Mechanism of Residence of Cytochrome b(5), a Tail-anchored Protein, in the Endoplasmic Reticulum

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Abstract. Endoplasmic reticulum (ER) proteins maintain their residency by static retention, dynamic retrieval, or a combination of the two. Tail-anchored proteins that contain a cytosolic domain associated with the lipid bilayer via a hydrophobic stretch close to the COOH terminus are sorted within the secretory pathway by largely unknown mechanisms. Here, we have investigated the mode of insertion in the bilayer and the intracellular trafficking of cytochrome b(5) (b[5]), taken as a model for ER-resident tail-anchored proteins. We first demonstrated that b(5) can acquire a transmembrane topology posttranslationally, and then used two tagged versions of b(5), N-glyc and O-glyc b(5), containing potential N- and O-glycosylation sites, respectively, at the COOH-terminal lumenal extremity, to discriminate between retention and retrieval mechanisms. Whereas the N-linked oligosaccharide provided no evidence for retrieval from a downstream compartment, a more stringent assay based on carbohydrate acquisition by O-glyc b(5) showed that b(5) gains access to enzymes catalyzing the first steps of O-glycosylation. These results suggest that b(5) slowly recycles between the ER and the cis-Golgi complex and that dynamic retrieval as well as retention are involved in sorting of tail-anchored proteins.

Key words: cell compartmentation • endoplasmic reticulum • glycosylation • Golgi apparatus • membrane proteins

Introduction

Notwithstanding the intense membrane traffic taking place within eukaryotic cells, subcellular compartments maintain their molecular identity with remarkable accuracy. Within the secretory pathway, proteins that reside in a given compartment must avoid being relocated to neighboring compartments in the face of ongoing transport. Two different mechanisms, static retention and dynamic retrieval, can, in principle, ensure that each compartment keeps its cohort of resident proteins. Retained proteins are thought to be simply excluded from transport out of their compartment of residence, whereas retrieved proteins do escape to a downstream organelle from which they are recycled by retrograde transport (Pelham and Munro, 1993).

Consistent with its diverse functions, the membrane of the ER has a complex molecular composition, and many studies have been devoted to the mechanisms through which it keeps its numerous resident polypeptides from following transported proteins down the secretory pathway. These studies have shown that both retrieval via coat protein I–coated vesicles and retention are operative in determining residence of ER membrane proteins (for review see Teasdale and Jackson, 1996). Of the two mechanisms, the former is better understood, and retrieval signals have been identified in the cytosolic tails of type I and type II ER resident membrane proteins (Cosson and Lletourneur, 1994; Teasdale and Jackson, 1996; Cosson et al., 1998). In addition, a number of membrane proteins are retrieved by interaction of their transmembrane domains (TMDs) with a recently identified receptor, rer1p (Sato et al., 1996, 1997). Less information is available on static retention. Although it could be argued that retention is ef-

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fected simply by the absence of positive signals required for efficient recruitment of cargo protein into transport vesicles (Springer et al., 1999). It is likely that specific retention signals or features are also necessary to prevent access of retained proteins into budding vesicles (Sonnewichen et al., 1994).

Although there is evidence that both retrieval and retention operate at the ER-Golgi complex boundary, an important question that is still largely unresolved are the relative roles played by these two mechanisms in determining the residency of ER membrane proteins. Post-translational modifications are generally used to follow intracellular trafficking between the ER and the Golgi. However, many ER membrane proteins do not have consensus sites for such modifications and are thus difficult to study. For instance, membrane proteins with the so-called tail-anchored (TA) topology, consisting of an NH$_2$-terminal active cytosolic domain and a hydrophobic membrane-anchoring region close to the COOH terminus, essentially lack a lumenal domain (for reviews see Borgese et al., 1991; Kutay et al., 1993). Many ER enzymes whose catalytic activity is at the cytosolic side of the membrane have a TA topology, e.g., cytochrome b(5) (b[5]), heme oxygenase, aldehyde dehydrogenase, and ubiquitin conjugating enzyme 6, and unraveling their sorting mechanisms would make an important contribution to our understanding of the genesis and maintenance of the molecular identity of the ER. In our laboratory we have been using b(5) as a model to study the targeting and sorting of ER-resident TA proteins. b(5) is an ~15,000-M$_r$ protein with an NH$_2$-terminal, globular cytosolic heme-binding domain, a short connecting region, and a hydrophobic anchoring domain followed by seven polar residues at the extreme COOH terminus (for review see Borgese et al., 1993). Since ER-resident TA enzymes generally have short hydrophobic anchoring domains (14–18 residues), we investigated a possible role of the length of the hydrophobic domain in sorting and demonstrated that a short anchoring domain is required to keep b(5) from escaping from the ER and from travelling down the secretory pathway to the cell surface (Pedrazzini et al., 1996). A similar observation was subsequently made on other ER-resident TA proteins (Ryner and Pelham, 1997; Yang et al., 1997), establishing the generality of tail length-dependent sorting. However, an open question remained as to whether the short hydrophobic domain effects ER residence of TA proteins by a static retention or by a dynamic retrieval mechanism. In this study, we have investigated this question. Using tagged versions of b(5) with N- or O-glycosylation sites attached at the COOH terminus, we show that b(5) gains access to enzymes catalyzing the first steps of O-glycosylation, suggesting that it slowly recycles between the ER and the cis-Golgi.

Materials and Methods

Plasmid Constructions

A cDNA coding for the rabbit ER form of b(5) (Giordano and Steggles, 1992) and subcloned into pGEM4 and into the mammalian expression vector pC86 (Brewer and Roth, 1991), has been described in previous work (Pedrazzini et al., 1996). We refer to the protein expressed from this plasmid as wild-type (wt) b(5).

To facilitate addition of sequence tags at the COOH terminus of b(5), we introduced a unique A gel site at the border between the hydrophobic domain and the extreme COOH-terminal polar region, which did not alter the encoded amino acid sequence, and XbaI and HindIII sites immediately after the stop codon. The three restriction sites were introduced by substituting a 3' terminal Aat2-Hind3 620-bp fragment of wt b(5) in pGEM4 (the Aat2 site at position 335 and the Hind3 site in the polylinker) with a 214-bp, PCR-generated fragment. The resulting plasmid (pgb[5]A) was used to construct the N- glyc b(5) and O-glyc b(5) mutants, which contained, close to their COOH termini, potential N- and O-glycosylation sites, respectively. The 25-bp A gel-XbaI fragment of pgb[5]A was replaced with an 89-bp duplex or 86-bp duplex obtained by annealing two complementary synthetic oligonucleotides coding for the sequences illustrated in Fig. 2A. The constructs were checked by sequencing and then subcloned into the Kpn1-Hind3 sites of pCB6 with a modified polylinker (De Silvestris et al., 1995).

Cell Culture and Transfection

Most of the experiments in this study were conducted on transiently transfected CV1 cells that were cultured and transfected with the Cad^PO$_4$ method as described previously (De Silvestris et al., 1995). The efficiency of transfection was usually monitored by cotransfecting with green fluorescent protein (Clontech). For the fractionation experiment (see Fig. 10), transfection was carried out with lipofectamine (GIBCO BRL) with 0.03 µg of b(5) cDNA/cm$^2$ of culture dish surface. Cells were used for immunofluorescence or biochemical experiments within 24 h after transfection. In one experiment (see Fig. 1), an MDCK line stably expressing wt b(5) was used. MDCK cells were cultured and transfected as described previously (Borgese et al., 1996). Several b(5)-expressing clones were obtained; however, the expression was completely lost after only a few passages in culture, so that further experiments with these clones could not be carried out.

For the experiment shown in Fig. 6B we used CHO-15B cells grown in a 5% CO$_2$ incubator in MEM with Earle's salts α-modification, supplemented with 7.5% FCS, 2 mM glutamine, and antibiotics. These cells were transfected with the lipofectamine (GIBCO BRL), using 0.2 µg of plasmid/cm$^2$ of culture dish surface.

Antibodies

The following antibodies (Abs) were produced in our laboratory: (a) affinity-purified polyclonal A bs against b(5) raised in rabbits in our laboratory (Borgese and Meldolesi, 1976; De Silvestris et al., 1995); (b) an affinity-purified polyclonal A b directed against the 10 COOH-terminal residues of b(5). 2.4 mg of the peptide (LMY R L Y M A D D) was conjugated to 3 mg of chicken ovalbumin with succinimidyl 4-[N-maleimido- methyl]-cyclohexane-1-carboxylate (Pierce) and used to immunize a rabbit. In addition, the following Abs were generously donated by the indicated investigators: a monoclonal anti-b(5) mAb from Dr. R. S. Park (Intracellular Research Institute, Inchon, Korea) (Park et al., 1992); an antipeptide polyclonal antiserum against the 10 COOH-terminal amino acids of ER GIC 53 (Q E A A K K F F) from Dr. Stefano Bonatti (Johannes-Gutenberg Universität, Mainz, Germany) (Bhakdi et al., 1994); and an antipeptide polyclonal antiserum against the 10 COOH-terminal amino acids of ER GIC 53 (Q E A A K K F F) from Dr. Stefano Bonatti (Johannes-Gutenberg Universität, Mainz, Germany) (Bhakdi et al., 1994).

Immunofluorescence

Paraformaldehyde-fixed cells were permeabilized with Triton X-100 and processed for immunofluorescence as described previously (De Silvestris et al., 1995). In some experiments, permeabilization of the plasma membrane was carried out with streptolysin O (SLO), obtained from Dr. S. Bhakdi (Institute of Immunology and Molecular Genetics, Karlsruhe, Germany) (Seelig et al., 1994). An anti-β tubulin mAb (DM 1a) from Dr. M. Kirschner (Harvard Medical School, Boston, MA); a mAb against the NH$_2$-terminal peptide of bovine opsin (R 2-15) from Dr. Paul Hargrave (University of Florida, Gainesville, FL) (A damus et al., 1993); an antilaminin polyclonal serum from Dr. M. Renz (Institute of Immunology and Molecular Genetics, Karlsruhe, Germany) (Seelig et al., 1994), and anti-G 528 mAb were from StressGen Biotechnologies.

The Journal of Cell Biology, Volume 148, 2000 900
4°C. A fter removal of excess SLO, incubation was continued in the same buffer lacking SLO at 37°C for 30 min. Cells were observed under a Zeiss Axioplan microscope equipped for epifluorescence or with a Bio-Rad MRC 1024 E laser confocal microscope.

**Velocity Sucrose Gradient Centrifugation**

CV1 cells transfected with wt b(5) in a 10-cm petri dish were washed free of medium with PBS, detached with a rubber policeman, sedimented, and resuspended in 200 μl of PBS and lysed for 10 min at 0°C with an equal volume of 2% Triton X-100, 40 mM NaCl, 50 mM Tris-Cl, pH 7.4, and protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1.4 μg/ml pepstatin, 2 μg/ml chymostatin) for 10 min on ice. A cleared lysate was obtained after centrifugation at 700 g for 10 min. The cleared lysate or a fraction of rat liver microsomes (1.3 mg of protein) solubilized under the same conditions as the cultured cells was loaded on top of a separate gradient centrifuged in parallel. 15 fractions were collected from the top with an Auto Densiflow probe (Buchler Instruments) and subjected to precipitation with TCA in the presence of 80% ethanol, 1.4 M sucrose, and layering under a linear 9.5-ml 8-22% isosmotic Optiprep gradient with the same ions as above. The gradients were topped with 0.3 ml of 8% sucrose plus ions, and centrifuged for 2 h at 28,000 rpm at 4°C in a Beckman SW 41 rotor. Fractions were collected and equal aliquots were subjected to immunoprecipitation followed by SDS-PAGE fluorography or to Western blot analysis.

**Biochemical Assays**

Most of the techniques used in this study (SDS-PAGE, Western blotting, NADPH-cytochrome c enzyme assay) are described in previous publications (Borgese and Pietrini, 1986; Borgese et al., 1996). Autoradiograms and fluorograms were scanned and band intensities determined using the NIH Image software.

For endoglycosidase (Endo) H treatment, immune complexes were detached from protein G-Sepharose beads by incubation for 3 min at 100°C in an equal volume of 40 mM Tris-Cl, pH 6.8, 4% SDS, 2% β-mercaptoethanol, and 17% glycerol. A fter sedimentation of the beads, the supernatant was supplemented with five volumes of 0.1 M Na+ citrate buffer, pH 5.5, 0.6 μl of a 100-mg/ml solution of BSA and protease inhibitors, and then incubated overnight at 37°C with or without 10 μl of Endo H (Boehringer Mannheim). 5-10 μl of in vitro translation mix was similarly denatured and digested. For Endo D treatment, samples were denatured as for Endo H digestions, but the SD-containing supernatants were diluted with five volumes of 0.25 M Na+ phosphate, pH 7.2, 10 mM EDTA plus BSA, and protease inhibitors. Digestion was carried out overnight with 3 μl of Endo D (Boehringer Mannheim).

**Results**

**Transmembrane Topology of b(5)**

To plan a strategy for the investigation of the mechanism of residence of b(5) in the ER, it was first necessary to define the topology of the hydrophobic membrane-ancho ring domain. Although b(5) is perhaps the first TA protein to have been characterized, there has been controversy as to whether it adopts a transmembrane disposition or whether it loops back into the cytosol. We investigated this problem with two different approaches as described below.

In the first approach, we used an Ab raised against the COOH terminus of b(5) (see Materials and Methods) to probe the accessibility of the COOH-terminal polar peptide by immunofluorescence in an MDCK cell line stably expressing b(5). Although this cell line originated from a nontransfected clone, only ~30% of the cells expressed detectable levels of b(5) at any one time. Cells were treated either with SLO to selectively permeabilize the plasma membrane (Bakdi et al., 1993), or with Triton X-100 after fixation to permeabilize intracellular membranes (compare Fig. 1, B and D). In contrast, the ER lumen of SLO-permeabilized cells was poorly accessible, as demonstrated by the staining with antitubulin mAb, which were equally effective with or without Triton X-100 permeabilization (compare Fig. 1, B and D). Under this same condition, the
COOH terminus of b(5) was not accessible to Abs (Fig. 1 A), which were, however, capable of binding their epitope after Triton X-100 treatment (Fig. 1 C). In contrast to the anti-COOH-terminal peptide Ab, Abs raised against the catalytic domain of b(5) were able to bind the antigen regardless of the permeabilization protocol (Fig. 1, E and G). The results illustrated in Fig. 1 are evaluated quantitatively in Table I, and are consistent with a lumenal location of the COOH-terminal polar peptide of b(5).

In the second approach, the COOH terminus of b(5) was extended with a sequence containing a consensus for N-glycosylation, as reporter for translocation into the ER lumen. The appended sequence (Fig. 2 A) was derived from the NH2 terminus of bovine opsin. The resulting construct, N-glyc b(5), was translated in vitro under different conditions. As shown in the upper panel of Fig. 2 B, lane 1, when N-glyc b(5) was translated in the presence of dog pancreas microsomes, two bands were obtained, of which one comigrated with the product obtained in the absence of membranes (lanes 3 and 4), and the other, indicated by the arrow, had a slower mobility and was Endo H-sensitive (lane 2), demonstrating that N-glyc b(5)’s COOH terminus is translocated across the membrane to become glycosylated. More importantly, if N-glyc b(5) was first...
incubation treated with CHX and ribosomes were removed by sedimentation. N-glyc b(5) was still glycosylated with high efficiency (lanes 5 and 6), demonstrating that the hydrophobic domain is capable of translocating downstream residues posttranslationally. A s a control, we analyzed the behavior of phaseolin, a plant vacuolar glycoprotein that is translocated across the ER cotranslationally (Ceriotti et al., 1991). A s shown in the lower panel of Fig. 2 B, lane 1, phaseolin was modified to the mono- and diglycosylated forms (marked by one and two asterisks, respectively) when microsomes were present during translation, but remained unglycosylated when membranes were added after translation (lanes 5 and 6).

**b(5) Overexpressed in CV1 Cells Is in the ER in a Nonaggregated State**

In mammalian cells, the first station after exit from the ER is a tubular vesicular compartment, also known as an intermediate compartment (IC) (Bannykh and Balch, 1997). Some proteins that are kept in the ER by a retrieval mechanism can be detected by immunofluorescence in the IC (Jackson et al., 1993; Martire et al., 1996; Lotti et al., 1999), and this IC localization can be increased by incubation at 15°C, a temperature that blocks exit from the IC (J ackson et al., 1993). We investigated whether this was also the case for b(5) by comparing its intracellular distribution with that of ERGIC 53, a marker for the IC (Schweizer et al., 1991), after incubation of transfected CV1 cells at 37 or 15°C. A s can be seen in Fig. 3, b(5) could not be detected in structures enriched in ERGIC 53 either under normal incubation conditions (Fig. 3, A–C), or after prolonged incubation at 15°C (Fig. 3, D–F). Under the latter condition, ERGIC 53-containing elements clustered in the Golgi area (compare Fig. 3, E and B) in agreement with the recent observations of Klumperman et al. (1998). It should be mentioned that at longer times after transfection (>24 h), b(5) was often seen to concentrate in large structures that were positive for ER markers (see Fig. 10). These structures may be related to the karmellae, which are induced in yeast cells by overexpression of b(5) (Vergères et al., 1993). To avoid excessive formation of these structures, all the experiments described in this paper were initiated within 24 h after transfection.

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| Triton X-100 | Antibodies against | Total cells counted | Positive cells | Number | % |
|-------------|-------------------|---------------------|----------------|--------|---|
| +           | Catalytic domain  | 303                 | 102            | 33.7   |
| –           | Catalytic domain  | 218                 | 77             | 35.3   |
| +           | COOH-terminal polar peptide | 282 | 71 | 25.2 |
| –           | COOH-terminal peptide | 168 | 0 | 0 |

MDCK cells expressing b(5) were permeabilized with 5 U/ml SLO and then fixed with 4% paraformaldehyde (see Materials and Methods). Coverslips were processed for immunofluorescence using primary Abs with the indicated specificities in the absence or presence of 0.6% Triton X-100. 10 photomicrographs of random fields, each in the phase-contrast and in epifluorescence, were taken with a 40× magnification lens with the same exposure for each sample, and total cells as well as positive immunofluorescent cells were counted.

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Table I. Quantitative Analysis of b(5) Immunofluorescence in Transfected MDCK Cells Permeabilized with SLO or with SLO + Triton X-100

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Figure 2. In vitro translated N-glyc b(5) becomes glycosylated in the presence of dog pancreas microsomes added either during or after translation. (A) COOH-terminal sequences of wt b(5) and of the N-glyc and O-glyc constructs. In all constructs, the hatched rectangle represents the COOH-terminal part of the membrane-anchoring domain. The underlined sequence in N-glyc b(5) corresponds to the first 19 amino acids of bovine opsin, and is connected to the COOH terminus of b(5) through a Ser and an Arg residue (italics). An N-glycosylation consensus sequence is boxed. The underlined sequence of O-glyc b(5), connected to the COOH terminus of b(5) via the SR sequence (italics), corresponds to residues 36-48 and residue 50 of mature human gly- coporphorin A. The residues in boldface (Y between V48 and T50 and the three COOH-terminal prolines) were included to create an optimal consensus for O-glycosylation (Yoshida et al., 1997). The boxed residues are those that are glycosylated in native glycoporphorin A (Tomita and M archesi, 1975). (B) N-glyc b(5) (upper panel) or bean phaseolin (lower panel) synthetic RNAs were translated for 1 h in the rabbit reticulocyte system in the presence (lanes 1 and 2; M bs co) or absence (lanes 3–6) of dog pancreas microsomes. Only samples, which was first incubated in the absence of microsomes, was then treated with CHX, subjected to ultracentrifugation to remove ribosomes (see Materials and Methods), and further incubated for 1 h with microsomes (lanes 5 and 6; M bs post). At the end of the incubations, samples were divided into two, denatured with SDS, and then incubated overnight in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of Endo H (see Materials and Methods). The arrow in the upper panel indicates the Endo H-sensitive polypeptide (glyco- sylated N-glyc b(5)) generated when microsomes were present during or after translation. The single and double asterisks in the lower panel indicate the mono- and diglycosylated forms of phaseolin, respectively (Ceriotti et al., 1991), which were generated only when membranes were present during the translation. Numbers on the left indicate the position and size (in kD) of M, markers.

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Pedrazzini et al. Intracellular Sorting of Cytochrome b(5)
Because a number of ER membrane proteins, including the ribophorins, are found in rapidly sedimenting aggregates after solubilization of microsomes with nonionic detergents, it is thought that in situ they form an intramembranous proteinaceous network, which could also be the basis for their retention in the ER (Yu et al., 1989). To investigate whether overexpressed b(5) might belong to such an aggregate, we analyzed its sedimentation on sucrose velocity gradients after detergent extraction from transfected CV1 cells and compared it with that of ribophorin I and of b(5) endogenous to rat liver microsomes. As can be seen from Fig. 4, under the conditions used, ribophorin I extracted both from rat liver microsomes (Fig. 4 A) and from transfected CV1 cells (Fig. 4 B) formed rapidly sedimenting aggregates, in agreement with published work (Hortsch et al., 1986; Yu et al., 1989). In contrast, b(5) from rat liver microsomes or transfected CV1 cells was recovered in the upper fractions of the gradient, cosedimenting with cytochrome c as expected for the monomeric form of the protein (Mr ~15,000), suggesting that it exists in a freely diffusible form within the ER of the transfected cells.

**Use of Tagged Versions of b(5) to Follow Its Intracellular Trafficking**

To investigate with biochemical methodology whether

(A) or CV1 cells transfected with wt b(5) cDNA were lysed and loaded onto a 5–20% sucrose gradient containing 0.2% Triton X-100 and low salt, as described in Materials and Methods. Fractions were analyzed by Western blotting. Ribophorin (R) forms aggregates which distribute throughout the gradient and sediment into the pellet, whereas b(5) is found only in the top fractions, cosedimenting with cytochrome c. The vertical and small arrows indicate the positions reached by cytochrome c (Mr ~12,300) and catalase (Mr ~232,000), respectively. Numbers on the left of the panels indicate the position and size (in kD) of Mr markers used in the Western blot analysis.
b(5) is retrieved from a downstream compartment, we used COOH-terminally appended tags to report on the intracellular compartments visited by b(5) during its lifetime. In addition to N-glyc b(5) described above, we constructed a mutant with potential O-glycosylation sites at the COOH terminus (O-glyc b[5]; Fig. 2 A). The sequence chosen was derived from human glycophorin A (residues 35–48), followed by the sequence YTPPP, in which the Thr residue is in an optimal consensus for O-glycosylation (Yoshida et al., 1997).

We first checked by immunofluorescence whether the added tags altered the intracellular distribution of b(5) in transiently transfected CV1 cells. As shown in Fig. 5, both polypeptides had a reticular distribution and a clear localization to the nuclear envelope, indicative of ER localization. Moreover, like the wt protein, they were not concentrated in structures enriched in ERGIC 53, and their catalytic domain was accessible to Abs restricted to the cytosol, as demonstrated by the good reaction obtained in cells permeabilized with SLO.

The Majority of N-glyc b(5) Does Not Reach the Compartment of Residence of Golgi Mannosidases I and II

To see whether the N-glycan of N-glyc b(5) is modified by mannosidase II, which is generally localized in the medial Golgi cisterna (Velasco et al., 1993), we investigated whether the metabolically labeled protein could acquire Endo H resistance. Cells were labeled with [35S]Met/Cys for 2 h, then chased for 6 h, and N-glyc b(5) was immunoprecipitated and analyzed by SDS-PAGE fluorography. As shown in Fig. 6 A, lanes 1 and 2, after the 2 h pulse, most of the in vivo synthesized N-glyc b(5) was glycosylated and Endo H–sensitive; Endo H sensitivity was completely retained also after 6 h of chase (Fig. 6 A, lanes 3 and 4). Since some N-linked glycans are not accessible to mannosidase II, even if they do transit through the Golgi, we checked whether the high mannose oligosaccharide attached to N-glyc b(5) could be modified by mannosidase II if forced into a common intracellular compartment. When cells were incubated with BFA to relocate Golgi enzymes to the ER (Lippincott-Schwartz et al., 1989), a portion of N-glyc b(5) was converted to a more slowly migrating Endo H–resistant polypeptide (Fig. 6 A, lanes 5 and 6). We conclude that under normal conditions N-glyc b(5) does not reach the compartment of residence of mannosidase II.

A n earlier step in N-glycan processing is trimming by mannosidase I, an event that can be monitored by assessing the acquisition of sensitivity to Endo D. We investigated the Endo D sensitivity of N-glyc b(5) in CHO 15B cells, which lack N-acetylglucosamine (GlcNAc) transferase I activity, to avoid a possible loss of Endo D sensitivity by further processing in the Golgi. As shown in Fig. 6 B, nearly all of the N-glyc b(5) remained Endo D–resistant after 6 h of chase (Fig. 6 B, lanes 1 and 2). However, compared with the nondigested sample, a small increase in the
Figure 6. The N-linked oligosaccharide of N-glyc b(5) retains Endo H sensitivity and Endo D resistance. (A) CV1 cells transfected with N-glyc b(5) cDNA were labeled for 2 h with [35S]Met/Cys, then chased for a further 6 h. One set of cells was exposed to BFA (10 μg/ml) 30 min before starting the labeling, and was kept with the drug for the entire pulse and chase period (lanes 5 and 6). At the end of the pulse (lanes 1 and 2) or after the chase (lanes 2–6), cells were harvested, lysed, and subjected to immunoprecipitation with antiopsin mAbs. A liquid containing the same amounts of incorporated radioactive amino acid were used for each immunoprecipitation. One half of each immunoprecipitate was treated with Endo H (lanes 2, 4, and 6), whereas the other half was left untreated (lanes 1, 3, and 5). The figure shows the result of the SDS-PAGE analysis of the Endo H– or Endo D–treated and untreated immunoprecipitates. The position of the 21-kD molecular mass marker is shown at the left of each panel. The vertical arrow indicates the position of the unglycosylated N-glyc b(5).

amount of deglycosylated polypeptide was visible after Endo D treatment (Fig. 6 B, arrow), suggesting that a small proportion of the tagged b(5) may have had access to mannosidase I. After incubation with BFA, all of the N-glyc b(5) became Endo D–sensitive (Fig. 6 B, lanes 3 and 4). These results indicate that the vast majority of N-glyc b(5) molecules do not reach the compartment of residence of mannosidase I during a 6-h chase.

Glycosylation of O-glyc b(5)

The results presented in Fig. 6 indicate that N-glyc b(5) does not recycle through the medial Golgi; however, it was possible that it reaches an earlier Golgi subcompartment. To investigate this possibility, we turned to the O-glyc b(5) construct. The first step in O-glycosylation is attachment of an N-acetylgalactosamine (GalNAc) residue to Thr or Ser residues, and the enzymes responsible for this reaction, the GalNAc transferases, have been localized to the cis-Golgi as well as to more distal cisternae (Röttinger et al., 1998).

We first compared the electrophoretic mobility of in vivo synthesized O-glyc b(5) with that of the in vitro generated primary translation product. A’s shown in Fig. 7 A, the product expressed in transfected cells (Fig. 7 A, lane 1) comigrated with the product synthesized in vitro in the absence (Fig. 7 A, lane 2) or presence (Fig. 7 A, lane 3) of membranes. However, if cells were incubated with BFA, O-glyc b(5) was shifted to a higher apparent Mr, (compare Fig. 7 B, lanes 3 and 4, or Fig. 7 B, lanes 5 and 6), indicating that at least one of the potential O-glycosylation sites could be used if the protein came into contact with the relevant enzymes. To see whether in the absence of BFA the initial steps of O-glycosylation were also taking place but were not resulting in reduced electrophoretic mobility of O-glyc b(5), we carried out metabolic labeling experiments with [3H]-labeled sugars. A’s shown in Fig. 7 B, incubation with [3H]Galactose, which serves as precursor both to UDP-GalNAc and to UDP-GalNAc, resulted in labeling of O-glyc b(5) both in the absence (Fig. 7 B, lane 3) and the presence (Fig. 7 B, lane 4) of BFA. The labeling was specific, because under the same conditions, wt b(5) was not labeled (Fig. 7 B, lanes 1 and 2), although it was efficiently expressed, as shown by the Western blot in the lower part of Fig. 7 B.

The second sugar to be added on O-linked oligosaccharides is galactose. With [3H]Galactose as radioactive precursor, O-glyc b(5) became labeled in the presence of BFA (Fig. 7 B, lane 6), and a weak signal was obtained also in the absence of the drug (Fig. 7 B, lane 5). Thus, at steady-state, a small portion of the O-glyc mutant has also undergone the second step of O-glycosylation.

To investigate how rapidly glycosylation of O-glyc b(5) occurs after its synthesis, we followed the time course of incorporation of [3H]-labeled sugar after blocking protein synthesis with CHX (Fig. 8). Cells were first incubated for 3 h with [3H]Galactose to allow equilibration between UDP-[3H]GalNAc and UDP-[3H]GalNAc. Incubation was then continued in the presence or absence of CHX. To control for the efficacy of the block in protein synthesis, a separate set of dishes of transfected cells was incubated in parallel with [35S]Met/Cys in the presence or absence of CHX, and O-glyc b(5) immunoprecipitates were then analyzed (Fig. 8 B). CHX efficiently blocked the synthesis of O-glyc b(5) (Fig. 8 B; compare lanes 2 and 4 with lanes 1 and 3). However, as shown in Fig. 8 A and on the graph on the right side of Fig. 8 C (obtained by normalizing the amount of immunoprecipitated [3H]-labeled O-glyc b(5) to the amount of O-glyc b(5) expressed, as determined by Western blotting), the block in protein synthesis had no effect at all on the rate of sugar incorporation, which continued at a linear rate for 4 h after addition of the drug. This result stands in sharp contrast to the strong inhibitory effect of CHX on the incorporation of labeled sugar into total cellular proteins (graph on the left side of Fig. 8 C). Thus, there is a large pool of unglycosylated O-glyc b(5) molecules that acquire O-linked sugar at a low rate. It should be mentioned that incubation with CHX for 4 h did not cause an appreciable decrease in the total amount of O-glyc b(5) or in the accumulation of the labeled species (compare Fig. 8 A, lanes 4 and 5) in this and other experiments, indicating that under these conditions O-glyc b(5) is stable. The variations in the amounts sometimes detected in Western blots (e.g., Fig. 8 A, lanes 3–5 vs. Fig. 8 A, lanes 1 and 2) are presumably due to differences in transfection efficiencies in the different dishes.

The bulk of the available evidence indicates that the
process of O-glycosylation occurs in the Golgi complex (Roth et al., 1994; Schweizer et al., 1994; Röttger et al., 1998). However, under some conditions, especially in infected cells, the initial steps may take place in a compartment upstream of the Golgi (discussed in Röttger et al., 1998). Moreover, recent evidence indicates that Golgi-resident enzymes slowly recycle through the ER (Cole et al., 1998; Storrie et al., 1998; Girod et al., 1999). To investigate whether O-glyc b(5) must leave the ER to become glycosylated, we treated cells with the phosphatase inhibitor OKA, a drug which blocks exit from the ER (Davidson et al., 1992). The results of Fig. 9 suggest that O-glyc b(5) must leave the ER to become glycosylated. We therefore performed experiments to determine whether OKA inhibits the process of O-glycosylation in vitro.

The results of Fig. 9 suggest that O-glyc b(5) must leave the ER to become glycosylated. We therefore performed experiments to determine whether OKA inhibits the process of O-glycosylation in vitro.

To exclude the possibility that OKA was decreasing O-glyc b(5) glycosylation through a direct or indirect inhibition of glycosyl transferases and not by a transport block, we analyzed its effect in the presence of BFA. A s can be seen in Fig. 9 A, the effect of OKA was reversed in the presence of BFA (Western blot; Fig. 9 A, lanes 2, 3, and 5). During a 1-h period of incubation with BFA, 41.7 ± 1.7% and 37.6 ± 1.6% (average ± half-range of two experiments) of O-glyc b(5) was converted to the slower migrating, fully processed form in the absence and presence of OKA, respectively.

A nother important piece of information in Fig. 9 can be obtained by examining the ratio of specific radioactivity (immunoprecipitate/Western) of O-glyc b(5) attained in the absence and presence of BFA (Fig. 9 A, lanes 2 and 3). By scanning a lower exposure of fluorographs in which the bands labeled in the presence of BFA had not saturated the film (data not shown), we found that the specific radioactivity attained in 1 h by O-glyc b(5) in the absence of drugs was ~7% that attained by the lower M₆ polypeptide of the doublet generated in the presence of BFA. Since the higher M₆ band presumably contains additional labeled sugar, it could not be compared with the more rapidly migrating polypeptide. If one assumes that the lower M₆ band of Fig. 9 A, lane 3 is 100% glycosylated, this would indicate a half-time for glycosylation of O-glyc b(5) (in the absence of BFA) of ~10 h, in agreement with the slow acquisition of sugar after the blockade of protein synthesis demonstrated in Fig. 8. This half-time may be an underestimate, since a fraction of the lower M₆ polypeptide molecules of Fig. 9 A, lane 3 may be unglycosylated.

The results of Fig. 9 suggest that O-glyc b(5) must leave the ER to become glycosylated. But does O-glyc b(5) return to its compartment of origin once it has exited? This question was investigated both by immunofluorescence and by cell fractionation (Fig. 10).
Figure 8. Time course of posttranslational addition of $^{3}$H-labeled sugar to O-glyc b(5). (A) Metabolic labeling with $[^{3}$H]GlcNH$_2$. Cells transfected with O-glyc b(5) were incubated for 3 h with $[^{3}$H]GlcNH$_2$ to allow for equilibration between UDP- $[^{3}$H]GlcNAc and UDP-$[^{3}$H]GalNAc. Cells from one dish were harvested at this point (lane 1), whereas the others were incubated for a further 1 (lanes 2 and 3) or 4 (lanes 4 and 5) h in the presence or absence of 15 $\mu$g/ml CHX as indicated. For each time point, cell lysates were prepared, and equal amounts of protein were used for immunoprecipitation with anti-b(5) Abs (upper panel). A proportional amount of each lysate (one fifth of the amount used for immunoprecipitation) was analyzed by Western blotting (lower panel). (B) Block of O-glyc b(5) synthesis by CHX. Cells transfected with O-glyc b(5) were incubated in parallel with those in A, but without added $[^{3}$H]GlcNH$_2$. After 3 h, $[^{35}$S]Met/Cys was added and incubation was continued in the presence or absence of 15 $\mu$g/ml CHX as indicated. At the indicated times, cell lysates were prepared and equal amounts of protein were used for immunoprecipitation, as in A. (C) Graphical representation of the results of A compared with the incorporation of $^{3}$H-labeled sugar into total protein. The graph on the right shows the ratios of band intensities of fluorographs (metabolically labeled O-glyc b(5)) to those of the Western blots (total O-glyc b(5)). Shown are the means from three sets of dishes ± SE M. The value for the ratio at 3 h (time of addition of CHX) has been normalized to 1. The graph on the left represents TCA-precipitable $^{3}$H-labeled sugar normalized to total protein content. Squares, no CHX; triangles, CHX.

Discussion

Both retention and retrieval mechanisms are thought to be involved in keeping ER resident proteins from escaping down the secretory pathway. The identification of specific retrieval sequences has spurred a large amount of work that has resulted in a significant increase in our understanding of the retrograde pathway (Pelham and Munro, 1993; Cosson and Letourneur, 1994; Teasdale and Jackson, 1996; Cosson et al., 1998). However, most of this work has been carried out on chimeras, consisting of a retrieval signal grafted onto a protein normally destined for export from the ER, and there is still not much information on the relative roles of retention and retrieval in determining the residence of bona fide ER proteins. A few mammalian ER membrane proteins have been studied by analyzing the processing of N-linked glycans attached to endogenous or engineered consensus sites. Due the failure of these proteins to acquire Endo H resistance, it has been con-
Pedrazzini et al. Intracellular Sorting of Cytochrome b(5)

Included that they reside in the ER by a true retention mechanism (Ahn et al., 1993; Masaki et al., 1996; Duvet et al., 1998; Honsho et al., 1998). However, since Endo H resistance is acquired in the medial Golgi, these studies could not exclude that retrieval from an earlier Golgi compartment was occurring. In this study, we have addressed this problem by attaching short sequences, containing either N- or O-glycosylation sites, to the COOH terminus of b(5), a TA protein of the ER. We show that in the O-glyc mutant at least one glycosylation site is used, indicating that the tagged protein comes in contact with cis-Golgi enzymes, an event which could not be detected by analysis of the oligosaccharide of the N-glyc–tagged version. The methodology used here should also be useful for the study of the trafficking of other ER membrane proteins, particularly of those lacking luminal domains with endogenous glycosylation sites.

Figure 9. OKA inhibits sugar acquisition by O-glyc b(5).
(A) CV1 cells transfected with O-glyc b(5) were incubated with [3H]GlcNH2 for 3 h to allow for equilibration between UDP-[3H]GlcNAc and UDP-[3H]GalNAc. At this time, one set of cells was harvested (lane 1), whereas the others were incubated for a further hour in the presence (lanes 4 and 5) or absence (lanes 2 and 3) of 1 μM OKA with or without 10 μg/ml BFA, as indicated. After clearing of lysates, equal amounts of protein were subjected to immunoprecipitation with anti-b(5) Abs (upper panel). A proportional amount of each lysate (one fifth of the amount used for immunoprecipitation) was analyzed by Western blotting (lower panel). (B) Graphical representation of the results of two experiments like the one in A. Two separate sets of dishes were labeled as described for A. The ratio of the intensities of the O-glyc b(5) bands detected by fluorography to that of the bands in corresponding samples detected by Western blotting is plotted. Shown are the means values ± half-range. (C) Immunofluorescence analysis of cells treated or not treated with 1 μM OKA. After 1 h with (C, panels d–f) or without (C, panels a–c) OKA, cells were fixed. Separate coverslips were analyzed for b(5) (C, panels a and d), ERGIC 53 (C, panels b and e), and giantin (C, panels c and f). Note the redistribution of ERGIC 53 induced by OKA, whereas O-glyc b(5) and giantin appear unaffected by the drug. Bar, 20 μm.

Posttranslational Translocation of the COOH Terminus of b(5) across the ER Membrane

Before undertaking an investigation of the intracellular
sorting of b(5), we judged it necessary to define the topology of its membrane-anchoring domain. Although this problem has been the object of numerous investigations, there were still conflicting views as to whether b(5)’s hydrophobic domain adopts a transmembrane disposition with the extreme COOH-terminal residues in the lumen of the ER, or whether it has a hairpin topology with the COOH terminus looping back into the cytosol (Dailey and

Figure 10. Intracellular distribution of O-glyc b(5). (A) O-glyc b(5) remains in the ER after prolonged incubation with CHX. CV1 cells transfected with O-glyc b(5) were incubated under the same conditions as in the experiments of Figs. 8 and 9, but without the labeled precursor and in the presence of 20 µg/ml of CHX for 4 (A, panels a–c) or 8 (A, panels d–f) h. Fixed cells were doubly labeled with anti-b(5) and anti-ribophorin I Abs (A, panels a–c), or with anti-b(5) and antigiantin Abs (A, panels d–f). A single confocal section is shown for the 4- and 8-h incubation. Images of the same field were acquired separately with the Texas red (A, panels a and d) and the fluorescein (A, panels b and e) filters. The corresponding merged images are shown in A, panels c and f (O-glyc b[5] in red, marker protein in green). Note the absence of colocalization of O-glyc b(5) with giantin and its similar distribution to ribophorin I. The arrowheads in A, panels a–c indicate structures in which O-glyc b(5) accumulates at long times after transfection, and which are also positive for ribophorin I. Bar: (panels a–c) 10 µm; (panels d–f) 20 µm. (B) Optiprep density gradient analysis of CV1 cells transfected with O-glyc b(5) and metabolically labeled with [3H]GlcNH₂. Cells were metabolically labeled with [3H]GlcNH₂ for 6 h, then returned to DME for 4 h, before collection, homogenization, and preparation of a PNS, which was loaded under an 8–22% Optiprep gradient (see Materials and Methods). After centrifugation, fractions were collected and analyzed for O-glyc b(5) by Western blotting (B, panel a), NADPH-cytochrome reductase enzyme activity (B, panel b), [3H]-labeled O-glyc

b(5) by immunoprecipitation followed by SDS-PAGE and fluorography (B, panel c), and GS28 by Western blotting (B, panel d). The thick line positioned at the right of each graph covers the fractions containing the load zone of the gradient.
Retention or Retrieval?

To investigate whether b(5) is retrieved from the IC or from the cis-Golgi, we first compared its distribution by immunofluorescence with that of the IC marker, ErgIC53, after incubation either under normal conditions or at 15°C, a temperature which interferes with trafficking between the ER and the Golgi, and which has been shown in some cases to cause accumulation of recycling ER proteins in the IC (Jackson et al., 1993). However, for b(5), this approach did not yield any evidence in favor of its movement out of the ER. Proteins that recycle slowly may not be captured in the IC by incubation at low temperature (Jackson et al., 1993), presumably because entry as well as exit from the IC may be slowed. Therefore, we turned to the use of luminaly tagged versions of b(5) (N-glyc and O-glyc b(5)), with the aim of using the attached oligosaccharide chains as reporters for the intracellular compartments visited by b(5). These tagged constructs were inserted into membranes with the correct topology, and had an intracellular distribution indistinguishable from that of transiently expressed wt b(5). During a 6-h chase, N-glyc b(5) did not acquire Endo H resistance, and also remained nearly completely Endo D-resistant, suggesting that it was not reaching the medial Golgi. However, when we turned to a more stringent assay based on the incorporation of sugar into the O-glyc mutant, we found that at least one O-glycosylation site was used, indicating that the protein was coming into contact with a GalNAc transferase, enzymes which are localized to the Golgi complex (Rötger et al., 1998). We believe that the acquisition of carbohydrate by O-glyc b(5) is reporting on a recycling event through the cis-Golgi, for the reasons detailed below.

Processing of a glycoprotein by Golgi enzymes has normally been taken as evidence for its passage through the Golgi complex. However, recent evidence showing that Golgi enzymes recycle slowly through the ER (Cole et al., 1998; Storrie et al., 1998; Grillo et al., 1999) makes it more difficult to interpret the significance of Golgi modifications on ER proteins. Nonetheless, since ER proteins generally do not carry Golgi modifications, it seems that these recycling Golgi enzymes are not active within the ER. Therefore, it is probable that O-glyc b(5) acquired its sugar after leaving the ER, and this idea is supported by the finding that glycosylation of O-glyc b(5) could be inhibited by the phosphatase inhibitor OKA, which blocks transport out of the ER (Davidson et al., 1992; Pryde et al., 1998). If O-glyc b(5) had been modified within the ER by recycling Golgi enzymes, OKA might actually have increased the processing of O-glyc b(5) by the trapped glycosyltransferases. A nother explanation for our data is that the tagged protein, after exiting the ER and becoming glycosylated, did not return to its compartment of origin. This interpretation is unlikely, since no redistribution of O-glyc b(5) from the ER to another compartment could be observed after a chase with CHX under conditions in which the proportion of glycosylated O-glyc b(5) molecules was increasing at a linear rate. Moreover, [3H]JGlcNAc2-labeled and total O-glyc b(5) had similar distributions on an Optiprep density gradient, suggesting that they coexist in the same intracellular compartment.

Does the behavior of overexpressed O-glyc b(5) reflect that of endogenous b(5) in mammalian cells? It is possible that overexpression causes leakage from the ER of the tagged b(5) or that the tag itself alters the behavior of b(5). A lthough we cannot exclude either of these possibilities, the important finding here is that b(5) appears to have the capacity to be captured and returned to the ER from a downstream compartment, even though the extent of exit from the ER for the endogenous molecule and the overexpressed tagged mutant may be different. The rate of acquisition of carbohydrate by O-glyc b(5) was in any case quite slow: by comparing the extent of labeling of the tagged protein in the presence or absence of BFA, we estimated a half-time for acquisition of O-linked GalNAc of ~10 h. This slow rate of glycosylation, as well as the lack of colo-
calization of wt and tagged versions of b(5) with markers for the IC, suggest that b(5) tends to be excluded from budding transport vesicles, and that retrieval from a downstream compartment represents a salvage mechanism for those molecules that occasionally leak out of the ER. In other words, it would appear that both retention and retrieval are operating together to keep b(5) in the ER. We do not know at present whether retention is based simply on the lack of a positive export signal and/or the presence of a positive retention signal or feature in b(5). Formation of oligomeric complexes has been suggested to result in retention of some ER proteins (Yu et al., 1989). However, this mechanism would not seem to be responsible for b(5) retention. Indeed, our sucrose gradient analysis did not reveal the presence of b(5) oligomers under conditions in which high-Mr complexes containing ribophorin I were preserved. Consistent with this result, preliminary experiments carried out with the fluorescence recovery after photobleaching technique suggest that green fluorescent protein–b(5) chimeras diffuse rapidly across the ER (Snapp, E., and J. Lippincott-Schwartz, personal communication).

Our previous work demonstrated that a short hydrophobic domain is required to keep b(5) in the ER: lengthening of the TMD results in escape of b(5) from the ER and its arrival at the plasma membrane (Pedrazzini et al., 1996). The extension of this finding by other laboratories to other TA proteins and reporter constructs (Rayner and Pelham, 1997; Yang et al., 1997; Honsho et al., 1998) suggests that TMD-dependent sorting may be a general phenomenon for TA proteins and may occur by a common mechanism. The results of the present study, which suggest that both retention and retrieval mechanisms are operating to keep b(5) in the ER, raise the question as to whether the short TMD is required for retention, retrieval, or for both these phenomena. If the TMD is involved in retrieval, a candidate recycling receptor involved in this process is rer1p, a protein demonstrated to be required for ER residence of a number of membrane polypeptides in yeast via recognition of their TMDs (Sato et al., 1996, 1997). This receptor, which also has been cloned from mammalian cells (Fullekrug et al., 1997), appears to prefer short and somewhat hydrophilic TMDs (Letourneur and Cosson, 1998). On the other hand, retrieval of TA proteins may occur by a completely different mechanism, since Ufe1p, a TA target soluble N-ethylnaleimide-sensitive factor attachment protein receptor of the ER, does not change its localization in rer1p-deficient yeast cells (Rayner and Pelham, 1997).

Further work with tagged versions of TA proteins like the ones described in this study should help to define at the molecular level the sorting mechanisms of this class of proteins.

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Pediartzii et al. Intracellular Sorting of Cytochrome b(5)