anti-RII antibody and the monoclonal mouse anti-human LAMP-2 antibody). After several washing steps,

cells were incubated with a mixture of secondary antibodies (donkey anti-rabbit IgG coupled to FITC, and donkey anti-mouse IgG coupled to Texas-red). Micrographs shown in panels a, b, i, and j of Fig. 4 were obtained with the Nikon PCM 2000 laser scanning confocal microscope (Nikon Inc., Melville, NY) using the Simple 32 software (Coprix Inc., Cranberry Township, NY). Images were prepared for publication using Adobe Photoshop software.

## Pulse-chase experiment

Twenty-four h after transfection, cells grown in a 10-cm dish were trypsinized, centrifuged and divided into two 60-mm dishes. Twenty h

later, cells were treated with 2 ml of methionine-free medium for 20 min, then incubated for 30 min with 2 ml of medium containing [<sup>35</sup>S]methionine (250 µCi per ml), and then chased for 0 or 3 h with medium containing non-labeled methionine before the cells were collected.

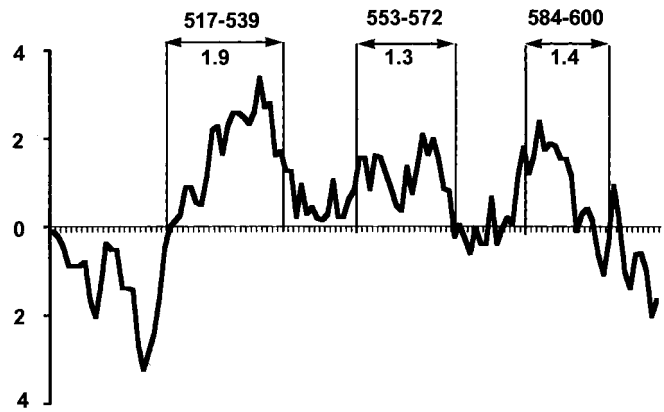
### Immunoprecipitation and digestion with glycosidases

[<sup>35</sup>S]methionine-labeled cells grown on a 60-mm dish were washed with cold PBS once, scraped with 1 ml of cold PBS and then transferred to an Eppendorf tube. Cells were lysed in 1 ml of lysis buffer (1% Triton X-100, 0.3 M NaCl, 50 mM Tris-Cl, pH 7.5) and sonicated 2 times for 10 s. The lysates were centrifuged for 15 min at 4 °C and the supernatant was transferred to a fresh tube. Hundred µl of the prepared protein A beads containing the 7G7B6 anti-Tac or the RIIL<sub>3</sub> antibody were then added to the lysates and samples were incubated on a rotator at 4 °C for 2 h. After centrifugation for 5 min, the pellets were washed three times with 1 ml of washing buffer (1% Triton X-100, 1 M NaCl, 50 mM Tris-Cl, pH 7.5) and subjected to Endo H treatment (Rosenfeld et al., 1984). The RIIL<sub>3</sub> antibody was used at a dilution of 1:250 (for details see also Yu et al., 1990). In order to remove complex N-linked oligosaccharides, immunoprecipitates were resuspended in 100 µl of buffer and after addition of 0.4 units of N-glycosidase F (Boehringer, Mannheim Indianapolis), the reaction mixture was incubated at 37 °C for 16 h. Similarly, for the removal of O-glycosidically linked sugars, immunoprecipitates were incubated with 2 mU neuraminidase and 2.5 mU O-glycosidase (Boehringer, Mannheim, Indianapolis) at 37 °C for 16 h.

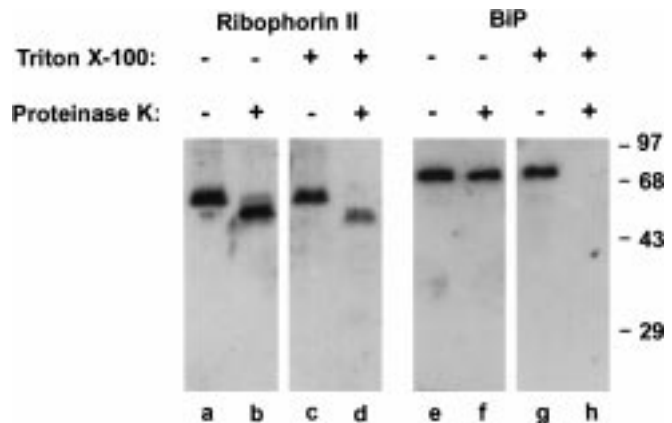
## Results

### Transmembrane disposition of RII

The primary sequence of this glycoprotein, derived from the corresponding cDNA (Pirozzi et al., 1991; Crimaudo et al., 1987), reveals that the nascent polypeptide contains a cleavable amino terminal signal sequence. This results in the ER-translocation and the N-glycosylation of the large amino terminal domain (516 residues). The putative transmembrane domain consists of 23 highly hydrophobic amino acids and it has an average hydrophobicity of 1.9, which is typical of polypeptide sequences expected to traverse the membrane in an alpha-helical conformation (Kyte and Doolittle, 1982) (Fig. 1; see also Fig. 2). The C-terminal portion of RII (aa 540 to 609) contains two additional weakly hydrophobic regions (aa 553 to 572 and 584 to 600) with average hydrophobicities of only 1.3 and 1.4, respectively, that cannot be regarded as potential membrane anchors (Kyte and Doolittle, 1982). It would appear, therefore, that RII, like RI, has a type I membrane disposition. Nevertheless, the cytoplasmic exposure of the C-terminal 70 amino acid segment of RII has not been clearly demonstrated. Indeed, previous studies (Crimaudo et al., 1987) with canine pancreatic rough microsomes (RM) found RII to be remarkably resistant to digestion with proteases. We found, however, that if the microsomes are first stripped of ribosomes by a pretreatment with K<sup>+</sup>-acetate and EDTA, digestion with proteinase K leads to the production of a 55 kDa protected fragment of RII that corresponds in size to a RII molecule from which the cytoplasmic domain has been removed (Fig. 3, lane b). In accord with previous findings (Crimaudo et al., 1987), we also found that, when digestion was carried out in the presence of Triton X-100, a small amount (30% compared with that in lane b) of a fragment of about 52 kDa (Fig. 3, lane d) was generated, which apparently corresponds to the amino terminal luminal domain of the



**Fig. 2.** The hydropathy plot of the C-terminal region of RII. The C-terminal portion of RII (aa 485–609) was subjected to hydropathy analysis according to Kyte and Doolittle (1982). Arrows define the three hydrophobic regions. The numbers above the arrows correspond to the amino acid numbers that define the borders of these regions, while the numbers under the arrows represent the average hydrophobicity within this region.



**Fig. 3.** Western blot analysis of ribophorin II in protease-treated microsomes. Rough microsomes stripped of ribosomes by treatment with EDTA and K<sup>+</sup> acetate (Walter and Blobel, 1983) were incubated on ice for 2 h without added protease (lanes a, c, e, g) or in the presence of 0.2 mg/ml proteinase K (lanes b, d, f, h). Samples in lanes c, d, g, h received, in addition, Triton X-100 at a final concentration of 0.5%. Each reaction was analyzed by SDS-PAGE and Western blotting with an antibody against RII (lanes a–d). The blot was then stripped and reprobed with an antibody against BiP (lanes e–h). Molecular masses of marker proteins (in kDa) are shown on the right.

protein which is at least partially resistant to proteolysis. The luminal protein BiP was completely resistant to proteolytic digestion, unless detergent was added to dissolve the membranes (Fig. 3, compare lanes f and h). We will refer to this segment (aa 540 to 609) as the cytoplasmic domain of RII, although we cannot rule out completely the possibility that the two moderately hydrophobic domains in the 70 amino acid C-terminal region of RII interact with the lipid bilayer.

In order to identify which domains within RII contribute to its retention in the ER we transfected HeLa cells with chimeric constructs in which one or more domains of RII were replaced by the corresponding domains of the Tac antigen, which is the alpha subunit of the interleukin-2 receptor (Leonard et al., 1984). The Tac antigen is a type I transmembrane protein of

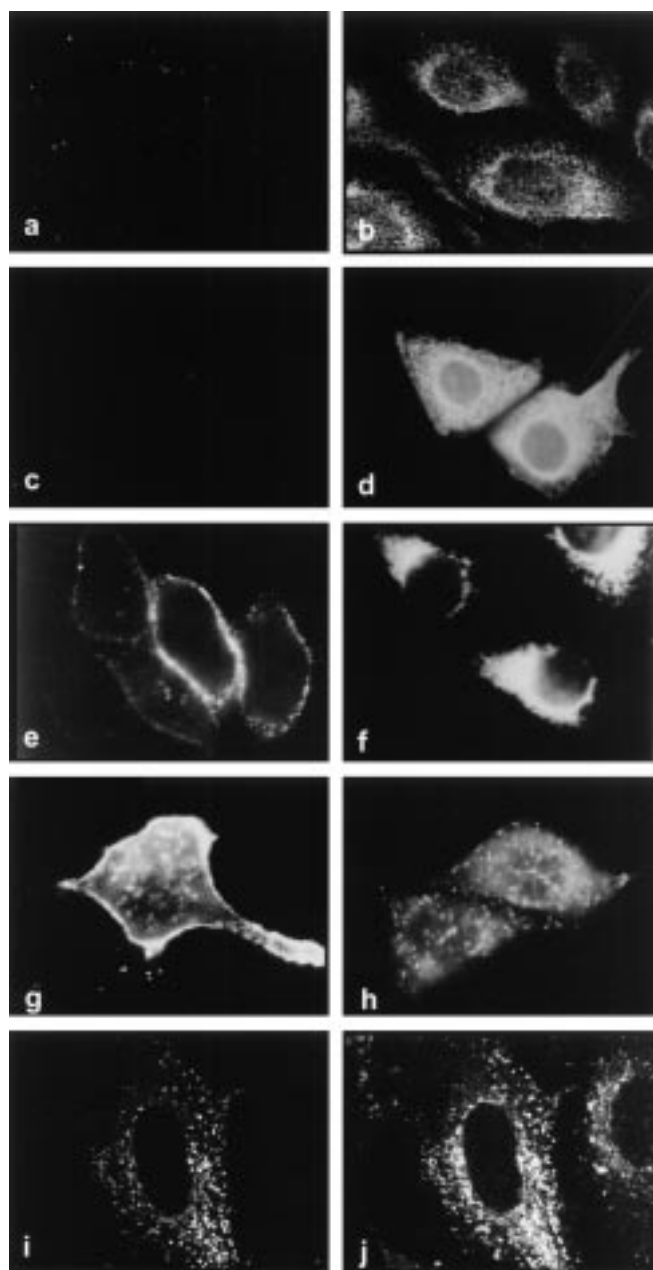
251 amino acids, with a short cytoplasmic domain of 13 amino acids (see also Fig. 1). Because the Tac antigen lacks intrinsic ER retention or retrieval signals and, when expressed in transfected cells, is rapidly and efficiently transported from the ER to the plasma membrane (Leonard et al., 1984), portions of this protein have frequently been used to construct chimeras in which they provide a neutral framework for the identification of sorting signals (Cosson et al., 1991; Manolios et al., 1990; Bonifacino et al., 1990a,b). Schematic representations of RII-Tac chimeras used in this work are shown in Fig. 1. The legend also explains the abbreviations used to designate these constructs.

### Constructs that contain only the luminal domain of RII exit from the ER

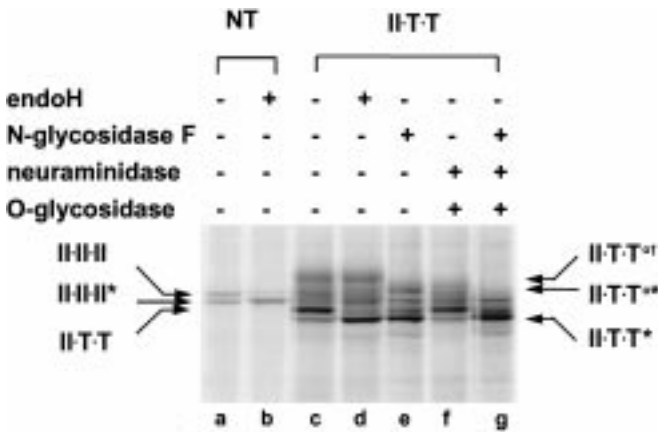
As indicated by the lace-like immunofluorescence pattern seen in permeabilized cells, not only the endogenous RII (Fig. 4b), but also overexpressed RII molecules are retained in the ER (Fig. 4d). Because of the limiting amounts of endogenous OST subunits in the transfected cells, most of the overexpressed RII polypeptides are not expected to be integrated into the hetero-oligomeric OST complex. The efficient retention of the unassembled RII, as indicated by the absence of cell surface immunofluorescence (Fig. 4c), suggests that this OST subunit may contain one or more intrinsic ER localization signals. In contrast to cells expressing the full-length RII, immunolabeling of non-permeabilized cells expressing the luminal domain of this OST subunit (II●●-) showed a punctate surface labeling, which is likely to correspond to secreted molecules that were retained in intercellular pockets near the sites of exocytosis at the plasma membrane (Fig. 4e). Immunolabeling after permeabilization showed not only the characteristic ER reticular staining pattern, but also an increased labeling in the perinuclear region of the transfected cells, as would be expected for II●●- molecules which traverse the Golgi apparatus (Fig. 4f).

The preceding experiments indicate that the RII luminal domain does not contain sufficient information to mediate the retention of RII in the ER. It was important, however, to demonstrate that this was also the case when the luminal domain was expressed as part of a membrane-anchored protein. Therefore, the behavior of a chimeric protein (II●T●T; see Fig. 1) was assessed, consisting of the complete luminal domain of RII fused to the transmembrane (19 amino acids) and cytoplasmic domain (13 amino acids: Thr-Trp-Gln-Arg-Arg-Gln-Arg-Lys-Ser-Arg-Arg-Thr-Ile) of the Tac antigen. Immunofluorescence analysis showed that the II●T●T chimera exited from the Golgi apparatus and reached the plasma membrane, where it could be labeled in non-permeabilized cells (Fig. 4g). In permeabilized cells, a significant fraction of the II●T●T chimera localized to vesicular structures as indicated by the pronounced punctate labeling pattern that suggest accumulation of the protein in lysosomes (Fig. 4h). Double immunofluorescence experiments labeling both RII (Fig. 4i) and the lysosomal membrane protein LAMP-2 (Fig. 4j) demonstrated that a large fraction of the chimera indeed accumulated in sites that contain the lysosomal marker.

That the II●T●T chimera exits from the ER and traverses the Golgi apparatus was also confirmed by pulse-chase experiments. In untransfected cells only approximately half of the endogenous RII molecules normally undergo N-glycosylation. Therefore, immunoprecipitation after pulse labeling yields two RII bands with slightly different electrophoretic mobilities



**Fig. 4.** The luminal domain of RII does not contain an ER localization signal. HeLa cells were kept as untransfected controls (**a** and **b**), or they were transfected with cDNAs encoding the full-length RII (**c** and **d**), the luminal domain of RII (II●●-; **e** and **f**) or the chimera consisting of the luminal domain of RII and the transmembrane and cytoplasmic domain of the Tac antigen (II●T●T; **g**-**j**). Cells were fixed with paraformaldehyde (**a**, **c**, **e** and **g**), or fixed with paraformaldehyde and permeabilized with Triton X-100 (**b**, **d**, **f**, **h**, **i**, and **j**), followed by incubation with a polyclonal rabbit antibody against RII (**a**-**i**) or with an monoclonal mouse antibody directed against LAMP-2 (**j**). As a secondary antibody, a fluorescein-conjugated donkey IgG directed against rabbit IgG (**a**-**i**) or a Texas red-conjugated donkey IgG directed against mouse IgG (**j**) was used. The ER staining (**b**, **d** and **f**) and the lysosomal staining (**h**, **i** and **j**) can be observed in the permeabilized cells, while plasma membrane staining (**e** and **g**) is seen in non-permeabilized cells. Fluorescence micrographs shown in panels **a**, **b**, **i**, and **j** were obtained with a confocal microscope.



**Fig. 5.** A construct that contains only the luminal domain of RII exits from the ER and acquires complex oligosaccharide modifications. Mock-transfected HeLa cells (lanes a and b) or cells transiently transfected with cDNA encoding II•T•T (c–g) for 48 hours were then labeled with [<sup>35</sup>S]methionine for 1 h. The cell lysates were immunoprecipitated using a polyclonal antibody against RII and digested with EndoH (lanes b and d) with N-glycosidase F (lane e), with neuraminidase and O-glycosidase (lane f), with N-glycosidase F, neuraminidase and O-glycosidase (lane g) or were mock digested (lanes a and c). Samples were analyzed by SDS-PAGE followed by autoradiography. Wild-type RII (II•II•II) or II•T•T marked by an asterisk “\*” (II•II•II\* and II•T•T\*, respectively) correspond to forms which do not carry an N-linked oligosaccharide. The band labelled II•T•T<sup>0+</sup> indicates that the chimera contains a complex N-linked oligosaccharide and O-linked sugar residues. The band labelled II•T•T<sup>0\*</sup> contains O-linked oligosaccharides (e) which can be removed by neuraminidase and O-glycosidase (g).

(II•II•II and II•II•II\* in Fig. 5, lane a) and Endo H treatment (lane b) converts the slower migrating form to the faster one (see Ivessa et al., 1992; Crimando et al., 1987). Similarly, in transfected cells that express the full-length RII polypeptide only approximately 50% of the exogenous molecules acquired N-linked oligosaccharide chains, and all of those remained Endo H sensitive, indicating that they did not reach the Golgi apparatus (Ivessa et al., 1992).

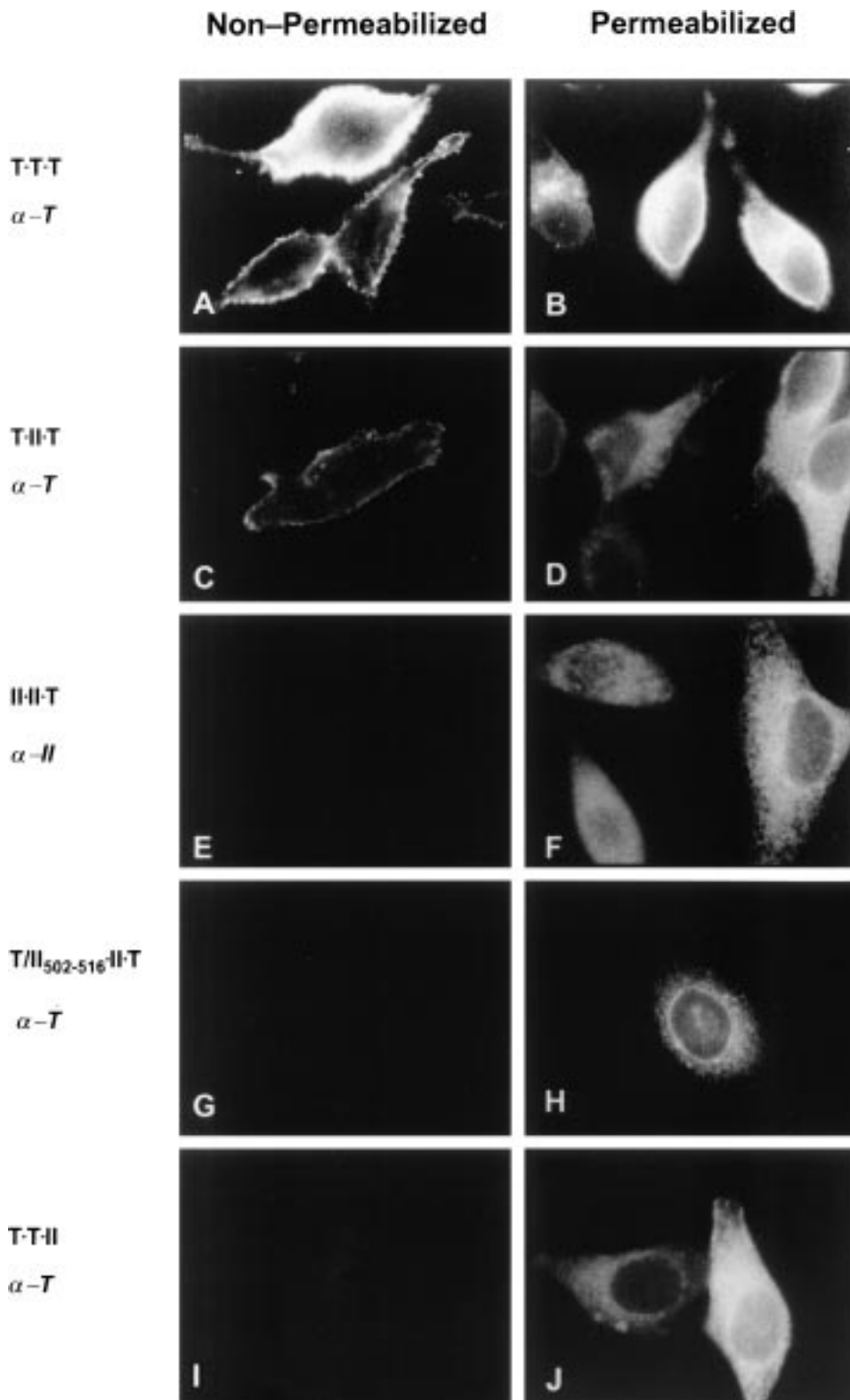
The analysis of immunoprecipitates obtained from cells expressing the chimera II•T•T showed that it exited efficiently from the ER, as indicated by its acquisition of Endo H resistance (Fig. 5, lane d). In addition to the N-glycosylated (II•II•II), and non-glycosylated forms (II•II•II\*) of the endogenous RII (see also lane a), three additional polypeptides were obtained when cell lysates were immunoprecipitated with anti-RII antibody (Fig. 5, lane c): a diffuse band of lower electrophoretic mobility (II•T•T<sup>0+</sup>;  $M_r \sim 72$  kDa) and two sharp bands of higher electrophoretic mobility (II•T•T;  $M_r \sim 60$  kDa and II•T•T\*;  $M_r \sim 51$  kDa). The band corresponding to II•T•T was sensitive to Endo H (Fig. 5, compare lanes c and d) indicating that it possessed a high-mannose oligosaccharide and had not yet exited from the ER. In contrast, the modified chimeric polypeptide (II•T•T<sup>0+</sup>) was resistant to Endo H (Fig. 5, lane d) but sensitive to endo F (II•T•T<sup>0\*</sup>, Fig. 5, lane e) and, therefore, had reached the medial region of the Golgi apparatus. The observation that even after Endo F digestion the modified polypeptide (II•T•T<sup>0\*</sup>) migrated as a diffuse band of lower electrophoretic mobility compared with II•T•T\*, suggested that, in addition to an N-linked oligosaccharide, it may also contain O-linked sugar residues. This was

confirmed by treatment of the immunoprecipitate with neuraminidase and O-glycosidase, which shifted most of the radioactivity into a band corresponding to II•T•T that carries only an N-linked oligosaccharide (lane f). Additional digestion with N-glycosidase F (lane g) resulted in the accumulation of the radioactivity in the band corresponding to the deglycosylated form (II•T•T\*). Since constructs which contain only the luminal domain of RII exit freely from the ER we conclude that this part of the molecule folds properly and binding of chaperones to the luminal domain plays no role in the retention of RII in the ER.

### The transmembrane domain of RII and a short luminal flanking region contain ER retention information

We next examined the localization of a chimera (T•II•T) containing the transmembrane domain of RII and the cytoplasmic and luminal domains of the Tac antigen. As expected, expression of the Tac antigen itself (T•T•T) resulted in intense surface labeling of all transfected cells (Fig. 6A and B). On the other hand, cells expressing T•II•T showed intense ER staining (Fig. 6D) and only about 20% of them showed also rather faint surface labeling (Fig. 6C). These observations suggest that the transmembrane domain of RII contains a retention signal, although it is unable to retain all the chimeric molecules in the ER. The rather efficient retention of T•II•T in the ER is strikingly demonstrated by the fact that after a 30 min pulse and 3 h chase period the chimeric molecules remain fully Endo H sensitive (Fig. 7, lanes g and h), in striking contrast to the Tac antigen (T•T•T) itself, which during the same chase period becomes almost fully Endo H resistant (Fig. 7, lanes c and d). Thus, although the surface fluorescence of non-permeabilized cells revealed that a significant amount of the chimeric protein (T•II•T) reached the cell surface (see Fig. 6C), the fraction of the total T•II•T molecules synthesized during the pulse period that exits from the ER is biochemically undetectable and corresponds to less than 5% of the labelled proteins.

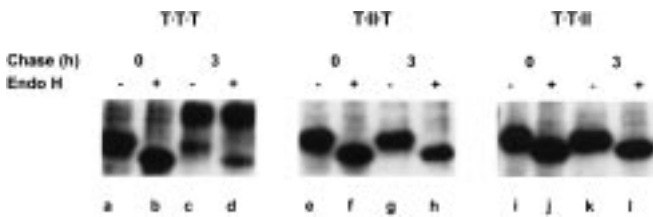
A chimera (II•II•T) in which only the cytoplasmic domain of RII was replaced by that of the Tac antigen was completely retained in the ER (Fig. 6F) and no cell surface fluorescence was detectable (Fig. 6E). It appears, therefore, that although the luminal domain of RII by itself does not contain a detectable ER localization signal (Figs. 4 and 5), it nonetheless augments the retention activity of the transmembrane domain (compare Fig. 6C and E). A similar localization enhancement function has been previously demonstrated for short luminal portions adjacent to the transmembrane domain of some Golgi enzymes (Machamer, 1991; Nilsson and Warren, 1994; Munro, 1995; Colley et al., 1992). As was suggested for the Golgi membrane proteins the short luminal segment of RII may serve to properly position the transmembrane domain within the lipid bilayer of the ER, which may enhance the retention capacity of the transmembrane domain [26]. We therefore expressed several T•II•T-related chimera in which the RII transmembrane domain was preceded by juxtamembranous luminal RII regions of decreasing length. The chimeric polypeptide containing a 15 amino acid segment of the luminal domain (aa<sub>502-516</sub>) in addition to the RII transmembrane domain was totally excluded from the cell surface and only detectable in the ER (Fig. 6H). This demonstrates that the transmembrane domain of RII together with a short luminal flanking region serves as an efficient ER retention motif.



**Fig. 6.** The transmembrane domain together with a short luminal flanking region contains the retention information. HeLa cells transiently transfected with cDNA encoding the Tac antigen (T•T•T, **A** and **B**), or T•II•T (**C** and **D**), or II•II•T (**E** and **F**), or T/II<sub>502-516</sub>•II•T (**G** and **H**), or T•T•II (**I** and **J**) were fixed with paraformaldehyde (**A**, **C**, **E**, **G**

and **I**), or fixed with paraformaldehyde and then permeabilized with Triton X-100 (**B**, **D**, **F**, **H** and **J**). For immunofluorescence staining, cells were incubated with a monoclonal antibody against the Tac antigen (**A**–**D** and **G**–**J**), or with anti-RII (**E** and **F**). For details, see Figure 4.





**Fig. 7.** Tac chimeras containing the transmembrane domain or the cytoplasmic domain of RII do not acquire Endo H resistance. HeLa cells transfected with cDNAs encoding the Tac antigen (T•T•T; lanes a–d), or the chimera T•II•T (lanes e–h), and T•T•II (lanes i–l) were pulse labeled and chased for 0 or 3 h. The lysates were immunoprecipitated with an antibody against Tac ( $\alpha$ -Tac), followed by Endo H treatment, SDS-PAGE and autoradiography. While most of T•T•T acquire Endo H resistance after a chase period of 3 h (lanes c and d), T•II•T (lanes g and h) or T•T•II (lanes k and l) were still sensitive to Endo H.

### The C-terminal domain of RII also contains ER retention information

The behavior of a chimera (T•T•II) containing the luminal and transmembrane domains of the Tac antigen and only the 70 amino acid cytoplasmic portion of RII was also examined to assess the contribution of the RII cytoplasmic domain to the retention of the protein in the ER. This chimera did not reach the cell surface, as revealed by immunofluorescence of non-permeabilized cells (Fig. 6I), but like the native RII it gave a typical ER staining in permeabilized cells (Fig. 6J). The retention properties of the cytoplasmic domain of RII were also demonstrated by pulse-chase experiments which showed that after a chase period of 3 h, the chimera was still fully sensitive to Endo H (Fig. 7, lanes k and l).

## Discussion

We have previously shown that two subunits of the oligosaccharyltransferase, RI and RII, are retained in the ER when overexpressed in transfected cells (Ivessa et al., 1992; Sander son et al., 1990). In this report we demonstrate that, in contrast to a truncated form of RI consisting only of its luminal domain, which is retained in the ER and subsequently degraded (Tsao et al., 1992; Ivessa et al., 1992), an analogously truncated RII polypeptide behaves as a secretory protein and is not retained to any significant extent in the ER. In addition, we show that both the cytoplasmic and transmembrane domains of RII independently contain ER retention information but, in order to be totally effective in mediating retention, the transmembrane domain must be accompanied by a short contiguous sequence of 15 amino acids from the luminal domain. Our observations, therefore, support the notion that the individual subunits that compose the oligomeric OST complex contain separate retention motifs, despite the fact that the function of these motifs may no longer be required after the polypeptides are integrated into the functional OST.

Our previous work has shown that components of both the OST and the translocation apparatus, including membrane bound ribosomes, are recovered in large raft-like arrays that remain sedimentable after solubilization of ER membrane lipids with neutral detergents (Kreibich et al., 1978a, b). It was suggested that their incorporation into a large proteinaceous network within the ER membranes provide a mechanism that

not only prevents the individual polypeptides from entering the smooth domains of the ER but also is responsible for the flattened cisternal morphology characteristic of rough ER membranes. Incorporation into a protein network within the rough ER membrane would also be expected to prevent resident ER polypeptides from entering vesicular carriers in which proteins destined for distal regions of the endomembrane system are transported. It also seems likely that the generation of vesicles by budding, which requires a pliable membrane, would be impeded by the presence of a rigid proteinaceous network in areas of the rough ER. In contrast to the OST and the components of the translocation apparatus (Kreibich et al., 1978a, b; Hortsch and Meyer, 1985; Wiedemann et al., 1989; Sitia and Meldolesi, 1992; Görlich et al., 1992; Kelleher and Gilmore, 1997; Kelleher et al., 1992; Hartman et al., 1993), other ER membrane proteins that participate in general metabolic functions, such as members of the electron transport chains that include cytochrome P450 and b5, are apparently not part of the RER-specific proteinaceous network and are found in both rough and smooth portions of the organelle (Teasdale and Jackson, 1996; Sitia and Meldolesi, 1992; Kreibich et al., 1983; Szczesna-Skorupa et al., 1994). Although the retention mechanism for these proteins has not been characterized, it is possible that these proteins are also part of higher order complexes that result in their retention in the ER.

Of the four subunits of the OST complex (Silberstein and Gilmore, 1996) only OST48 has a canonical dilysine motif at position -3 and -4 from the C-terminus, which normally functions in the retrieval of escaped membrane proteins from distal regions of the endomembrane system (Letourneur et al., 1994). Since we have shown that the luminal domains of both RI and RII interact with that of OST48 (Fu et al., 1997), a conceivable explanation for the retention of newly synthesized ribophorins in the ER would have been that they simply associate with OST48. Such a piggy-back retention mechanism cannot account for the localization of newly synthesized ribophorins in the ER, since we have shown that the ribophorins remain in the ER even when overexpressed and produced at levels far higher than that of endogenous OST48. Moreover, a variant of OST48 in which the two lysine residues close to the C-terminus are replaced by serine – which allows this OST48 variant to be efficiently transported to the cell surface – does not lead to the concomitant exit of endogenous ribophorins from the ER (Fu and Kreibich, unpublished observations). In fact, coexpression of RI or RII with the OST48 variant results in their complete retention in the ER. This finding supports the presence of independent retention information in the ribophorin polypeptides.

The retention in the ER of the unassembled ribophorin II could result from the interaction of its luminal domain with chaperones, such as BiP, calreticulin, or calnexin. These chaperones, which may be involved in the assembly of the oligomeric OST, themselves contain retrieval signals that assure that their ligands are available in the ER for the assembly of the oligomers (Teasdale and Jackson, 1996). Interaction with ER chaperones may, in fact, be the mechanism for retention of a truncated form of ribophorin I that consists only of the luminal domain of the protein (Tsao et al., 1992). It is clear, however, that the interaction of chaperones with the luminal domain of RII cannot be responsible for retention of the protein in the ER, since we found that, when synthesized as a truncated polypeptide or as part of a chimera

(II●T●T), the luminal domain of RII exited the ER and underwent a series of posttranslational modifications, including processing of the N-linked oligosaccharide chain to a complex form and the addition of O-linked sugar residues.

The II●T●T chimera exited from the ER and traversed the Golgi apparatus to reach the plasma membrane but also accumulated in lysosomes. Indeed, we found that the II●T●T chimera was degraded with a  $T_{1/2}$  of 2 h and that this degradation was prevented by leupeptin, a well known lysosomal protease inhibitor (Pirozzi and Kreibich, unpublished results). We do not yet have information on the route followed by this chimera to reach the lysosomal compartment after its processing in the Golgi apparatus. Some lysosomal membrane proteins, like Igp120 are delivered directly from the Golgi apparatus to lysosomes (Hart and Mellman, 1992), while other proteins are known to reach the lysosome only after they are delivered to the plasma membrane and then internalized by endocytosis (Mellman, 1996). No internalization signal has been demonstrated in the  $\alpha$ -subunit of the IL-2 receptor (for review see Mellman, 1996). The two possible routes to the lysosome are not mutually exclusive and a combination of the two may account for the transport of II●T●T to lysosomes. However, the indirect route involving passage through the plasma membrane should be a minor one, since in a pulse chase experiment we found no measurable levels of labeled chimera at the cell surface, as indicated by its susceptibility to protease digestion.

We found that the transmembrane domain of RII was also capable of localizing a chimera to the ER. The presence of localization signals within transmembrane domains has been demonstrated for a number of type II membrane proteins of the Golgi apparatus (Swift and Machamer, 1991; Machamer, 1991; Nilsson et al., 1991; Nilsson and Warren, 1994), as well as for some proteins of the ER (Szczesna-Skorupa, 1995) and the nuclear envelope (Wozniak and Blobel, 1992; Smith and Blobel, 1993). Although the mechanism responsible for the specific localization of these proteins in the different subcellular compartments is unclear, it has been suggested that either a lateral aggregation of the transmembrane domains serves as a sorting mechanism (Machamer, 1991; Munro, 1991; but see Cole et al., 1996) or that the thickness of the lipid bilayer of a given membrane, which is determined by the specific lipid composition, plays a role in selecting for proteins that contain hydrophobic domains of a suitable length (Munro, 1995).

In yeast cells it has been recently shown that transmembrane domains of type I transmembrane proteins function as ER retention signals (Letourneur and Cosson, 1998) and that these proteins, similar to the ER membrane proteins carrying a dibasic amino acid motif close to the C-terminus, are in fact retrieved from a post Golgi compartment (Letourneur and Cosson, 1998). From the experiments shown here it is clear, however, that constructs such as T●II●T or II●II●T do not accumulate in the ER by a retrieval mechanism, but rather that they apparently never exit from the ER, since the luminal Tac or RII domains are not modified by Golgi glycosyltransferases.

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