Sarco/endoplasmic-reticulum calcium ATPase SERCA1 is maintained in the endoplasmic reticulum by a retrieval signal located between residues 1 and 211

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The location of sarco/endoplasmic-reticulum calcium ATPase (SERCA) retention/retrieval motifs in the sequence of the SERCA1 has been investigated by examining the subcellular location in COS-7 cells of enhanced-green-fluorescent-protein-tagged calcium-pump chimaeras. These chimaeras have been constructed from the fast-twitch SERCA1 and the plasma-membrane calcium ATPase PMCA3. The N-terminal, central and C-terminal segments of these calcium pumps were exchanged between SERCA1 and PMCA3. The segments exchanged correspond to residues 1–211, 212–711 and 712–994 of SERCA1, and residues 1–264, 265–788 and 789–1159 of PMCA3 respectively. Only chimaeras containing the N-terminal segment of SERCA1 were located in the endoplasmic reticulum (ER), whereas chimaeras containing the N-terminal segment from PMCA3

were able to escape from the ER and enter the endomembrane pathway en route for the plasma membrane. Co-localization of SERCA1 in COS-7 cells with the ER/Golgi-intermediate compartment marker ERGIC53 indicates that SERCA1 is maintained in the ER by a process of retrieval. These results indicate that the N-terminal region of SERCA1, containing transmembrane helices M1 and M2, contains an ER-retrieval signal.

Key words: endoplasmic reticulum (ER), ER/Golgi-intermediate compartment (ERGIC), plasma-membrane calcium ATPase (PMCA), retrieval, *trans*-Golgi.

INTRODUCTION

Calcium is a major intracellular second messenger that controls a wide range of cellular events, and the calcium ATPases of the sarco/endoplasmic reticulum (SERCAs) and plasma membrane (PMCA) have a key role in controlling the levels of intracellular free calcium [1]. SERCAs are responsible for recharging endoplasmic reticulum (ER) calcium stores, as well as removing calcium from the cytoplasm [2], whereas PMCA pumps calcium out of the cell across the plasma membrane [3]. Both types of pump belong to the P-type ATPase family of cation transporters and show very high sequence identity in the key areas of the pump involved in nucleotide binding and phosphorylation, the overall sequence similarity being approx. 30% [4]. What is not clear is how these two types of calcium pump are targeted, one to the ER and the other to the plasma membrane.

Two lysine residues positioned four residues from a cytoplasmically exposed C-terminus (i.e. KKXX) can act as an ER-retrieval signal for type I membrane proteins [5]. A cytoplasmically exposed N-terminal double arginine motif has also been implicated in maintaining type II membrane proteins in ER [6]. The N- and the C-termini of SERCAs, except SERCA2b, which has an extra transmembranous loop at the C-terminus [7], are thought to be cytoplasmically exposed and so could, in principle, contain ER-retrieval sequences. In fact, only SERCA3 from rat contains the KKXX motif at its C-terminus [4], but since it is not a conserved feature (the human sequence does not have the motif [8]), its role in targeting is doubtful.

The mechanism by which SERCAs are retained by the ER could involve novel specific motifs, which interact with retention/

retrieval machinery. However, it is also possible that retention is not a receptor-mediated event [9]. For example, models proposed for the retention of single transmembrane proteins could apply to the SERCAs. These models include retention by partitioning into bilayers of optimal thickness, which has been proposed to account for targeting of Golgi and ER proteins [10–13]. The plasma membrane is significantly thicker than the ER and Golgi membranes, due in part to the high cholesterol content of the plasma membrane. The transmembrane domains of some ER and Golgi-resident proteins are shorter than those in the plasma membrane, so that the ER and Golgi proteins may be excluded from partitioning into the thicker plasma membrane, thus preventing them from leaving their compartments. Alternatively, proteins could be prevented from leaving a compartment if they oligomerized to form complexes that were too large to enter the transport vesicles budding off from the ER, as has been suggested to occur in Golgi cisternae [14–16].

In order to identify the segments of SERCAs that are responsible for their location in the ER, we have produced a series of SERCA1–PMCA3 chimaeras. Although this has been attempted previously [17], we have refined the technique to ensure that ER targeting cannot be confused with the retention of misfolded proteins by the ER. Incorrectly folded glycoproteins are retained in ER [18] and, for example, the Na⁺/K⁺ pump, which is closely related to SERCA1 and PMCA3 in structure, is retained in ER when it fails to achieve its native conformation because of the absence of its β -subunit [19]. Three pairs of SERCA1–PMCA3 chimaeras have been produced (Figure 1) in which, for each pair, a different segment of SERCA1 has been transferred to PMCA3 and vice versa. The proteins have been joined in areas where

Abbreviations used: EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; ERGIC, ER/Golgi-intermediate compartment; PBST, PBS plus Triton X-100; PMCA, plasma-membrane calcium ATPase; SERCA, sarco/endoplasmic-reticulum calcium ATPase.

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Figure 1 Organization of the SERCA1-PMCA3 chimaeras

Horizontal bars represent the amino-acid sequence of SERCA1 (grey bar) and PMCA3 (black bar). Vertical bars indicate the positions of the ten transmembrane helices. All chimaeras were tagged at the C-terminal end with EGFP.

there is absolute conservation of amino acid sequence in order to reduce the risk of producing a misfolded protein. Using this approach, provided the chimaeras are not retained by the quality-control apparatus of the ER, one of the pair should be located in the plasma membrane and the other in ER. The advantage of this approach lies in the fact that the presence of an ER-retention/retrieval sequence in one construct is confirmed by the loss of an ER-retention/retrieval sequence by its obverse partner.

In addition, the location of enhanced green fluorescent protein (EGFP)-tagged SERCA1 relative to markers of the ER/Golgi-intermediate compartment (ERGIC) has been examined. The ERGIC is a tubulo-vesicular compartment containing material from the ER en route to the Golgi apparatus [20]. Evidence is presented that shows that SERCA1 is able to enter the ERGIC, and so its prolonged residence in ER is due to a process of retrieval from a post-ER compartment.

MATERIALS AND METHODS

Construction of EGFP-tagged SERCA1-PMCA3 chimaeras

The cDNA clone pPA8 containing the rabbit skeletal muscle *SERCA1* sequence modified for optimal expression in yeast was provided by Dr P. Adams (Upstate, Dundee Technology Park, Dundee, Scotland, U.K.) [21], and a second plasmid containing the rat *PMCA3* sequence was provided by Professor Gary Shull (Department of Physiology, College of Medicine, University of Cincinnati, Cincinnati, OH, U.S.A.) [22].

The cDNA sequence coding for EGFP was introduced at the 3' end of the *SERCA1* sequence in expression plasmid pPA8 by oligonucleotide-directed mutagenesis. Briefly, the stop codon in *SERCA1* was removed and the coding sequence was ligated in frame to cDNA encoding the c-myc epitope. A unique *AgeI* site was introduced at the 3' end of the cDNA to facilitate the addition of a further segment of sequence encoding EGFP amplified by PCR from pEGFP-1 (Invitrogen). The linking sequence between *SERCA1* and *EGFP* was *ctcgagcaaaagctgactctgaagaggacctaccggtcgccacc* (the sequence encoding the c-myc epitope is in italics and the flanking restriction

sites for *XhoI* and *AgeI* are underlined). The entire sequence was transferred into the expression vector pcDNA3.1(+) (Invitrogen). PCR was used to introduce silent mutations for unique *AflIII* and *KpnI* sites in the coding sequence of *PMCA3* at positions 790–795 and 2362–2367 respectively. A silent mutation was introduced between nucleotides 631 and 636 to introduce a unique *AflIII* site into the *SERCA1* sequence (an appropriately located *KpnI* site already exists in the *SERCA1* sequence). PCR was used to remove the stop codon from the *PMCA3* sequence, and the linker described above was used to extend the coding sequence to incorporate the c-myc epitope and EGFP sequences. The restriction-endonuclease sites for *KpnI* and *AflIII* allow the isolation of cassettes corresponding to sequences coding for residues 1–264, residues 265–788 and 789–1159 of *PMCA3* and residues 1–211, 212–711 and 712–994 for *SERCA1*. These cassettes were used to produce the chimaeras used in this study. The junction sites for the chimaeras correspond to sequences within *PMCA3* and *SERCA1* that show absolute sequence identity. Figure 1 provides a summary of the chimaeras produced.

Transfection of COS-7 cells

COS-7 cells were grown on 25 mm glass coverslips in 24-well culture plates for immunofluorescence studies and in tissue-culture dishes for immunoblotting and functional studies. The cells were grown to approx. 80% confluence before transfecting with plasmid DNA, using either LIPOFECTAMINE™ (Gibco) or Fugene 6 (Roche), according to the manufacturer's instructions.

Western blotting of SERCA1, PMCA3 and the chimaeras tagged with EGFP

COS-7 cells growing in 57 cm² tissue-culture dishes were transfected with the appropriate constructs. At 3 days after transfection, the culture medium was removed from the cells, which were washed twice in PBS and the cells were solubilized in 0.5 ml of double-strength Laemmli sample buffer [23], supplemented with 1 mM 2-mercaptoethanol and 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride at 90 °C. If necessary, the samples were briefly sonicated using a sonic probe to reduce the viscosity and were then rapidly frozen in liquid nitrogen and stored at –70 °C. The proteins of the solubilized transfected COS-7 cells were resolved by SDS/PAGE (10% gel), transferred on to nitrocellulose [24] and probed using mouse monoclonal anti-EGFP antibodies (diluted 1:5000; Roche) and sheep anti-mouse IgG conjugated with horseradish peroxidase (diluted 1:20000; Amersham Biosciences). The peroxidase activity was detected by enhanced chemiluminescence (SuperSignal kit, Pierce, or ECL® kit, Amersham Biosciences).

Laser confocal fluorescence microscopy

For the examination of EGFP-tagged constructs, coverslips with adherent transfected COS-7 cells were washed three times with ice-cold PBS. The coverslips were mounted on glass slides using Mowiol (Calbiochem) with 0.1% (v/v) Citifluor (Citifluor Ltd). Fluorescence was visualized using a Bio-Rad MRC-600 Confocal Imaging System equipped with a krypton/argon laser.

For immunofluorescence microscopy, following the PBS-wash step, the coverslips were removed from the 24-well plate and were fixed in ice-cold methanol for 10 min. For the simultaneous

localization of SERCA1–EGFP and the *trans*-Golgi marker TGN46 [26], the cells were treated with brefeldin A (5 μ g/ml) for 1 h to enhance the separation between the ER and the *trans*-Golgi network [27]. The coverslips were incubated with PBS with 0.1 % (v/v) Triton X-100 (PBST) for 15 min and were then incubated with primary antibodies. For localization of SERCA1, mouse monoclonal antibody Y/1F4 that recognizes residues 510–515 of SERCA1 [28] was used at 1:10 dilution of conditioned medium. The mouse monoclonal antibody directed against ERGIC-53 (kindly provided by J.-P. Hauri, University of Basel, Basel, Switzerland) was used at a 1 in 50 dilution and the rabbit antiserum directed against TGN46 (Serotec) was used at a 1 in 50 dilution. The coverslips were then washed three times with PBST and incubated with the appropriate secondary antibody conjugated with Texas Red (diluted 1:100; Amersham Biosciences) or rhodamine (diluted 1:100; Chemicon). After repeating the wash steps, the coverslips were mounted on glass slides using Mowiol with 0.1 % (v/v) Citifluor. Fluorescence was visualized using a Bio-Rad MRC-600 Confocal Imaging System equipped with a krypton/argon laser (excitation wavelength 488 nm). Emission filters of 552 nm and 585 nm were used to visualize the EGFP and Texas Red/rhodamine-labelled secondary antibodies respectively.

Subcellular fractionation

COS-7 cells were grown to 80 % confluence in 145 cm² dishes, washed twice with PBS and were harvested by scraping into 2 ml of buffer (10 mM triethanolamine, 10 mM acetic acid, 250 mM sucrose, 1 mM EDTA, 1 mM DTT and 10 μ g/ml each of PMSF, antipain, pepstatin A and leupeptin). Cells were disrupted by passing them five times through each of 19-, 23- and 25-gauge syringe needles. The disrupted cells were then centrifuged (1500 *g* for 5 min at 4 °C) and the supernatant was fractionated using a Nycodenz gradient as described in [29]. Following fractionation, the gradient was collected as nine fractions of equal volume. The protein in these fractions was precipitated by the addition of an equal volume of 50 % perchloric acid and following centrifugation on a microfuge (16000 *g* for 5 min), the pellets were solubilized in Laemmli sample buffer [23] containing 8 M urea. Proteins were separated by SDS/PAGE (10 % gel) and subjected to Western blotting using antibodies directed against SERCA1 (Y/1F4; [24]), the human-KDEL receptor (StressGen Biotechnologies Corp., Victoria, BC, Canada) and calnexin (StressGen), as outlined above.

RESULTS

Western blotting of SERCA1, PMCA3 and the chimaeras tagged with EGFP

Figure 2 shows a Western blot of the SERCA1–PMCA3 chimaeras following expression in COS-7 cells. SERCA1–EGFP was expressed in much larger amounts than any of the other constructs and so, to prevent over-saturation of the image, this lane comes from an earlier exposure time of 2 min, compared with 10 min for the rest of the blot. All of the tagged proteins migrate on SDS/polyacrylamide gels with apparent molecular masses of approx. 140–160 kDa, as anticipated. These results indicate that the EGFP observed in the confocal studies is the result of EGFP-tagged SERCA1, PMCA3 or SERCA1–PMCA3 chimaeras. Densitometry analysis of the Western blotting data obtained after 2 min exposure (results not shown) reveals that

Figure 2 Western blot of SERCA1–EGFP, PMCA3–EGFP and EGFP-tagged chimaeras

COS-7 cells growing in 10 cm diameter dishes were transfected with mammalian expression plasmids encoding the EGFP-tagged chimaeras, and SERCA1 and PMCA3. After 2 days, the cells were solubilized in 500 μ l of Laemmli sample buffer [23] and 10 μ l aliquots were resolved by SDS/PAGE (10 % gels). The proteins were transferred on to nitrocellulose membranes, and were then probed with anti-EGFP antibody followed by anti-mouse IgG conjugated to horseradish peroxidase. The horseradish peroxidase activity was detected by enhanced chemiluminescence. Exposure time was 10 min for all samples except for SERCA1–EGFP, which was exposed for 2 min.

chimaeras A and B are expressed at approx. 25 % of the level of SERCA1–EGFP, whereas the corresponding values for chimaeras C, E and F, and PMCA–EGFP are between approx. 10 and 15 %. Chimaera D is the least well expressed and is at the limits of detection here, at approx. 3 % relative to SERCA1–EGFP.

Immunolocalization of SERCA1, PMCA3 and their chimaeras

All of the constructs used in the present study produced significant amounts of heterologously expressed proteins within 24 h of transfection, as judged from immunofluorescence microscopy (results not shown). Figure 3 shows confocal immunolocalization results 3 days post-transfection. SERCA1–EGFP (Figure 3a) shows a clear reticular pattern, which has a perinuclear organization. This is typical of an ER-located protein, as shown by the membrane-bound ER-resident proteins, calnexin and Sec61 [30,31]. In contrast, PMCA3–EGFP localization (Figure 3b) is more widespread and occupies the whole of the cell volume, including the cell boundary (see arrows in Figure 3b). Again, this pattern is frequently observed when proteins destined for the plasma membrane, such as the Na⁺/K⁺-ATPase or membrane-bound receptors, are overexpressed in cells [32,33], and the protein is found throughout the ER/Golgi complex. However, in Figure 3(b), it is not possible to say with certainty that the tagged PMCA3 construct has inserted into the plasma membrane, because the internal membranes are highly enriched in the fluorescently tagged PMCA3 and there is no clear increase in the fluorescence signal at the plasma membrane, though it is possible to say that the EGFP-tagged PMCA3 construct has clearly escaped from the ER into the wider endomembrane compartments. These two profiles for localization seen for SERCA1–EGFP and PMCA3–EGFP can then be used to discriminate between ER-located chimaeras and those that have exited the ER and are en route to the plasma membrane. Inspection of the immunolocalization of chimaeras A, C and E (Figures 3c, 3e and 3g) shows that they have a similar distribution to that of SERCA1, being located on a reticular structure, which does not include the boundary of the cell, indicating that they are localized to the ER. Chimaeras B, D and F, on the other hand, have a similar cellular location to PMCA3 (Figures 3d, 3f and 3h) and so have escaped from the ER. The fact that pairs of chimaeras A and B, C and D, and E and F are located such that one member of the pair is in the ER and the other clearly translocates to post-ER locations

Figure 3 Location of EGFP-tagged SERCA1-PMCA3 chimaeras by laser confocal fluorescence microscopy

COS-7 cells grown on glass coverslips were transfected with constructs coding for (a) SERCA1-EGFP, (b) PMCA3-EGFP, (c) chimaera A, (d) chimaera B, (e) chimaera C, (f) chimaera D, (g) chimaera E and (h) chimaera F. At 3 days post-transfection, the cells attached to the coverslips were rinsed in PBS and were mounted in Mowiol with 0.1% (v/v) Citifluor. The EGFP fluorescence was visualized using a Bio-Rad MRC-600 confocal microscope. The arrows indicate the boundary of the cells. Scale bar, 15 μm .

indicates that retention of misfolded proteins is not responsible for the apparent targeting that is shown in Figure 3.

Co-localization of SERCA1-EGFP and ERGIC53

To study whether or not SERCA1 can leave the ER and enter the ERGIC, the location of SERCA1-EGFP relative to the ERGIC marker, ERGIC-53 [34], was examined. Figures 4(A), 4(C) and 4(E) shows the location of SERCA1 relative to ERGIC-53. SERCA1-EGFP is located in the ER, but there is also a

large amount of fluorescence associated within the perinuclear region (Figure 4A). There are four cells in the field shown in Figure 4(A), but only two appear to be transfected by the *SERCA1-EGFP*-containing plasmid. The anti-(ERGIC-53) antibody, which is a marker for the ERGIC, also highlights a membranous compartment close to one pole of the nucleus; this marker does not extend out towards the periphery of the cell in the same way that EGFP-tagged SERCA1 does. (Figure 4C). This is typical of the location of the ERGIC [34]. When the two signals are merged (Figure 4E), it can be seen that ERGIC-53 and SERCA1-EGFP are co-localized, as shown by the yellow colour,

Figure 4 Subcellular localization of EGFP-tagged SERCA1

COS-7 cells, grown on coverslips, were transfected with the expression construct containing the sequence for EGFP-tagged SERCA1. The transfected cells were fixed after 2 days using ice-cold methanol and mounted using Mowiol with 0.1 % (v/v) Citifluor. (A) and (B) Fluorescence from SERCA1-EGFP recorded with a 552 nm emission filter. (C) Texas Red fluorescence recorded with a 585 nm emission filter, indicating the position of ERGIC53 and the ERGIC compartment. (D) Rhodamine fluorescence recorded with a 585 nm emission filter, indicating the location of TGN46 and the *trans*-Golgi network. (E) Merged fluorescence signals of (A) and (C) demonstrating the co-localization of SERCA1-EGFP and ERGIC53. (F) Merged fluorescence signals from (B) and (D) showing that SERCA1-EGFP and TGN46 are not co-localized. Scale bar, 20 μ m.

where the signals overlap. These experiments show that a significant amount of SERCA1-EGFP is able to escape from the ER to the ERGIC.

To study whether SERCA1-EGFP can proceed from the ERGIC as far as the *trans*-Golgi, the localization of SERCA1-EGFP relative to the *trans*-Golgi marker [26] was examined. In the presence of brefeldin A, the *trans*-Golgi collapses around the microtubule-organizing centre [27] and, in Figure 4(D), it can be seen that the *trans*-Golgi marker, TGN46, has aggregated tightly around one pole of the nucleus in all three cells. Only one of these cells is expressing SERCA1-EGFP (Figure 4B), and the distribution of SERCA1-EGFP is clearly different from that of TGN46. When the signals for TGN46 and SERCA1-EGFP are merged (Figure 4F), the red signal of the TGN48 is still clearly visible showing that SERCA1-EGFP and TGN48 are not co-localized. Thus, although SERCA1-EGFP reaches the ERGIC, it does not translocate as far as the *trans*-Golgi network.

Cell fractionation

Figure 5 shows that when COS-7 cells are fractionated by density-gradient centrifugation, heterologously expressed SERCA1-EGFP locates to two distinct zones of the Nycodenz gradient. It is located in the light fractions, mainly fraction 8, as well as the heavy fraction, fraction 1, and to a lesser extent in fractions 2 and 3 (Figure 5A). Fractions 1–3 correspond to the ER, as indicated by the presence of the strongly retained ER marker, calnexin [30] (Figure 5C). The KDEL receptor, a marker for the ERGIC [35], is found mainly in the light fractions 7–9, with a small amount of material being located in fraction 1. Thus SERCA1-EGFP is located in both the ER and the ERGIC. The cell fractionation results thus support the information obtained from the fluorescence-microscopy results, which indicates that significant amounts of SERCA1 can be located in the ERGIC (Figure 4, see above).

Figure 5 Subcellular fractionation

COS-7 cells transfected with EGFP-tagged SERCA1 were disrupted as described in the Materials and methods section and, after removing the nuclei and undisrupted material with a preliminary centrifugation step, the material was applied to and centrifuged through a Nycodenz gradient to produce nine fractions (fraction 1 is the most dense sample). Protein from these fractions was precipitated using perchloric acid and the pellets obtained by centrifugation were solubilized in Laemmli sample buffer containing 8 M urea. Proteins from the nine samples were separated by SDS/PAGE (10% gel) and subjected to Western blotting using antibodies directed against (A) SERCA1 (Y1F4; [24]), (B) KDEL receptor and (C) calnexin. These antibodies were detected using secondary antibodies conjugated to horseradish peroxidase, and peroxidase activity was visualized by enhanced chemiluminescence. Each blot is the product of a single Nycodenz gradient.

DISCUSSION

Identification of ER-targeting motifs using chimaeras or mutation techniques suffers from the potential drawback that altering the ER-targeted protein to locate such motifs may also lead to misfolding of the protein, which in itself could lead to ER retention. In the present study, chimaeras have been designed based on two calcium pumps, SERCA1 and PMCA3, which are ER-located and plasma-membrane-located respectively. The present study has been designed so that each chimaera has an obverse form in which SERCA1 domains are replaced with PMCA3 domains and vice versa (Figure 1). In this way, the loss of an ER-targeting sequence in one of a pair of chimaeras should result in the gain of an ER-targeting sequence by the obverse partner, provided that the formation of the chimaeras does not cut across a targeting sequence. In the present study, the chimaeras have been joined in segments of conserved sequence, so it is unlikely that targeting signals would be interrupted by the joinings. This means that, for each pair of chimaeras, one would be expected to be maintained in the ER while the other will lose its retention/retrieval signal(s) and be translocated towards the plasma membrane, provided that the chimaeras are not misfolded to the extent that the quality-control apparatus in the ER prevents the constructs from exiting the ER. Figure 2 shows that the constructs are being expressed as full-length fusion proteins, and no free EGFP is in evidence (results not shown) that might interfere with the interpretation of the results.

The EGFP-tagged SERCA1 expressed in COS-7 cells is seen in the ER, with no indication of it being translocated to the plasma membrane, despite overexpression and its EGFP tag (Figure 3a). The distribution of tagged PMCA3 is quite different from that of SERCA1 (Figure 3b). There is a significant amount of fluorescence associated with the internal membranes, and it is clear that the location of the EGFP-tagged PMCA3 extends beyond the limits of the ER to other endomembranes. Whether or not significant amounts of EGFP-tagged PMCA3 reach the plasma membrane is unclear, but Figure 3(b) shows that most of the PMCA is located within the endomembranes of these COS cells and not on the cell surface.

The fate of the chimaeras can now be discerned, with reference to the patterns of expression of SERCA1-EGFP (Figure 3a)

and PMCA3-EGFP (Figure 3b). Chimaeras possessing the N-terminal segment of SERCA1 are located exclusively to the ER, i.e. chimaeras A, C and E (Figures 3c, 3e and 3g), whereas EGFP-tagged chimaeras in which the N-terminal segment of SERCA1 has been replaced with that of PMCA3 (chimaeras B, D and F) are able to exit the ER and enter other endomembrane compartments (Figures 3d, 3f and 3h). These results show that the residues responsible for the retention/retrieval of SERCA1, and thereby its maintenance in the ER, are located within the first 211 residues of SERCA1. The finding that only chimaeras B, D and F are able to leave the ER is concrete evidence that an ER-retention/retrieval motif is absent from these chimaeras and the only consistent feature of these chimaeras is the lack of the SERCA1 N-terminus.

Since a retention/retrieval sequence for SERCA1 appears to be located at the N-terminus, and since the structure of SERCA1 has been elucidated, it is of interest to speculate where a retention/retrieval motif might be located within the sequence. The 211 N-terminal residues of SERCA1 contain a cytoplasmic N-terminus (residues 1–59), two transmembrane α -helices, joined by a short luminal loop, and a cytoplasmic segment (residues 108–211) [36]. Both cytoplasmic regions make up part of the activation, or A, domain of SERCA1, which has a functional role. It therefore seems unlikely that these segments will be available to have a role in targeting, because the A domain undergoes significant movement during calcium pumping [36]. However, the two transmembrane domains M1 and M2 are located on the periphery of SERCA1 in an ideal position to interact with the transmembranous domains of a retention/retrieval receptor. Since there are no KKXX or double arginine motifs in SERCAs, the only remaining mechanism so far elucidated is that supported by Rer1p. Rer1p has been shown to interact directly with the transmembrane domains of target proteins to effect their retrieval [37].

SERCA1 could be maintained in the ER by a process of retention or retrieval, or a combination of the two. It has been demonstrated that a number of transmembrane ER-resident proteins are maintained in the ER by retrieval from the ERGIC. For Sec61, it is evident from the amount of Sec61 in the ERGIC, that significant amounts of the protein escape the ER and have to be retrieved [30]. However, in the case of cytochrome *b₅*, although no protein can be detected in the ERGIC, a painstaking analysis of glycosylation patterns of modified cytochrome *b₅* indicates that some of the protein has entered post-ER compartments and is presumably recycled to the ER [38]. Similarly, despite the fact that calnexin has a KKXX ER-retrieval motif, calnexin is undetectable in the ERGIC [30], and it has been suggested that the KKXX motif may also act as an ER-retention signal [39]. Thus it seems that ER-resident proteins may have ER-retention sequences of different strengths. The retention sequence for Sec61 appears to be relatively weak in comparison with that of cytochrome *b₅* and calnexin. Figure 4(C) shows the location of the ERGIC, as revealed by antibodies directed against ERGIC-53, an ERGIC marker, and Figure 4(D) demonstrates that a significant amount of SERCA1 has left the ER and is located in the ERGIC. However, the SERCA1 has not progressed as far as the *trans*-Golgi, because it does not co-localize with the Golgi marker TGN46 (Figure 4F). Hence the SERCA1 is maintained in the ER by a process of retrieval. Since a significant amount of SERCA1 appears in the ERGIC, it would seem that the strength of any retention motif is relatively weak. The localization of SERCA1 in the ERGIC is supported by the cell-fractionation study (Figure 5). SERCA1 is located mainly in the heavy fraction of membranes, separated by Nycodenz density-gradient centrifugation, along with calnexin, which, as discussed above,

serves as an exclusive marker of the ER. However, SERCA1 is also found in the heavier fractions containing the KDEL receptor, which is a marker for the ERGIC [35], confirming the ERGIC location observed for SERCA1-EGFP in the immunofluorescence study.

Concerning the issue as to whether residues 1–211 of SERCA1 contain a retention or a retrieval sequence, it is clear that any retention signal in this segment must be relatively weak, because, as outlined above, significant amounts of SERCA1 leave the ER and enter the ERGIC. It seems unlikely then that the retargeting of chimaeras that lack the N-terminus of SERCA is due to the loss of a retention signal, because the loss of a relatively weak signal would still allow chimaeras B, D and F to be returned to the ER using the retrieval pathway. It seems more likely that the loss of a retrieval signal (embedded within residues 1–211 of SERCA1) is the explanation for the failure of chimaeras B, D and F to be maintained in the ER.

A number of studies have indicated that membrane thickness and the hydrophobic dimensions of the transmembrane regions of intrinsic membrane proteins interact to influence the targeting of transmembrane proteins [10–13]. This appears to be important in the maintenance of Golgi-resident proteins, where proteins with shorter transmembrane helices may be prevented from partitioning into thicker cholesterol-rich membranes en route to the plasma membrane [10,11]. Similarly, increasing the hydrophobic thickness of the transmembrane region by four residues leads to the translocation of ER-resident protein cytochrome *b₅* to the plasma membrane [12]. An analysis of the hydrophobic segments of the transmembrane α -helices of SERCA1 indicates that they may be unusually narrow [40]. It is difficult to assign a precise thickness, because there is no bound lipid in the high-resolution structure of SERCA1. Instead, the position of the bilayer is inferred from the position of belts of tryptophan and charged residues, which suggests a thickness of 21 Å (1 Å = 0.1 nm) for SERCA1 [40]. This would be compatible with the thinner bilayers of the ER and the *cis*-Golgi [10]. However, the observation that the ER retains a construct with just the first two transmembrane α -helices of SERCA1 and the remaining eight transmembrane α -helices from PMCA3, argues against hydrophobic thickness being important in targeting, in this case. It seems that, for the sorting of polytopic membrane proteins, a mechanism, more complex than the simple partitioning of hydrophobic helices into lipids of different composition, must be involved.

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