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A mutant cytochrome b_5 with a lengthened membrane anchor escapes from the endoplasmic reticulum and reaches the plasma membrane

(brefeldin A/mammalian cells/protein sorting/retention mechanisms/tail-anchored proteins)

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ABSTRACT **Many resident membrane proteins of the endoplasmic reticulum (ER) do not have known retrieval sequences. Among these are the so-called tail-anchored proteins, which are bound to membranes by a hydrophobic tail close to the C terminus and have most of their sequence as a cytosolically exposed N-terminal domain. Because ER tail-anchored proteins generally have short (≤17 residues) hydrophobic domains, we tested whether this feature is important for localization, using cytochrome b_5 as a model. The hydrophobic domain of cytochrome b_5 was lengthened by insertion of five amino acids (ILAAV), and the localization of the mutant was analyzed by immunofluorescence in transiently transfected mammalian cells. While the wild-type cytochrome was localized to the ER, the mutant was relocated to the surface. This relocation was not due to the specific sequence introduced, as demonstrated by the ER localization of a second mutant, in which the original length of the membrane anchor was restored, while maintaining the inserted ILAAV sequence. Experiments with brefeldin A and with cycloheximide demonstrated that the extended anchor mutant reached the plasma membrane by transport along the secretory pathway. We conclude that the short membrane anchor of cytochrome b_5 is important for its ER residency, and we discuss the relevance of this finding for other ER tail-anchored proteins.**

Proteins inserted into the membrane or translocated into the lumen of the endoplasmic reticulum (ER) either remain in the ER or travel to another destination along the secretory pathway. According to the default hypothesis, proteins must carry specific signals to maintain their residence in the ER, while proteins lacking any topogenic sequence are transported to the plasma membrane after transiting through the Golgi complex (1).

As predicted by the default hypothesis, topogenic sequences determining ER residence have indeed been identified both for lumenal and membrane proteins. These signals generally appear to operate in the retrieval of proteins that have escaped from the ER rather than in directly determining their retention (2). Many lumenal proteins carry a KDEL or KDEL-like sequence at their C terminus, which is recognized by a recycling receptor (3). Type I and type II ER membrane proteins commonly carry a double lysine- or a double arginine-containing motif at the extremity of their cytoplasmic tails (4, 5). However, many ER membrane proteins do not carry identified residence signals, and the mechanisms by which they escape vesicular transport to the surface are unknown.

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A well-known class of ER resident proteins is constituted by enzymes with cytosolically exposed N-terminal catalytic domains and C terminal membrane anchors. Proteins with this kind of topology, referred to as "tail-anchored" (6), are inserted posttranslationally into the ER membrane by a signal recognition particle-independent mechanism (7, 8). Known ER retrieval/retention signals have not been identified in these proteins. However, a feature common to them is the relatively short length of the hydrophobic domain through which they are anchored to the phospholipid bilayer, as compared to typical transmembrane domains of plasma membrane proteins (7). A short transmembrane domain is a feature which has also been observed for Golgi resident proteins (9), and it has been demonstrated that this feature is important for retention of trans-Golgi enzymes (10–12).

In the present study, we investigated whether the length of the membrane anchor plays a role in retention of resident ER proteins. For our study we used the ER isoform of mammalian cytochrome (cyt) b_5, one of the most well-known tail-anchored proteins. Although there has been much debate over the topology of its membrane anchor, current evidence favors the trans-bilayer hypothesis (8). The folding of the N-terminal catalytic domain is in any case not influenced by the presence of the membrane anchor (for review, see ref. 7). Here, we show that the extension of this membrane by five residues results in cyt b_5 being transported to the cell surface, and suggest that a short membrane anchor may provide a general mechanism for the exclusion of ER tail-anchored proteins from transport down the secretory pathway.

MATERIALS AND METHODS

Plasmid Construction. DNA manipulations were carried out by standard techniques (13, 14). The absence of errors in fragments generated by PCR was checked by sequencing.

An 893-nt cDNA fragment containing the coding sequence for rabbit cyt b_5 (15) was subcloned into the *Sma* I–*Hin*dII sites of the pGEM4 vector (Promega), generating a plasmid called pG$b_5$wt (wt, wild type). The cyt b_5 extended-anchor mutant (pG$b_5$ext) was constructed by substituting the *Aat* II/*Eae* I fragment in cyt $b_5$wt cDNA (nt 335–530) with a PCR-generated fragment containing a 15-nt insertion (coding for amino acids ILAAV; see Fig. 1) starting from position 510. The upper and lower primers were 5′-ACTTTGAGGACGTCGGGCAC-3′ and 5′-GTCGGCCATGTAGAGGCGATACATCAGTACTGCTGCTAATATTGCCACGATCAG-3′, respectively (inserted sequence in the lower, mutagenic primer is underlined).

Abbreviations: BFA, brefeldin A; cyt, cytochrome; ER, endoplasmic reticulum; PDI, protein disulfide isomerase; FITC, fluorescein isothiocyanate; wt, wild type.

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The cyt b_5 revertant mutant (pG$b_5$rev) was made by substituting a 3′-terminal 557-nt fragment of pG$b_5$ext (starting from the *Aat* II site at position 335) with a PCR fragment containing a 15-nt deletion (nt 487–501), generated on pG$b_5$ext as template. The upper primer was the same as that used for the construction of pG$b_5$ext. The lower, mutagenic primer spanning nt 558–493 of pG$b_5$ext was 5′-AAAGGATCCTCAG-TCGTCGGCCATGTAGAGGCGATACATCAGTACTGCT-GCTAATATTGCCACGAT---GGGGATCAC-3′ (underlined nucleotides are extrasequence containing a *Bam*HI site, boldface nucleotides are the stop codon, and dashes are in the position of the deleted 15 nt. The resulting construct specifies a protein with a membrane anchor of the same length as the wild type, but with altered sequence (see Fig. 1).

For expression in mammalian cells, the cDNAs coding for the three cyt b_5 forms were subcloned into pCB6 (16) that had been modified in the polylinker (17).

Cell Culture and Transient Transfections. CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics under a 10% CO_2/90% air atmosphere. MDCK II cells were grown in minimum essential medium with Earle's salts, supplemented as above, under a 5% CO_2/95% air atmosphere.

Cells, grown on 1.7 × 1.7-cm glass coverslips or on a plastic substrate to ≈50% confluence, were transfected by the calcium phosphate method (18) using 1 μg of Qiagen-purified plasmid per cm^2 of monolayer (Qiagen, Chatsworth, CA). Cells were incubated at 37°C (3–4 h for CV-1 cells, 6–9 h for MDCK cells) with the DNA precipitate in the presence of 0.1 mM chloroquine diphosphate (Sigma). After removal of the DNA-containing medium, MDCK cells were subjected to a glycerol shock (19).

Treatment with Brefeldin A (BFA) and Cycloheximide. For BFA treatment, transfected CV-1 cells were rinsed twice with DMEM after incubation with DNA and chloroquine and then placed in complete medium supplemented with 10 μg/ml of the drug (Boehringer Mannheim). Incubation at 37°C was continued for 20 h.

For cycloheximide treatment, transfected MDCK cells were first incubated at 37°C for 18 h to permit the expression of the heterologous proteins. Cycloheximide (Sigma) was then added at a final concentration of 15 μg/ml, and incubation was continued for the time intervals specified in the legend to Fig. 6.

Immunofluorescence and Lectin Labeling. Transfected cells grown on glass coverslips were fixed for 30 min with 4% paraformaldehyde in 0.120 M sodium phosphate (pH 7.4), permeabilized, and processed for immunofluorescence as described (17). Primary antibodies used were (*i*) an affinity-purified polyclonal antibody against a bacterially expressed fusion protein of rabbit cyt b_5 (17) and (*ii*) a monoclonal antibody against bovine protein disulfide isomerase (PDI) from StressGen Biotechnologies (Victoria, BC, Canada).

For surface labeling with Con A, the time of fixation of the monolayers with paraformaldehyde was reduced to 10 min. Coverslips were incubated in 2 ml of 10 mM Hepes, pH 7.2/0.15 M NaCl/0.1 mM $MnSO_4$/0.1 mM $CaCl_2$/5 μg of biotinylated Con A per ml (Pierce). Bound Con A was revealed with fluorescein isothiocyanate (FITC)-conjugated streptavidin. Cells were then permeabilized and standard immunofluorescence labeling with anti-cyt b_5 antibodies was performed. For double labeling with lentil lectin, permeabilized cells were first stained for cyt b_5, and then incubated in the same buffer as used for Con A, but containing 3 μg/ml FITC-conjugated lentil lectin (EY Laboratories).

Cells were observed under a Zeiss Axioplan microscope equipped for epifluorescence or with a Bio-Rad MRC 1000 laser confocal microscope.

Radioimmunoblot Analysis. Transfected CV-1 cells, plated in 3.5-cm wells, were washed free of medium with phosphate-buffered saline (PBS) containing 5 mM EDTA, scraped from the plastic with a rubber policeman, and concentrated by sedimentation and resuspension in 50 μl of PBS/5 mM EDTA. Cell suspensions were analyzed on high salt SDS/12.5% polyacrylamide minigels (20), followed by radioimmunoblot analysis as described (21).

RESULTS

Construction of b_5 Mutants. To study the influence of the length of the transmembrane domain of cyt b_5 on its retention in the ER, we constructed a cyt b_5 mutant with an extended hydrophobic anchor ($b_5$ext). Fig. 1 shows the hydrophilicity plots of the C-terminal portions of $b_5$wt and b_5 mutants calculated with the scale of Engelman *et al.* (22) over a window of seven residues. With this method, $b_5$wt had a stretch of 17 residues with negative hydrophilicity values. This membrane anchor was extended by inserting five amino acids (ILAAV, underlined in Fig. 1 *Middle* and *Bottom*) already present in the hydrophobic domain (but in different order) and known not to interfere with α-helix formation (23). To exclude the possibility that the inserted sequence contained an unknown targeting signal, we constructed a second mutant, in which we deleted five amino acids (AISAL, underlined with interrupted line in Fig. 1 *Top* and *Middle*), different from the inserted ones, from the anchor of $b_5$ext (Fig. 1 *Bottom*). This mutant was called $b_5$rev because the length of the membrane anchor reverted

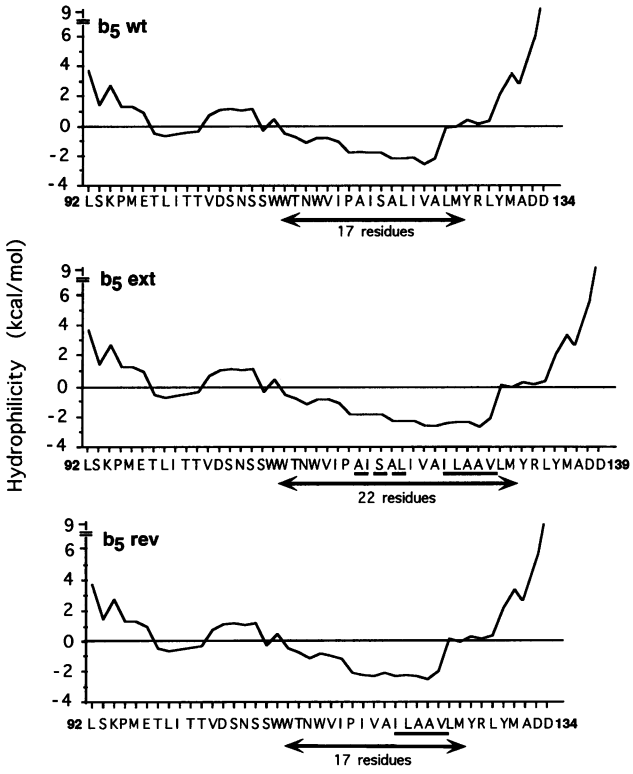

FIG. 1. Hydrophilicity analysis of the the C-terminal portion of cyt b_5 and cyt b_5 mutants. The analysis was carried out from residue 92 to the C terminus, using the scale of Engelman *et al.* (22) over a window of seven residues. Amino acids are indicated on the abscissa with the one-letter code. The double arrow in all three panels encompasses residues scoring negative hydrophilicity, which presumably constitute the membrane anchor. Residues underlined with the interrupted line in $b_5$ext are those which are deleted in the $b_5$rev mutant. Residues underlined with the continuous line in $b_5$ext and $b_5$rev are those that are inserted in the two mutants. The figure illustrates how $b_5$rev and $b_5$wt have similar hydrophilicity profiles, whereas the hydrophobic portion of $b_5$ext is extended by five residues.

to that of the wild type, although the inserted sequence of $b_5$ext was still present.

Expression of cyt b_5 Forms in Mammalian Cells. The wild-type and the two mutant forms of cyt b_5 were transiently expressed in CV-1 cells. To check the integrity of the expressed products as well as the specificity of our anti-cyt b_5 antibodies, we performed a radioimmunoblot analysis on transfected and mock-transfected cells. As shown in Fig. 2, endogenous cyt b_5 was not detectable in the control cells (lane 1), while samples from the the transfected cells (lanes 2–4) all contained an anti-cyt b_5 antibody reactive polypeptide with the expected mobility ($M_r \approx 16{,}000$). The cyt $b_5$ext polypeptide (lane 3) had slightly retarded mobility compared to that of the wild type (lane 2) and of cyt $b_5$rev (lane 4), as expected.

The localization of cyt b_5 forms in transfected cells was analyzed by immunofluorescence (Fig. 3). At the dilutions of primary antibody used, endogenous cyt b_5 was not detectable (see cells marked by asterisks in Fig. 5). $b_5$wt showed a typical ER staining pattern, as expected (Fig. 3*A*), while the distribution of $b_5$ext was drastically changed, with many cells showing bright surface staining (Fig. 3*B*). In other cells, ER staining was also clearly visible (e.g., cell at upper left in *B*). In contrast to $b_5$ext, the $b_5$rev mutant showed a localization indistinguishable from that of the wild type, as exemplified in Fig. 3*C*, indicating that the change in localization of $b_5$ext was due to the altered length of the membrane anchor and not to the amino acid sequence of the inserted peptide. Similar results to the ones shown in Fig. 3 were obtained on transfected COS (not shown) and MDCK cells (see Fig. 6).

To more precisely define the localization of the cyt b_5 forms, a double-labeling experiment with anti-b_5 antibody and Con A was performed. Briefly fixed, nonpermeabilized, transfected CV-1 cells were first incubated with biotinylated Con A to label surface glycoproteins. The cells were then permeabilized and incubated with anti-b_5 antibody. As can be seen from Fig. 4*A*, there was no colocalization between $b_5$wt and Con A. In contrast, $b_5$ext, in addition to showing intracellular labeling, also clearly colocalized with Con A (Fig. 4*B*).

The $b_5$ext Mutant Reaches the Plasma Membrane Along the Secretory Pathway. Because cyt b_5 has no N-terminal signal sequence and is translated on free polysomes in the cytosol (6, 24), the $b_5$ext mutant could theoretically have two alternative routes to reach the plasma membrane—i.e., either it could be directly targeted from the cytosol to the cytoplasmic face of the plasma membrane, or it could first be inserted into the ER membrane and then travel to the plasma membrane along the secretory pathway. To distinguish between these two possibilities, we used BFA to block transport from the ER to the Golgi complex (25). After transfection, CV1 cells were allowed to express cyt b_5 forms for 20 h in the presence or absence of BFA and then doubly immunostained for cyt b_5 and for the ER marker PDI (Fig. 5). As expected, $b_5$wt colocalized with PDI in cells treated (Fig. 5 *E* and *F*) or not treated (Fig. 5 *A* and *B*) with BFA. In the absence of BFA, $b_5$ext showed its usual intracellular plus surface staining (Fig. 5*C*) Treatment with BFA abolished the surface staining and caused colocalization of the mutant with PDI (Fig. 5 *G* and *H*), indicating that $b_5$ext must transit from the ER through the Golgi complex to reach the plasma membrane.

To further investigate the relationship between intracellular and plasma membrane cyt $b_5$ext, we performed a cycloheximide chase experiment. Eighteen hours after transfection, MDCK cells expressing $b_5$wt or $b_5$ext were incubated with cycloheximide and then fixed at different times from the start of the treatment. Cells were double labeled with anti-cyt b_5 antibodies and lentil lectin, used as a specific stain for the Golgi complex (26). The results are shown in Fig. 6. During cycloheximide treatment $b_5$wt did not change its ER localization (red in Fig. 6 *A–C*). A small amount of apparent colocalization (yellow) with lentil lectin binding sites could be seen in some cells at all times; however, at least part of the lectin label (green) was not superimposed on the b_5 stain. In contrast to the situation with $b_5$wt, the localization of $b_5$ext changed dramatically during the cycloheximide chase (Fig. 6 *D–F*). At the start of the chase (Fig. 6*D*), $b_5$ext showed its usual intracellular localization. In the absence of staining of a surface marker, and because of the bright intracellular staining, plasma membrane localization of the mutant cytochrome was not easily discernable in the optical sections. In some cells, like the one shown in Fig. 6D, lentil lectin binding sites only partially colocalized with the cyt b_5 mutant, suggesting that in these cells most of the $b_5$ext had not yet left the ER. After 4 h of cycloheximide chase, $b_5$ext was clearly present in the Golgi complex in all the cells (Fig. 6*E*). After 9 h in cycloheximide, the mutant was almost completely on the plasma membrane and not present on the ER and Golgi (Fig. 6*F*), suggesting that all of the expressed $b_5$ext is competent for transport to the plasma membrane via the Golgi complex, albeit with slow kinetics.

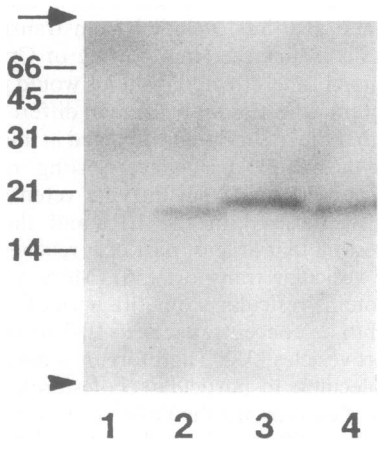

FIG. 2. Radioimmunoblot analysis of CV-1 cells transfected with different cyt b_5 forms. Protein (50 μg) from mock-transfected CV-1 cells (lane 1) or CV-1 cells transfected with $b_5$wt (lane 2), $b_5$ext (lane 3), or $b_5$rev (lane 4) was analyzed by SDS/PAGE radioimmunoblot analysis. The blot was probed with anti-cyt b_5 antibodies. Numbers on the left refer to the molecular masses (in kDa) of standards from Bio-Rad. Arrow and arrowhead refer to origin and front of the gel, respectively.

DISCUSSION

Although the factors determining protein retention in or retrieval to the ER are the object of investigation in many laboratories, numerous proteins reside in this organelle by unknown mechanisms. In the present study we have shown that the addition of five hydrophobic amino acids to the 17-residue-long membrane anchor of cyt b_5, a well-known ER-resident tail-anchored protein, results in its relocation to the plasma membrane. This relocation was not due to direct targeting of the altered cyt b_5 from the cytosol to the plasma membrane, but rather to its transport out of the ER along the secretory pathway, as indicated by the ability of BFA to suppress its appearance at the surface and as suggested by the observation that a cycloheximide chase resulted in the gradual depletion of the intracellular pool of cyt $b_5$ext, while plasma membrane staining for the mutant cytochrome remained strong over the chase period.

The most straightforward interpretation of our results was that the mere lengthening of the membrane anchor of cyt b_5 was responsible for its escape from the ER. To formally exclude that the sequence ILAAV contained a positive signal for transport to the plasma membrane, we constructed a mutant (cyt $b_5$rev) in which the original length of the membrane anchor was restored but the inserted sequence of cyt

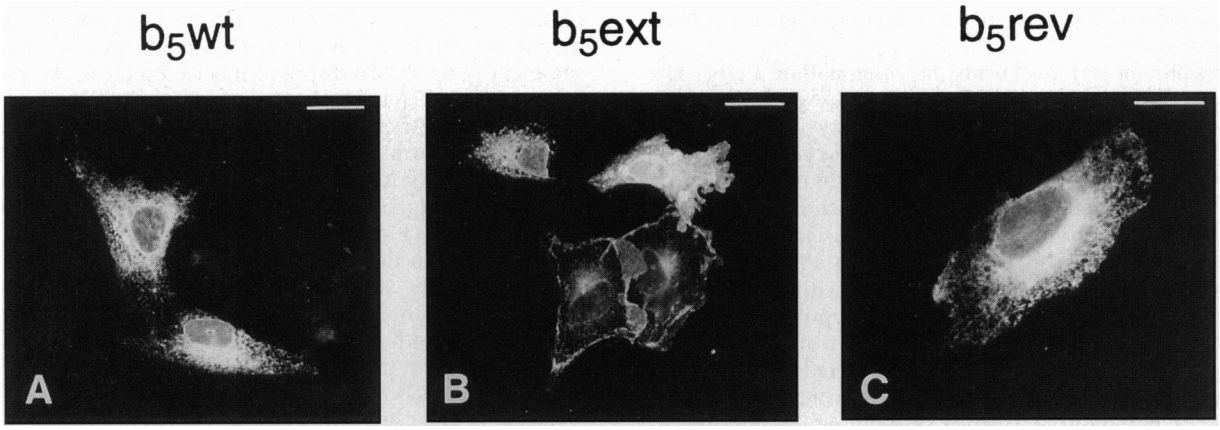

FIG. 3. The length of the hydrophobic anchor of cyt b_5, but not the amino acid sequence, is important for residence in the ER. Twenty-four hours after transfection with $b_5$wt (*A*), $b_5$ext (*B*), or $b_5$rev (*C*), CV-1 cells were fixed, permeabilized, stained with anti-cyt b_5 antibodies, and observed by normal epifluorescence microscopy. Note apparent surface staining of cells transfected with the $b_5$ext mutant (*B*), whereas the $b_5$wt and $b_5$rev mutant show a similar ER-like pattern. (Bars = 20 μm.)

$b_5$ext was retained while five upstream residues (AISAL) were removed. This mutant was localized to the ER, indicating that the anchor length in itself is a factor involved in determining the subcellular localization of cyt b_5.

How could a short membrane anchor keep cyt b_5 in the ER? Does it play a direct role or is it affecting interactions mediated by other protein domains? According to the latter hypothesis, the altered length of the hydrophobic segment could determine an alteration in the position of the N-terminal hydrophilic domain or of the C-terminal polar residues with respect to the membrane. This altered geometry could interfere with protein–protein interactions necessary for the ER retention of cyt b_5. For instance, cyt b_5 could normally form aggregates which interfere with its entry into transport vesicles, as hypothesized for medial Golgi enzymes (27), or it could interact tightly with protein(s) residing in the ER by a retention or retrieval mechanism, and these interactions would be disrupted in the $b_5$ext mutant. Although we cannot exclude this possibility, we think that it is unlikely on the following grounds. First, there is no evidence suggesting that cyt b_5 forms large oligomers or aggregates; rather, it appears to be distributed randomly and to be free to diffuse within the bilayer (28). It is capable of entering into complexes with some of its functional partners (some cyt P-450 isoforms and NADPH–cyt P-450 reductase; ref. 29); however, these functional partners are not present or are present at very low levels in the cells we transfected, making it extremely unlikely that they could account for the ER retention of overexpressed cyt b_5. Second, and perhaps more importantly, a short membrane anchor appears to be a common feature of ER-resident tail-anchored proteins, suggesting that it plays a direct role in determining localization. Protein tyrosine phosphatase 1B, heme oxygenase I, aldehyde dehydrogenase, dimethylaniline monooxygenase 1, and ubiquitin-conjugating enzyme E2 are examples of microsomal tail-anchored enzymes with membrane anchors even shorter than that of cyt b_5.

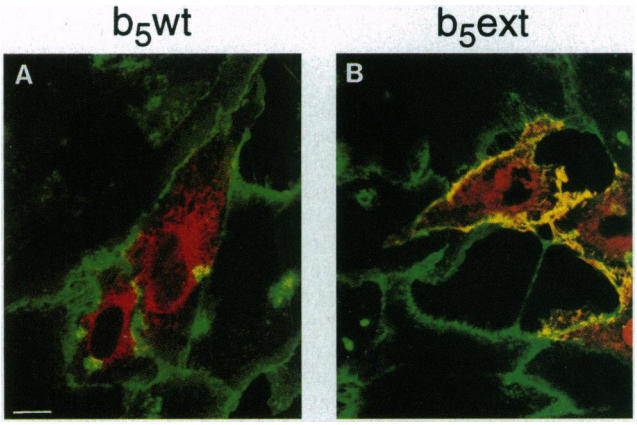

FIG. 4. Colocalization of $b_5$ext mutant with surface glycoproteins. Twenty-four hours after transfection with $b_5$wt (*A*) or $b_5$ext (*B*), CV-1 cells were fixed mildly and exposed to biotinylated Con A to label surface glycoproteins. After incubation with FITC-conjugated streptavidin, the cells were permeabilized, processed for immunofluorescence with anti-cyt b_5 antibodies and rhodamine-conjugated secondary antibodies, and observed by confocal microscopy. The superposition of images viewed under the rhodamine or FITC filter is shown. Red and green show cyt b_5 and surface glycoprotein localization, respectively. Yellow indicates colocalization between $b_5$ext and surface glycoproteins. (Bar = 20 μm.)

A model to explain how a short transmembrane domain could influence membrane protein localization has been proposed for Golgi enzymes (2, 9, 12). This model takes into account the observation that the plasma membrane is much richer than intracellular membranes in cholesterol and sphingolipids, lipids which are known to result in bilayer thickening. It is proposed that, within Golgi cisternae, resident enzymes, because of their short transmembrane domains, are prevented from entering lipid microdomains enriched in cholesterol and sphingolipids and are thus excluded from transport vesicles directed to the cell surface. Thus, sorting of Golgi enzymes from plasma membrane-directed proteins would be mediated simply by different affinities for bilayers of different thickness.

We suggest that the observations reported in this study could be explained in the context of a "bilayer-sorting" model similar to the one proposed for Golgi enzyme retention. Indeed, cholesterol is synthesized in the ER, and there is some evidence suggesting that at least part of it reaches the plasma membrane by vesicular transport (30). Moreover, a plasma membrane protein, vesicular stomatitis virus G protein, has been observed to be concentrated 5- to 10-fold as it exists the ER in transport vesicles (31). Therefore, it is also conceivable that other molecules, in particular cholesterol, are concentrated at ports of exit toward the Golgi, resulting in buds with a lipid composition different from that of the bulk of the ER, with the exclusion of a class of ER resident membrane proteins on purely physicochemical grounds. Alternatively, bilayer-mediated sorting could operate after exit of short tail-anchored proteins from the ER, which would be preferentially concentrated in recycling vesicles returning to the ER from the Golgi and differing in lipid composition from the bulk of the early Golgi bilayer.

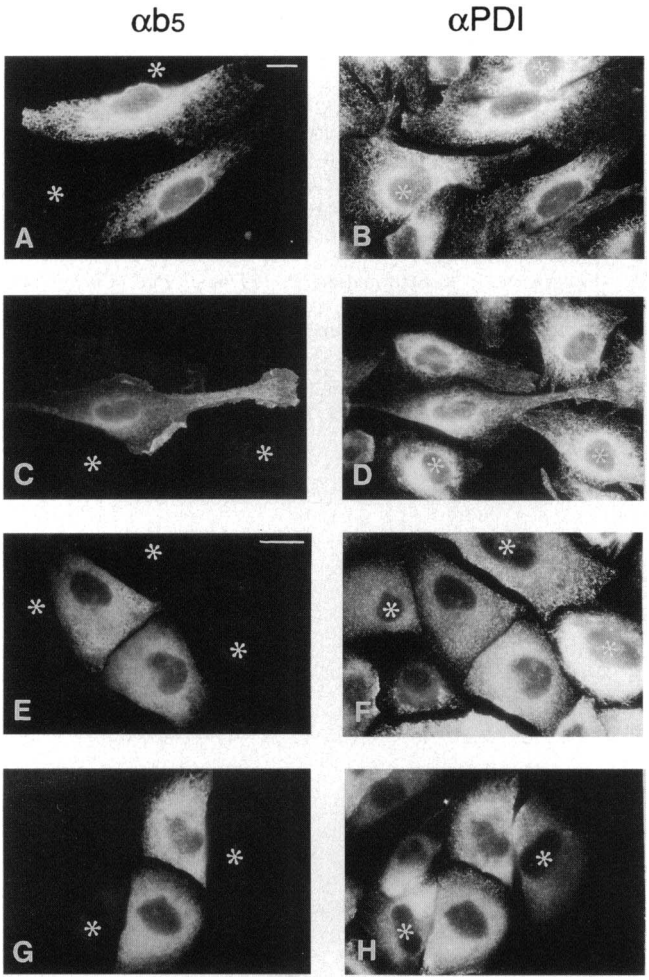

FIG. 5. BFA blocks the transport of b_5 to the plasma membrane. CV-1 cells transfected with $b_5$wt (*A, B, E,* and *F*) or $b_5$ext (*C,D, G,* and *H*) were incubated in the presence (*E–H*) or in the absence (*A–D*) of BFA for 20 h, and then fixed and doubly labeled with polyclonal anti-b_5 (*A, C, E,* and *G*) and monoclonal anti-PDI (*B, D, F,* and *H*) antibodies, which were revealed with rhodamine- or FITC-labeled anti-rabbit or anti-mouse IgG antibodies, respectively. Cells were observed by normal epifluorescence microscopy. *B, D, F,* and *H* (FITC filter, revealing PDI) show the same field as *A, C, E,* and *F* (rhodamine filter, revealing cyt b_5), respectively. After BFA treatment $b_5$wt and $b_5$ext are indistinguishable from each other and from PDI (*E–H*). Asterisks indicate the positions of nontransfected cells, which are positive for the PDI stain but negative for cyt b_5. [Bars in *A* (referring to *A–D*) and *E* (referring to *E–H*) = 20 μm.]

An obvious question that arises from this interpretation of our results is how cyt b_5 is distinguished from Golgi resident proteins, since the length of its membrane anchor is the same as that of the average transmembrane domain of type II Golgi membrane proteins (9). In other words, what keeps cyt b_5 from becoming a Golgi protein? We envisage two, nonmutually exclusive possible explanations. First, differences in amino acid sequence of the anchor could have a subtle role in modulating the effect of sheer length. The second possibility is based on the conclusions which can be reached from the studies on Golgi enzymes—i.e., that while the length of the transmembrane domain is important in determining Golgi residency, it is not the only factor involved. For example, Sed5, a cis-Golgi located tail-anchored protein involved in vesicular traffic, has a short transmembrane domain that can confer Golgi localization when transferred to the plasma membrane protein syntaxin. However, the longer transmembrane domain of syntaxin does not result in the relocation of Sed5 to the plasma membrane, which therefore possesses additional Golgi retention information (32). It appears that membrane anchor length operates in conjunction with other signals, and that in different proteins, the strength and location of these additional signals varies. Thus, cyt b_5 may have ER retention signals in its short lumenal or catalytic cytoplasmic domain which are not strong enough to function alone. A long transmembrane domain would override these signals, allowing the release of the protein from the ER.

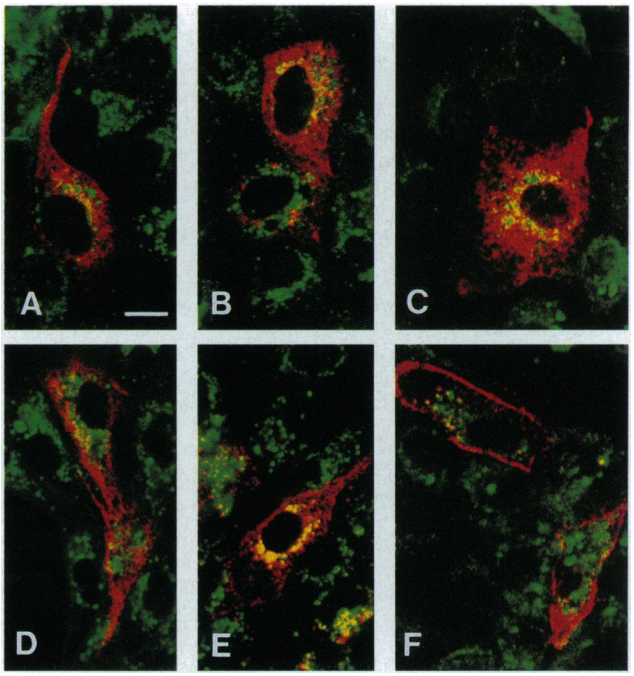

FIG. 6. A cycloheximide chase causes depletion of the intracellular pool of $b_5$ext. MDCK cells transfected with $b_5$wt (*A–C*) or $b_5$ext (*D–F*) were allowed to express the exogenous cDNA for 18 h, after which incubation was continued in the presence of cycloheximide for the following times: *A* and *C*, 0 h; *B*, 7 h; *E*, 4 h; *C* and *F*, 9 h. Cells were fixed and doubly labeled with anti-cyt b_5 (red) and FITC-labeled lentil lectin (green) to stain the Golgi complex and observed by confocal microscopy. Yellow indicates colocalization of cyt b_5 and Golgi-located lectin binding sites and is evident in $b_5$ext-transfected cells chased for 4 h with cycloheximide (*E*). Note how after 9 h of cycloheximide chase the intracellular pool of $b_5$ext appears depleted (*F*). (Bar = 20 μm.)

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