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Retrieval From the ER–Golgi Intermediate Compartment Is Key to the Targeting of C-Terminally Anchored ER-Resident Proteins

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ABSTRACT

Endoplasmic reticulum (ER) resident proteins may be maintained in the ER by retention, where the leak into post-ER compartments is absent or slow, or retrieval, where a significant leak is countered by retrieval from post-ER compartments. Here the targeting of the C-terminally anchored protein ER-resident protein, cytochrome b5a (cytb5a), considered to be maintained in the ER mainly by the process of retention, is compared with that of sarcolipin (SLN) and phospholamban (PLB); also C-terminally anchored ER-residents. Laser confocal microscopy, and cell fractionation of green fluorescent protein-tagged constructs expressed in COS 7 cells indicate that while calnexin appears to be retained in the ER with no evidence of leak into the ER–Golgi intermediate compartment (ERGIC), significant amounts of cytb5a, SLN, and PLB are detectable in the ERGIC, indicating that there is considerable leak from the ER. This is supported by an in vitro budding assay that shows that while small amounts of calnexin appear in the transport vesicles budding off from the ER, significant amounts of cytb5a and SLN are found in such vesicles. These data support the hypothesis that retrieval plays a major role in ensuring that C-terminally anchored proteins are maintained in the ER. J. Cell. Biochem. 112: 3543–3548, 2011.

KEY WORDS: CALNEXIN; CYTOCHROME b5; PHOSPHOLAMBAN; RETENTION; RETRIEVAL; TARGETING

Membrane proteins are synthesized on the rough endoplasmic reticulum (RER) and then sorted according to their required destination. However, some proteins are required for the functioning of the ER and so must be maintained in this compartment either by a process of retention, in which their removal from the ER is prevented or slowed, or by retrieval, in which they leave the ER and are retrieved from post-ER compartments [see reviews, Teasdale and Jackson, 1996; Lee et al., 2004]. Among the proteins maintained in the ER are a group that are anchored to the membrane by their C-termini. Such proteins include, phospholamban (PLB) and sarcolipin (SLN [Butler et al., 2007]) sec61α, β, and γ [Greenfield and High, 1999] and microsomal cytochrome b5 (cytb5a [Pedrazzini et al., 1996, 2000; Honsho et al., 1998]). We have shown that PLB and SLN are maintained in the ER by a process of retrieval, as significant amounts of these proteins leave the ER and enter the ER–Golgi intermediate compartment (ERGIC), but fail to progress to the trans-Golgi network (TGN [Butler et al., 2007]); this appears also to be the case for sec61 [Greenfield and High, 1999]. However, it has been reported that cytb5a is retained by the ER, with only a low level leak into the ERGIC [Pedrazzini et al., 2000]. Calnexin, an N-terminally anchored transmembrane protein, also appears to be retained in the ER with barely any of the protein being detectable beyond the confines of the ER [Hammond and Helenius, 1994; though see, Okazaki et al., 2000].

To investigate whether cytb5a is unusual in its mechanism of ER maintenance among the C-terminally anchored ER-resident proteins, cytb5a was tagged at its N-terminus with the enhanced green fluorescent protein (GFP) and expressed in COS 7 cells so that its targeting could be compared with retrieved proteins (GFP-tagged PLB and SLN) and the retained protein (GFP-tagged calnexin). These

Abbreviations used: GFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; ERGIC, endoplasmic reticulum–Golgi intermediate compartment; cytb5a, cytochrome b5a; TGN, trans-Golgi network; DAPI, 4',6-diamidino-2-phenylindole; HRP, horseradish peroxidase; RER, rough endoplasmic reticulum; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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proteins were localized by confocal microscopy and by cell fractionation and their exit from the ER was investigated using an in vitro budding assay. In addition, to ensure that tagging of cytb5a with GFP did not lead to any mis-targeting, untagged cytb5a was localized by immunofluorescence microscopy.

These studies show that although GFP-tagged calnexin is retained by the ER, with undetectable ERGIC localization and barely detectable rates of transfer into ER transport vesicles, in contrast GFP-tagged cytb5a has a profile similar, in terms of its distribution between ER and ERGIC, to GFP-tagged PLB and SLN. The ability of GFP–cytb5a to be packaged into ER exit vesicles is also similar to that of GFP-tagged PLB. These data indicate that the targeting of cytb5a is similar to that found for all of the C-terminally targeted ER-resident proteins that have been investigated [Greenfield and High, 1999; Butler et al., 2007] and that retrieval plays a major role in their ER maintenance.

METHODS

CONSTRUCTION OF cytb5a EXPRESSION CONSTRUCTS

cDNA coding for GFP was amplified from the pGFP-N1 vector (Clontech) using the polymerase chain reaction (PCR) with the following primers: GAT TCT AAG CTT ACC ATG GTG AGC AAG GGC GA; ACA GTT GGT ACC CTT GTA CAG CTC GTC CAT GC. The amplified gene was cloned into pcDNA 3.1(+) (Invitrogen) to produce pcDNA3.1GFP.

The cDNA coding for the human cytb5a (accession number M22865) was amplified by PCR from clone IRATp970E0218D (RZPD) using primers: ATT AGT GAA TTC CAT GGC AGA GCA GTC GGA CG; AGT TAC TCT AGA TGT TCA GTC CTC TGC CAT GTA TAG. The gene was then cloned into pcDNA3.1GFP downstream of GFP to produce pcDNA3.1GFPCYB5. GFP was linked to cytb5a by the sequence GTELGSTSPVWWNS.

To produce the construct coding for untagged cytb5a the following primers were used: GAT CTC AAG CTT ACC ATG GCA GAG CAG TCG GAC GAG; GAT GTT GGT ACC TCA GTC CTC TGC CAT GTA TAG. The amplified product was cloned (*Hin*dIII, *Kpn*I) into pcDNA3.1(+).

GFP-TAGGED CALNEXIN

A clone coding for calnexin–GFP (calnexin accession number NM_001746) was purchased from Genecopoeia (catalog. number EX-K4904-M03).

TRANSFECTION OF COS 7 CELLS

COS 7 cells were grown to 80% confluence on 25 mm coverslips for immunofluorescence studies and in tissue culture dishes for immunoblotting and functional studies before transfecting with plasmid DNA using Fugene 6 (Roche), according to the manufacturer's instructions.

SUBCELLULAR FRACTIONATION

Subcellular fractionation was carried out as described previously [Butler et al., 2007]. Briefly, COS 7 transfected with the appropriate construct were incubated for 2 days. The cells were harvested and then disrupted by sequential passage through 19-, 23-, and 25-gauge needles. The disrupted cells were centrifuged (1,500*g* for 5 min at 4°C) and the resulting supernatant was fractionated using a Nycodenz gradient. Following fractionation the gradient was collected as nine 1.5 ml fractions and the protein precipitated with perchloric acid. The precipitates were solubilized in Laemmli sample buffer containing 8 M urea. The protein samples were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose. Western blotting was performed using antibodies directed against GFP (Roche), calnexin (Stressgene), β COP (Abcam), and TGN 46 (Serotec). The primary antibodies were detected with the appropriate secondary antibodies conjugated to horseradish peroxidase (HRP; Amersham Life Science) and ECL super signal substrate (Pierce).

VISUALIZATION OF GFP-TAGGED CONSTRUCTS IN COS 7 CELLS

Transfected cells were viewed under a Leica digital epifluorescence microscope (DM IRBE) fitted with standard FITC and rhodamine filter sets for the excitation of both GFP and Texas Red fluorophores. Images were also obtained with a Zeiss confocal microscope (LSM 510 META); the GFP fluorophore was excited at 488 nm with a band pass filter of 505–530 nm and Texas Red and Alexa Fluor 594 at 543 nm with a long pass filter of 560 nm.

IMMUNOFLUORESCENCE MICROSCOPY

To examine the subcellular location of GFP-tagged cytb5a, transfected COS 7 cells were treated with a cold block or brefeldin A to enhance the visualization of the appropriate compartments [Butler et al., 2007] before fixation in ice-cold methanol for 15 min. The cells were preincubated for 30 min in PBS Triton X-100 (0.01%) with 2% milk powder. The primary antibodies used were sheep anti-human TGN 46 (Serotech), diluted 1:50 and mouse anti-human ERGIC 53 (a gift from H.P. Hauri, University of Basel) diluted 1:100. The secondary antibody for TGN 46 was donkey anti-sheep IgG conjugated to Texas Red (Amersham Life Sciences) diluted 1:100 and for ERGIC 53 was sheep anti-mouse Texas Red antibody (Amersham Life Sciences) diluted 1:50.

Non-tagged cytb5a was visualized using rabbit anti-cytb5a (Santa Cruz Biotechology) diluted 1:100, followed by a secondary antibody, donkey anti-rabbit conjugated to FITC (Amersham Life Sciences) diluted 1:50.

IN VITRO VESICLE BUDDING ASSAY

Microsomes were prepared as described previously [Rowe et al., 1996], with the following modifications. For each of the EGFP chimeric proteins, cells were scraped from eight 140 cm^2 plates of transfected COS 7 cells. Cells were homogenized by passing them 20 times through a tight fitting 1 ml glass homogenizer. The final microsomal pellet was resuspended in 200–300 μl of transport buffer giving a microsomal protein concentration of between 1 and 3 mg/ml. Rat liver cytosol was prepared as described previously [Davidson and Balch, 1993]. The in vitro budding assay and separation of the transport vesicles by differential centrifugation was performed as described by Rowe et al. [1996], except that the control reactions were incubated on ice and the experimental reactions at 32°C for 20 min. Samples were separated using SDS–PAGE and detected by Western blot analysis using Roche anti-GFP

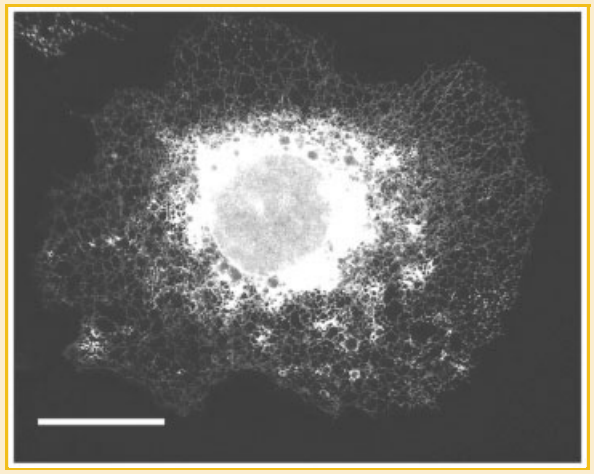

Fig. 1. Location of GFP-tagged cytb5a. COS 7 cells were seeded onto coverslips and transfected with an expression construct coding for GFP-tagged cytb5a. The cells were incubated for 2 days before viewing the cells with a Zeiss confocal microscope. The white scale bar is 10 μm.

antibody 1:1,000 and anti-mouse HRP conjugate GE Healthcare 1:2,000.

RESULTS

LOCATION OF GFP-TAGGED cytb5a AND CALNEXIN BY FLUORESCENCE MICROSCOPY

Figure 1 shows the expression of GFP-tagged cytb5a in a COS 7 cell. As previously reported the distribution of cytb5a is consistent with its location in the ER [Kuroda et al., 1996]. A similar distribution is shown in cells in Figure 2a. However, when the ERGIC is revealed using antibodies directed against ERGIC 53 ([Schindler et al., 1993] (Fig. 2b) there is demonstrable co-localization of GFP-tagged cytb5a and the ERGIC maker (compare areas indicated by white arrows in Fig. 2a and Fig. 2c). In a similar study, where TGN is identified using antibodies directed against TGN 46 [Banting and Ponnambalam, 1997] (Fig. 2e) it is apparent that the GFP-tagged cytb5a (Fig. 2d) does not co-localize with the TGN marker (Fig. 2f).

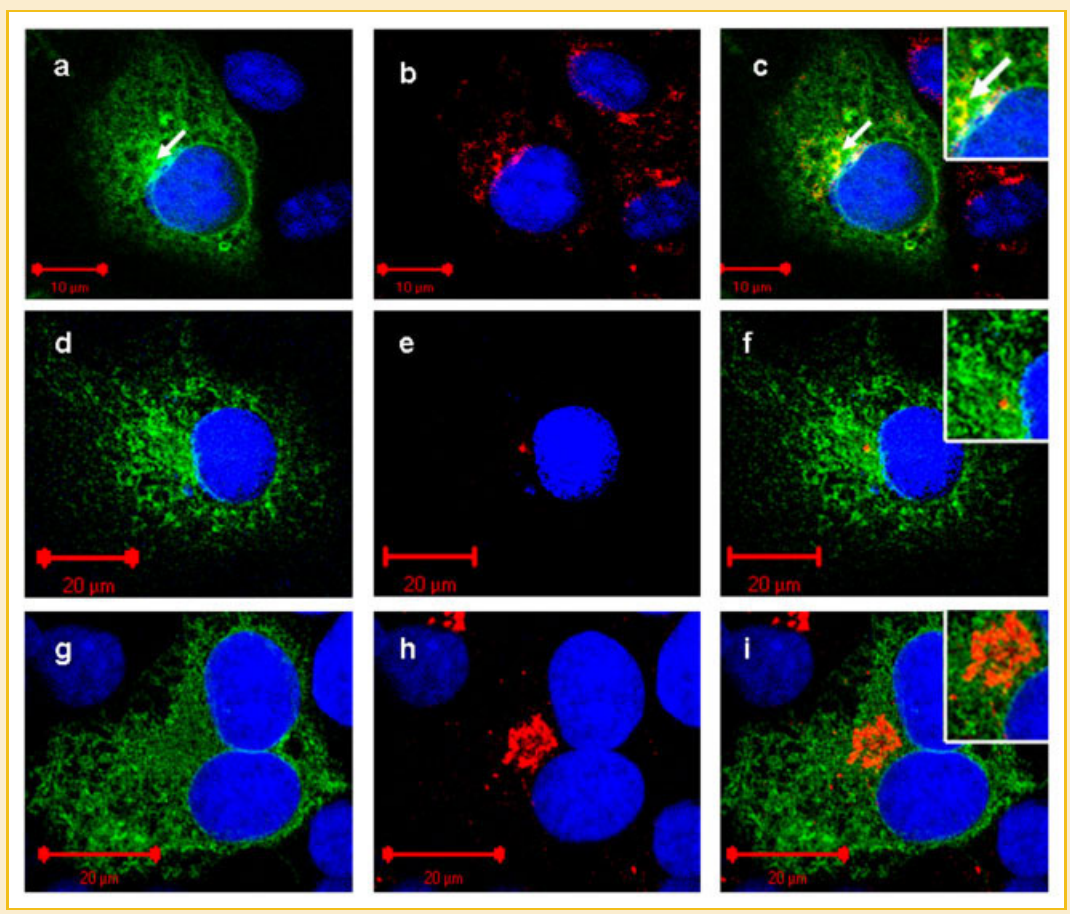

Fig. 2. GFP-tagged cytb5a in COS 7 cells is located in the ERGIC but not the trans-Golgi. COS 7 cells were seeded onto coverslips and transfected with an expression construct coding for GFP-tagged cytb5a (panels a–c and d–f) or GFP-tagged calnexin (panels g–i). The cells were incubated for 2 days before using an anti-ERGIC 53 antibody to reveal the ERGIC compartment (panels b and e). Panel a shows GFP-tagged cytb5a fluorescence. Panel b shows the same cells treated with an antibody against the ERGIC marker followed by a Texas Red conjugated secondary antibody and panel c shows an overlay of panels a and b. Panel d shows GFP-tagged cytb5a fluorescence. The trans-Golgi network was identified using an anti-TGN 46 antibody visualized with a Texas Red conjugated secondary antibody. Panel e reveals the TGN marker and panel f shows an overlay of panels d and e. Panel g shows GFP-tagged calnexin fluorescence. Panel h shows the same cells using the ERGIC marker. Panel i shows an overlay of panels g and h. Nuclei have been revealed using DAPI fluorescence. Cells were viewed with a Zeiss confocal microscope. The red scale bars are 10 μm in panels a–c and 20 μm in panels d–i. The white arrows in panels a, c, g, and i indicate structures where GFP-tagged cytb5a and the ERGIC marker are co-localized. The inset panels in panels c, f, and i show the localization of the ERGIC and trans-Golgi network in greater detail. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

The ER marker calnexin [Bergeron et al., 1994], when tagged with GFP maintains its ER localization (Fig. 2g), but in the same cells it is clear that GFP-tagged calnexin is excluded from the ERGIC (Fig. 2h) since there is no apparent co-localization of GFP-tagged calnexin and the ERGIC 53 (Fig. 2i); Thus, the rate of leak from ER to ERGIC in these COS 7 cells is undetectable using fluorescence microscopy.

LOCATION OF GFP-TAGGED cytb5a AND CALNEXIN BY SUBCELLULLAR FRACTIONATION

Figure 3 shows the subcellular localization of calnexin and GFP-tagged cytb5a. Calnexin is located in the densest fractions (fractions 1 and 2) of the Nycodenz gradient, whereas GFP-tagged cytb5a is also located in the lighter fractions (fractions 6–8). These lighter fractions are where the markers for the ERGIC [identified using an antibody against β-COP [Tang et al., 1997]; mainly fractions 7–8] and TGN [identified using an antibody against TGN 46 [Ponnambalam et al., 1996]; mainly fraction 6] are located.

LOCATION OF UNTAGGED cytb5a BY IMMUNOFLUORESCENCE MICROSCOPY

To confirm that the presence of the GFP-tagged cytb5a in the ERGIC is not an artefact due to the presence of the tag, the localization of untagged cytb5a was also studied. The fluorescence micrographs of ERGIC 53 (Fig. 4b) and heterologously expressed cytb5a (Fig. 4a) in COS 7 cells when merged indicate that an appreciable amount of cytb5a is located in the ERGIC (compare Fig. 4a and Fig. 4c; regions indicated by the white arrows).

DEMONSTRATION OF GFP–cytb5a, CALNEXIN, AND PHOSPHOLAMBAN EXIT FROM THE ER USING AN IN VITRO BUDDING ASSAY

Figure 5 shows the results of the in vitro budding assay used to assess the movement of material from the ER into COP II coated vesicles en route for the ERGIC [Rowe et al., 1996]. After incubating rat liver cytosol with microsomes containing GFP-tagged calnexin, cytb5a, or PLB for 20 min at 0°C almost all of the GFP-tagged material remained in the medium speed microsome pellet (M) following centrifugation. Only a trace of GFP-tagged protein was located in the high-speed pellets (H) that contain the transport vesicles that have budded off from the microsomes. However, performing the incubation at 32°C resulted in significant amounts of GFP-labeled cytb5a and PLB being located in the high-speed pellet, indicating that these proteins have migrated from the microsomes into the budding transport vesicles. In contrast, although there is some evidence for the migration of GFP-tagged calnexin migration from the microsome into the vesicles found in the high-speed pellet, the amount is much smaller than seen for EGFP-tagged cytb5 and PLB.

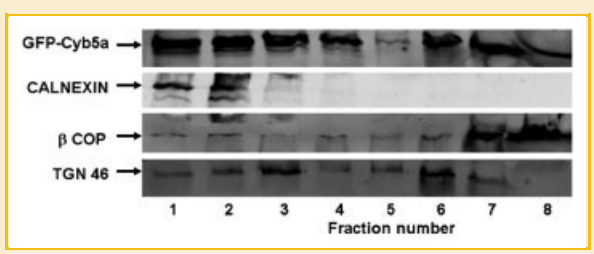

Fig. 3. Localization of GFP-tagged cytb5a by cell fractionation. COS 7 cells transfected with a construct coding for GFP-tagged cytb5a were disrupted as described in the Methods Section. After removing the nuclei and undisrupted cells with a preliminary centrifugation step, material was centrifuged on a Nycodenz gradient. Fractions were collected, fraction 1 being the densest. Proteins from each fraction were precipitated with perchloric acid and the pellets obtained by centrifugation were solubilized and the component proteins separated by SDS–PAGE. The proteins were then subjected to Western blot analysis using antibodies directed to GFP, calnexin, β COP, and TGN 46. The primary antibodies were detected using secondary antibodies conjugated to horseradish peroxidase in a chemiluminescence protocol. The signals were detected and recorded using a Biorad Versa Doc system.

DISCUSSION

Most of the C-terminally anchored proteins of the internal membranes of the cell appear to translocate from the ER into the endomembrane system on completion of synthesis. Some proteins, such as giantin, migrate only as far as the Golgi apparatus [Linstedt et al., 1995], but C-terminally anchored SNARE proteins are found throughout the endomembrane system (see review [Burri and Lithgow, 2004]. PLB and SLN, while maintained by the ER, nonetheless leave the ER to be retrieved from the ERGIC [Butler et al., 2007] as indeed are GFP-tagged Sec61α and wild-type Sec61β and Sec61γ [Greenfield and High, 1999]. The one apparent exception to this is the ER targeted form of cytb5a which was reported to be maintained in the ER by a retention mechanism albeit backed up by a retrieval mechanism that scavenges the small amounts of cytb5a (undetectable by immunofluorescence) leaking to the ERGIC [Pedrazzini et al., 2000]. Here, we show that in COS 7 cells significant amounts GFP-tagged cytb5a accumulate in the ERGIC (Fig. 2a–c), which contrasts with what has previously been reported for cytb5a and variants thereof, though the previous studies were conducted in CV1 cells [Pedrazzini et al., 2000]. It seems unlikely that the cytb5a is mis-targeted as a result of the GFP-tag, since Figure 4 shows by fluorescence microscopy that untagged cytb5a is also located in the ERGIC. In addition, GFP tagging has been used a method for the localization of proteins since 1995 and there are no reports in the literature of interactions of GFP with any of the intracellular compartments; if GFP is expressed in the cytoplasm, for example, it is uniformly distributed throughout this compartment [Ogawa et al., 1995]. Also in a previous study [Watson et al., 2011], we tagged a plasma membrane calcium pump with GFP. This construct was not retained by the ERGIC and entered the wider endomembrane system, indicating that retention of GFP–cytb5a is not the result of the GFP-tag.

In contrast GFP-tagged calnexin appears to be largely maintained in the ER by a process of retention [Greenfield and High, 1999; Newton et al., 2003; Butler et al., 2007], although in immature thymocytes significant amounts of calnexin appear on the cell surface [Wiest et al., 1997; Okazaki et al., 2000]. In this study, GFP-tagged calnexin is undetectable in the ERGIC or TGN, by fluorescence microscopy or cell fractionation/Western blotting (Figs. 2g,h and 3). In addition, the vesicle budding assay (Fig. 5)

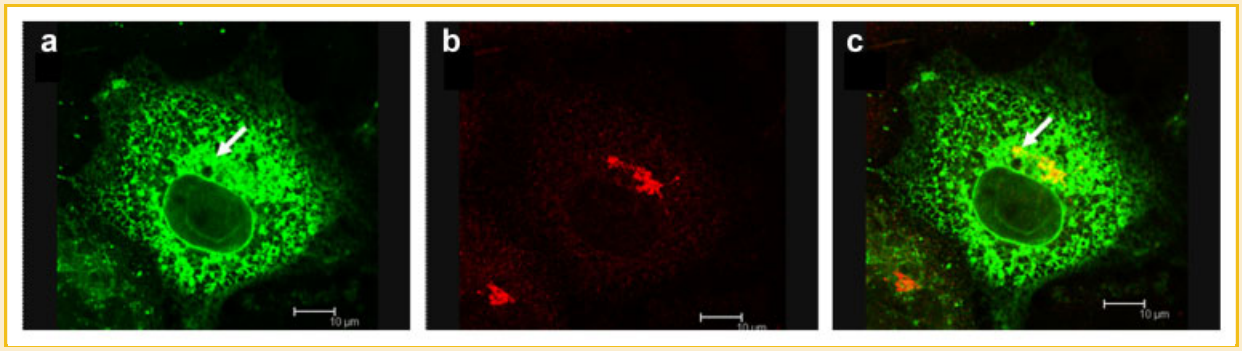

Fig. 4. Cytb5a in COS 7 cells is located in the ERGIC. COS 7 cells were seeded onto coverslips and transfected with an expression construct coding for cytb5a (panels a–c). The cells were incubated for 2 days before using an anti-ERGIC 53 antibody to reveal the ERGIC compartment (panel b). Panel a shows the location of cytb5a visualized using an antibody directed against it, followed by a FITC-conjugated secondary antibody. Panel b shows the same cells treated with an antibody against the ERGIC marker followed by a Texas Red conjugated secondary antibody and panel c shows an overlay of panels a and b. Cells were viewed with a Leica confocal microscope. The white arrow indicates structures where GFP-tagged cytb5a and the ERGIC marker are co-localized. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

shows that compared with GFP-tagged cytb5a and PLB very little GFP-tagged calnexin buds off from microsomes into transport vesicles indicating that like PLB, but unlike calnexin, significant amounts of cytb5a exit the ER en route to the ERGIC and must therefore be maintained in the ER by a process of retrieval.

That GFP-tagged cytb5a is maintained in the ER by retrieval is also supported by the finding that it fails to progress further along the endomembrane pathway to the TGN, indicating that it must be recycled back to the ER (Fig. 2d–f). The cell fractionation study also provides additional support for retrieval (Fig. 3). It that while the GFP-tagged cytb5a is located in the compartment occupied by calnexin in the densest Nycodenz fractions (the ER), it is also found in the lighter fractions where the markers of the TGN and ERGIC are located. Though it is not possible to discriminate between ERGIC and TGN in such gradients the study serves to emphasize that significant amounts of GFP-tagged cytb5a leave the ER and enter the wider endomembrane system.

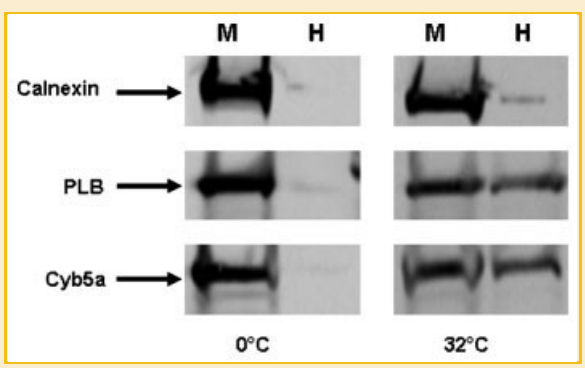

Fig. 5. In vitro budding assay. Microsomes from COS 7 cells expressing GFP-tagged constructs of calnexin, phospholamban, and cytb5a were incubated with rat liver cytosol for 20 min at either 0 or 32°C. Centrifugation was used to separate microsomes (M) from transport vesicles (H) and the proteins were subjected to SDS–PAGE and Western blotting using antibodies directed against the GFP-tag. The amount of M analyzed was 10% of that loaded for H.

These data indicate that cytb5a along with all the other C-terminally anchored membrane proteins of the endomembrane system examined thus far are able to leave their site of insertion in the ER and enter the ERGIC in significant quantities. For those C-terminally anchored proteins resident in the ER, it appears that retrieval from the ERGIC plays a major role in their targeting.

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