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Steady-state free Ca^{2+} in the yeast endoplasmic reticulum reaches only 10 μM and is mainly controlled by the secretory pathway pump Pmr1

Jochen Strayle, Tullio Pozzan[1] and Hans K.Rudolph[2]

Institut für Biochemie der Universität Stuttgart, Pfaffenwaldring 55, D-70569 Stuttgart, Germany and [1]Department of Biomedical Sciences and CNR Centre for the Study of Biomembranes, University of Padova, Via Colombo 3, 35121 Padova, Italy

[2]Corresponding author
e-mail: rudolph@po.uni-stuttgart.de

Over recent decades, diverse intracellular organelles have been recognized as key determinants of Ca^{2+} signaling in eukaryotes. In yeast however, information on intra-organellar Ca^{2+} concentrations is scarce, despite the demonstrated importance of Ca^{2+} signals for this microorganism. Here, we directly monitored free Ca^{2+} in the lumen of the endoplasmic reticulum (ER) of yeast cells, using a specifically targeted version of the Ca^{2+}-sensitive photoprotein aequorin. Ca^{2+} uptake into the yeast ER displayed characteristics distinctly different from the mammalian ER. At steady-state, the free Ca^{2+} concentration in the ER lumen was limited to ~10 μM, and ER Ca^{2+} sequestration was insensitive to thapsigargin, an inhibitor specific for mammalian ER Ca^{2+} pumps. In *pmr1* null mutants, free Ca^{2+} in the ER was reduced by 50%. Our findings identify the secretory pathway pump Pmr1, predominantly localized in the Golgi, as a major component of ER Ca^{2+} uptake activity in yeast.

Keywords: aequorin/Ca^{2+} homeostasis/Ca^{2+} stores/endoplasmic reticulum/Golgi apparatus

Introduction

All eukaryotic cells display some mechanism that exploits the unique chemical properties of the Ca^{2+} ion to transduce a cellular signal. Depending on cell type, local changes in the intracellular Ca^{2+} distribution trigger a variety of physiological responses, indicating the existence of distinct Ca^{2+} signaling pathways (Clapham, 1995). Due to the inherent stability of Ca^{2+} in any biological environment, access of this second messenger to the cytoplasm of cells must be strictly controlled. Thus, Ca^{2+} ions are actively transported out of the cell and into internal organelles, which in turn may release Ca^{2+} upon appropriate stimuli. Extensive studies in animal cells identified the endoplasmic reticulum (ER) as a major site of intracellular Ca^{2+} storage and release (reviewed by Berridge, 1994). To fulfill its role as a dynamic Ca^{2+} reservoir, the ER is suitably equipped with pumps for Ca^{2+} uptake, channels for Ca^{2+} release, and Ca^{2+}-binding proteins for Ca^{2+} buffering (see Pozzan *et al*., 1994).

The main Ca^{2+} store of the yeast *Saccharomyces cerevisiae* (at least with respect to overall Ca^{2+} content) is the vacuole, with a total Ca^{2+} concentration of ~2 mM. Buffered by inorganic polyphosphates, the concentration of free Ca^{2+} in the vacuolar lumen is reduced to ~30 μM (Dunn *et al*., 1994). Two vacuolar Ca^{2+} uptake systems have been identified: Vcx1, an H^+/Ca^{2+} antiporter (Cunningham and Fink, 1996; Pozos *et al*., 1996) and Pmc1, a P-type ATPase (Cunningham and Fink, 1994). A third yeast Ca^{2+} transporter, the secretory pathway ion pump Pmr1 (Rudolph *et al*., 1989), is predominantly localized in the Golgi (Antebi and Fink, 1992). Over the last decade, many components related to mammalian Ca^{2+} signaling pathways have been identified in yeast. Despite these efforts, functions of Ca^{2+} ions in the yeast ER and Ca^{2+} handling by this organelle remained elusive. Only recently, some progress was made with regard to possible functions. First, mutants in sphingolipid biosynthetic steps hosted in early secretory organelles (ER, Golgi) are sensitive to elevated Ca^{2+} levels (Zhao *et al*., 1994) and secondly, *pmr1* mutants are sensitive to Ca^{2+} starvation and stabilize a misfolded luminal ER protein (Dürr *et al*., 1998), which normally undergoes retrograde transport from the ER into the cytosol for ubiquitination and subsequent degradation (reviewed by Sommer and Wolf, 1997). However, no Ca^{2+} transport systems have been identified in yeast for two major control sites of mammalian Ca^{2+} regulation, i.e. the ER and the plasma membrane. Moreover, no data are available about Ca^{2+} concentrations in the lumen of early secretory organelles in yeast. Evidently, methods to monitor free Ca^{2+} directly within the ER of living yeast cells could provide invaluable insights into the role of this organelle in Ca^{2+} homeostasis and signaling.

Here, we report the development of a yeast ER Ca^{2+} probe based on aequorin, a Ca^{2+}-sensitive photoprotein that emits light when exposed to Ca^{2+} in the presence of its prosthetic group coelenterazine (Allen *et al*., 1977; Inouye *et al*., 1985). Targeting of functional aequorin into the ER lumen of mammalian cells has been accomplished by several groups (Montero *et al*., 1995; Button and Eidsath, 1996; Montero *et al*., 1997; Alonso *et al*., 1998; Pinton *et al*., 1998). In yeast however, aequorin has only been used to monitor cytosolic Ca^{2+} (Nakajima-Shimada *et al*., 1991). To construct a yeast ER Ca^{2+} probe, we fused aequorin onto Stt3, a subunit of the oligosaccharyl transferase complex in the ER membrane. This approach strictly localized aequorin to the ER lumen under all our experimental conditions. Using this probe, we measured a steady-state concentration of 10 μM free Ca^{2+} for the ER of wild-type yeast strains. The ER Ca^{2+} level was unaffected by treatment with thapsigargin, indicating that yeast lacks the sarco/endoplasmic reticulum (SERCA)-type Ca^{2+} pump present in the ER of higher eukaryotes. However, ER Ca^{2+} was severely reduced in cells lacking the secretory pathway pump Pmr1, demonstrating that this

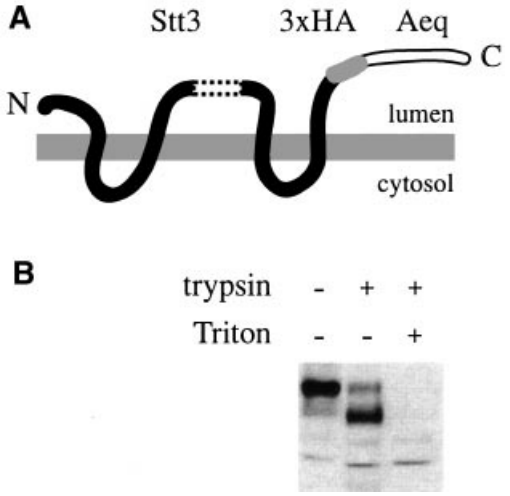

Fig. 1. ER–Aeq has correct membrane topology. (**A**) A cartoon of the membrane topology of ER–Aeq. (**B**) The aequorin moiety has luminal orientation. Crude lysates of strain YS256 expressing ER–Aeq were treated with trypsin and Triton X-100 at 4°C for 20 min as indicated; a control remained untreated (- -). Treatment with trypsin resulted in digestion of cytosol-facing parts of the chimeric protein, but the HA-epitope was efficiently protected. Permeabilization of membranes in the presence of Triton resulted in complete digest.

'Golgi' ion pump also controls, at least in part, the luminal Ca^{2+} concentration of the yeast ER.

Results

A Stt3/aequorin fusion protein as a potential ER Ca^{2+} probe

Stt3 is a transmembrane protein with a hydrophilic C-terminal domain, which protrudes into the ER lumen and tolerates additional sequences at the C-terminus without abolishing Stt3 function (Zufferey *et al*., 1995). These facts, and its relative abundance make Stt3 an ideal candidate to anchor aequorin on the luminal side of the ER membrane. Expecting a high Ca^{2+} concentration ($> \mu M$) within the ER, we used an aequorin variant with reduced Ca^{2+} affinity (Kendall *et al*., 1992; Montero *et al*., 1995) and fused its cDNA to the entire *STT3* coding region (Figure 1A), including a triple HA-epitope tag at the Stt3/aequorin junction (Wilson *et al*., 1984). The entire construct was transplaced at the chromosomal *STT3* locus under control of the *STT3* promoter (see Materials and methods). Strains expressing ER–Aeq were viable and grew indistinguishably from their parental *STT3* strains, demonstrating a functional replacement of the essential *STT3* gene and suggesting a correct membrane topology/localization of the Ca^{2+} probe. A protease protection experiment (Figure 1B) confirmed that the aequorin domain of ER–Aeq was indeed located in the lumen of an organelle, presumably within the ER.

The intracellular localization of ER–Aeq was first examined by immunofluorescence microscopy. The staining pattern obtained with the anti-HA antibody clearly coincided with that of the ER, as visualized by anti-Kar2 antibody in the same cells (Figure 2A). A typical ring-like staining pattern around the nucleus, plus some staining below the plasma membrane was observed, exactly as for the ER-marker Kar2 (Rose *et al*., 1989). Strains lacking ER–Aeq did not stain with the anti-HA antibody (data not shown). To verify ER–Aeq localization by a second method, cells expressing ER–Aeq were broken and fractionated by sedimentation on a sucrose gradient, essentially as described by Schröder *et al*. (1995). Gradient fractions were analyzed for the presence of marker enzymes by appropriate enzymatic assays and immunoblotting (Figure 2B). In excellent agreement with the immunofluorescence data, ER–Aeq co-fractionated with all ER-markers tested: Kar2, Wbp1 (te Heesen *et al*., 1992), Sec61 (Stirling *et al*., 1992) and Dpm1 (Preuss *et al*., 1991), all of which migrated together in the bottom fractions of the gradient. ER–Aeq was separated almost completely from the Golgi markers GDPase (Abeijon *et al*., 1989), Emp47 (Schröder *et al*., 1995) and Och1 (Nakayama *et al*., 1992) which occupied middle fractions of the gradient. Och1, the *cis*-most Golgi marker functionally identified thus far, peaked in fractions 7–8 and was clearly displaced from the bulk of ER–Aeq. It is evident from the distribution of α-mannosidase activity that vacuolar membranes hardly entered the gradient and were well separated from ER–Aeq. Taken together, our data clearly demonstrate that the steady-state pool of ER–Aeq resides in the membrane of the ER, thus confining aequorin to the lumen of this organelle.

On denaturing gels, we observed a slightly faster mobility for ER–Aeq from cells treated with tunicamycin (see Figure 3A), an inhibitor of N-linked protein glycosylation, suggesting that ER–Aeq was carrying carbohydrates at asparagine residues within the Stt3 moiety. This finding allowed us to examine further the potential overspill of ER–Aeq from the ER into a *cis*-Golgi compartment, where α-1,6-mannosyltransferase activity is known to attach α-1,6-linked mannose residues to the carbohydrate chains of core-glycosylated proteins (Nakanishi-Shindo *et al*., 1993). To test whether ER–Aeq was exposed to the α-1,6-mannosyltransferase activity of early Golgi compartments, cells expressing ER–Aeq and HA-tagged Och1 were radiolabeled briefly with [^{35}S]methionine and chased for 30 min prior to immunoprecipitation with anti-HA antibody. The immunoprecipitated proteins were eluted from the anti-HA antibody and equal aliquots were subjected to a second immunoprecipitation with antibodies specific for α-1,6-mannose linkages and the HA epitope, respectively. As seen in Figure 3B, *de novo*-synthesized ER–Aeq was not precipitable with the linkage-specific antibody, indicating that ER–Aeq bore no α-1,6-mannose linkages. HA-tagged Och1, a *cis*-Golgi protein for which we found a similar fraction of α-1,6-linked mannose modification as reported in other studies (Harris and Waters, 1996), served as a positive control in this experiment. These results are consistent with our localization studies, i.e. immunofluorescence microscopy and subcellular fractionation found that ER–Aeq was restricted to the ER, and strongly suggest that ER–Aeq is virtually absent from Golgi compartments.

As a first indication of functionality of the Ca^{2+}-responsive photoprotein domain in ER–Aeq, we tested for Ca^{2+}-dependent chemiluminescence in crude cell extracts treated with coelenterazine to allow reconstitution of aequorin holoenzyme (Nakajima-Shimada *et al*., 1991; Rizzuto *et al*., 1994). Crude lysates from cells expressing ER–Aeq, when incubated for 4 h on ice with coelenterazine in the presence of the chelator EDTA and 2-mercapto-

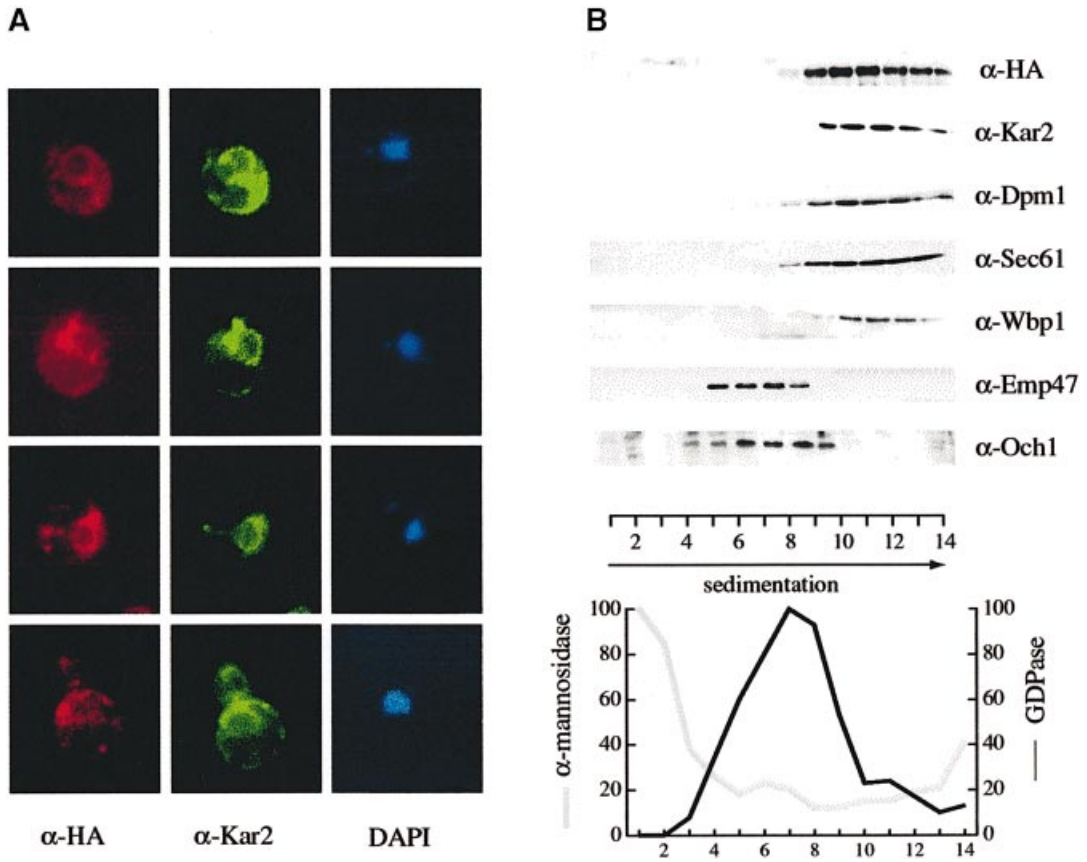

Fig. 2. ER–Aeq localization in wild-type cells. (**A**) ER–Aeq colocalized with Kar2. Fixed and permeabilized YS256 cells were simultaneously incubated with 12CA5 (directed against the HA-epitope of ER–Aeq; left panel) and anti-Kar2 antibody (middle). 12CA5 was visualized with Cy3-conjugated goat anti-mouse IgG and anti-Kar2 antibody with FITC-conjugated goat anti-rabbit IgG. DNA was stained with DAPI (right). (**B**) Migration of organelle-specific markers on sucrose density gradients. A spheroblast homogenate of strain YS256 was fractionated on an 11-step sucrose gradient (18–60%). Aliquots of the collected fractions were subjected to Western blotting analysis and used to assay the marker enzymes α-mannosidase (vacuole) and guanosine diphosphatase (GDPase, Golgi). Activities are given as a percentage activity of the highest values measured. The arrow indicates the direction of sedimentation.

ethanol (2-ME), produced light upon addition of Ca^{2+} (5000 ± 250 counts; the background before adding Ca^{2+} was 5.5 ± 2.5 counts). In contrast, lysates made from a control strain lacking ER–Aeq produced no increase in light emission after addition of Ca^{2+} (background was 5 ± 2 counts). These experiments indicated that ER–Aeq was functional and prompted us to evaluate further its use as an ER Ca^{2+} probe in living yeast cells.

Reconstitution of ER–Aeq in vivo requires treatment with Ca^{2+} ionophore/EGTA

Reconstitution of active aequorin holoenzyme and subsequent light emission upon Ca^{2+} challenge of intact cells was previously shown for a yeast strain expressing cytosolic aequorin (Nakajima-Shimada *et al*., 1991). Using our own expression system, we also observed efficient reconstitution of cytosolic aequorin ($>10^7$ counts per coverslip; J.Strayle and H.K.Rudolph, unpublished results). However, cells expressing ER–Aeq produced only a modest chemiluminescence signal ($<10^5$ counts per coverslip) upon cell lysis and addition of Ca^{2+}, even when a divalent cation chelator (EGTA) was present during the entire procedure. To prevent premature aequorin consumption during the reconstitution period, we pretreated cells with the Ca^{2+} ionophore A23187 and EGTA to deplete intralumenal Ca^{2+} stores before addition of coelenterazine. A significant 10- to 15-fold increase in total count yield ($>10^6$ counts per coverslip) was observed with this treatment (see Materials and methods).

To verify persistent ER localization of our Ca^{2+} sensor under the newly developed conditions, we performed immunofluorescence microscopy and subcellular fractionation studies on cells treated with A23187/EGTA/coelenterazine as for reconstitution. Curiously, the new protocol produced a somewhat altered distribution of Golgi markers (Figure 4B): whereas GDPase activity displayed a relatively sharp peak centered around fraction 7, the bulk of Emp47 was now more spread out and shifted towards lesser densities (fractions 3–7). However, as with untreated cells, ER–Aeq still co-fractionated with several ER-marker proteins (Figure 4B) and produced in immunoflourescence microscopy a typical Kar2-like ER staining pattern (Figure 4A). These data demonstrate a firm association of the Ca^{2+} sensor with the ER, which persists even under ion depletion/reconstitution conditions and thus validates our protocol for reconstitution of ER–Aeq into a functional ER Ca^{2+} probe in living yeast cells.

Measuring the concentration of free Ca^{2+} in the ER

For measurements of ER Ca^{2+} with ER–Aeq, we used an experimental set-up consisting of a perfusion chamber

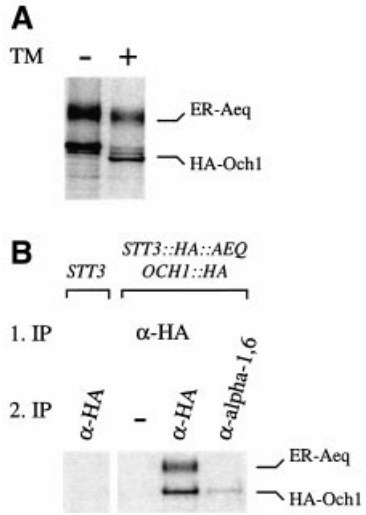

Fig. 3. ER–Aeq is an ER-resident protein. (**A**) ER–Aeq is core-glycosylated. Immunoprecipitation (IP) of metabolically labeled ER–Aeq and HA–Och1 (from strain YS256 transformed with plasmid pOH; Harris and Waters, 1996) respectively, in the presence (+) or absence (–) of tunicamycin (TM). Reduced mobility in the absence of TM indicates core-glycosylation of ER–Aeq and HA–Och1. (**B**) No escape of ER–Aeq to post-ER compartments. Cells expressing ER–Aeq and HA–Och1 were labeled for 5 min at 30°C and chased for 30 min. Protein was immunoprecipitated with anti-HA antibody. The immunocomplex was dissociated by heating in the presence of 2-mercaptoethanol. After dividing the precipitated proteins into three equivalent aliquots, they were then subjected to a second immunoprecipitation with either the anti-HA antibody or the α-1,6-mannose linkage-specific antibody. In a mock control we tested for the complete inactivation of anti-HA antibody used in the first IP. The proteins were separated by SDS–PAGE and visualized by autoradiography.

connected to a photon counting device (described by Rizzuto *et al*., 1994). In order to fix yeast cells in the perfusion chamber, we embedded the ion depleted/reconstituted spheroblasts in low melting agarose on a coverslip. In a typical experiment, the mounted spheroblasts were perfused first with an EGTA solution containing the lipophilic agent bovine serum albumin (BSA) to extract remaining traces of ionophore and to wash out loosely attached cells. Subsequently, a Ca^{2+}-containing standard yeast medium was applied to allow refilling of Ca^{2+}-depleted intracellular stores. The consumption of reconstituted ER–Aeq accompanying this process was quantitatively monitored by photon counting. At steady-state, usually after 3–5 min, all cells were lysed by the addition of digitonin in the presence of excess Ca^{2+} to discharge all residual holoaequorin. Details regarding the conversion of aequorin chemiluminescence data into Ca^{2+} concentrations are described in Materials and methods.

Figure 5A (inset) shows the kinetics of chemiluminescence obtained with wild-type yeast cells expressing ER–Aeq and reconstituted in the presence of 10 μM ionophore A23187. Photon emission, close to background levels during perfusion with EGTA-containing medium, showed a rapid increase after switching to the Ca^{2+}-containing medium, and reached a plateau after ~1–2 min. Subsequent lysis led to a transient increase in photon counts and the discharge of residual aequorin, demonstrating that ~40% of reconstituted aequorin outlasted consumption during refilling. Conversion of the luminescence signal into Ca^{2+} values using the Ca^{2+} affinity for mutated aequorin determined by Montero *et al*. (1995), revealed a steady-state concentration of ~10 μM Ca^{2+} which was reached within 1–2 min after the switch to Ca^{2+}-containing medium and remained constant for at least 4 min (Figure 5A). We obtained very similar kinetics of chemiluminescence from two different wild-type strains expressing ER–Aeq (Figure 5B), strongly suggesting that the steady-state concentration of free Ca^{2+} in the ER lumen of wild-type yeast cells is indeed ~10 μM.

Since traces of A23187, if not adequately removed before the switch to Ca^{2+}-rich medium, could antagonize or limit refilling of the ER with Ca^{2+}, we tested a range of different ionophore concentrations. As seen in Figure 5C, the use of 100 nM or 100 μM ionophore A23187 produced the same steady-state Ca^{2+} concentration of ~10 μM upon refilling, strongly arguing against any influence of A23187 on these measurements. Another potential artefact we considered was 'wash-out' of spheroblasts during perfusion, which would reduce total counts and thus lead to an overestimation of Ca^{2+} levels. However, a comparison of the light emission during extended perfusion (5 min, $1.6\times10^6 \pm 2\times10^4$ counts/coverslip) with the total counts obtained during a short perfusion period (30 s, $1.6\times10^6 \pm 3\times10^4$ counts/coverslip) revealed no significant difference. Thus, 'wash-out' of cells could be disregarded in our analysis.

To address whether a fraction of reconstituted ER–Aeq might escape from the ER into a potentially Ca^{2+}-rich post-ER compartment, we introduced our Ca^{2+} probe into a *ret2-1* mutant with a conditional block in Golgi-to-ER retrograde transport due to a defective coatamer (COPI) (Cosson *et al*., 1996) which might enhance post-ER accumulation of ER–Aeq and thus alter the refilling profile. *ret2-1* cells were grown at permissive temperature and processed for reconstitution as usual. The batch was then split into two aliquots: one, which was shifted to 37°C for 1 h before refilling, was monitored at this restrictive temperature, and the other aliquot was assayed at room temperature. As shown in Figure 5D, there was no remarkable difference in the refilling profiles of the COPI mutant at permissive or restrictive temperature. These findings corroborate our conclusion that the ER–Aeq probe senses ER Ca^{2+} levels without significant influence from post-ER compartments.

The secretory pathway pump Pmr1 contributes to thapsigargin-resistant, vanadate-sensitive Ca^{2+} uptake into the ER

Thapsigargin, a potent and specific inhibitor of SERCA Ca^{2+} pumps, blocks Ca^{2+} uptake into the mammalian ER (Thastrup *et al*., 1989). As shown in Figure 5E, treatment of spheroblasts during reconstitution (1 h) with 5 μM thapsigargin, a concentration several magnitudes higher than that required to block SERCA pumps (Sagara and Inesi, 1991), had no effect on ER refilling to a steady-state level of 10 μM Ca^{2+}. We reported previously that expression of rabbit SERCA1a suppresses the EGTA-hypersensitivity of mutants lacking the Golgi ion pump Pmr1 (Dürr *et al*., 1998). As seen in Figure 6, such a *pmr1* mutant strain expressing SERCA1a is sensitive to thapsigargin, illustrating that thapsigargin can enter intact yeast cells and block an appropriate target. Thus our data imply that SERCA homologs are not involved in ER Ca^{2+}

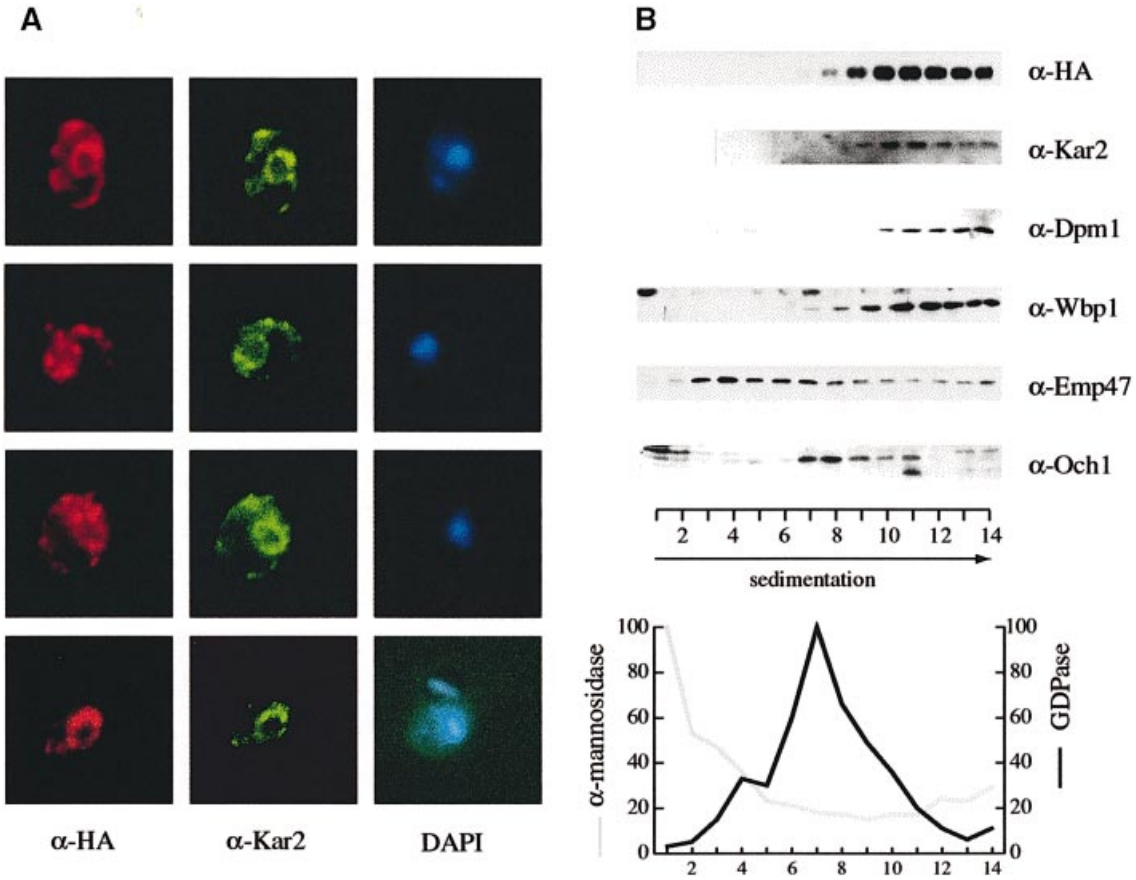

Fig. 4. Localization of ER–Aeq is inert to Ca^{2+} deprivation in wild-type cells. (**A**) ER–Aeq still colocalizes with Kar2 in Ca^{2+}-depleted cells. Cells were treated virtually the same as those used for Ca^{2+} measurements. Spheroblasts were incubated in the presence of 10 μM A23187 for 1 h at 4°C in the presence of 5 mM EGTA. After fixation, immunofluorescence was performed as described. (**B**) Under Ca^{2+}-depletion conditions ER–Aeq cofractionated with all ER markers. Cells treated in the presence of the ion chelator EGTA with Ca^{2+} ionophore were spheroblasted. After homogenization and subcellular fractionation on a discontinuous sucrose density gradient, aliquots were analyzed by Western blotting and enzymatic assays as described previously.

homeostasis in yeast, as previously suggested from the analysis of the yeast genome (Sorin *et al*., 1997).

All known ATP-driven Ca^{2+} transporters, including the two Ca^{2+} pumps characterized in yeast (Pmr1, Pmc1), belong to the superfamily of P-type ATPases and thus show some sensitivity to vanadate, which blocks formation of an auto-catalytic phosphoenzyme intermediate. As illustrated in Figure 5F, the steady-state Ca^{2+} concentration reached upon refilling was reduced by almost 50% when cells were preincubated with 1 mM vanadate. However, further addition of monensin to collapse H^+ transmembrane gradients had no effect on refilling in the presence of vanadate. These results suggest that the ER membrane harbors at least one P-type Ca^{2+} pump, but lacks a Ca^{2+}/H^+ antiport under our experimental conditions. Strikingly, *pmr1* mutants displayed a reduced steady-state ER Ca^{2+} level of ~6 μM (Figure 5G), a value very similar to wild-type assayed in the presence of vanadate. This result directly implicates Pmr1 in ER Ca^{2+} homeostasis. We verified that ER–Aeq was properly localized to the ER of *pmr1* cells, well separated from Golgi markers (see Figure 7). The only noteworthy difference from wild-type cells (see Figures 2 and 4) was altered fractionation profiles for α-mannosidase and GDPase. Finally, inhibition of protein synthesis with cycloheximide, either during the entire reconstitution period (Figure 5H) or just before switching to Ca^{2+}-rich medium (data not shown), had no effect on ER refilling in wild-type cells. Thus, the main contribution of Pmr1 to ER Ca^{2+} uptake can not be ascribed to newly synthesized protein, but rather reflects activity of a pre-existing Pmr1 pool.

Discussion

Here, we describe *in vivo* measurements of free Ca^{2+} in the lumen of the yeast ER using a protein chimera which consists of the Ca^{2+}-sensitive photoprotein aequorin fused onto Stt3, an ER-resident oligosaccharyl transferase subunit. Measurements with this sensor reveal a steady-state free Ca^{2+} level of ~10 μM for the yeast ER, a concentration significantly lower than free Ca^{2+} in the mammalian ER. Our experiments identify the yeast ER as a thapsigargin-resistant, but vanadate-sensitive, Ca^{2+} store lacking the SERCA-type Ca^{2+} ATPases known to control mammalian ER Ca^{2+}. Most importantly, we demonstrate that the secretory pathway ion pump Pmr1, a P-type ATPase prominently localized in the Golgi complex, is a major component of ER Ca^{2+} uptake activity in yeast.

ER–Aeq is a suitable ER Ca^{2+} indicator

Our approach to target aequorin to the ER lumen by virtue of an endogenous ER membrane protein (Stt3) avoids addition of an ER retention signal (i.e. the carboxy-terminal tetrapeptide sequence His-Asp-Glu-Leu HDEL)

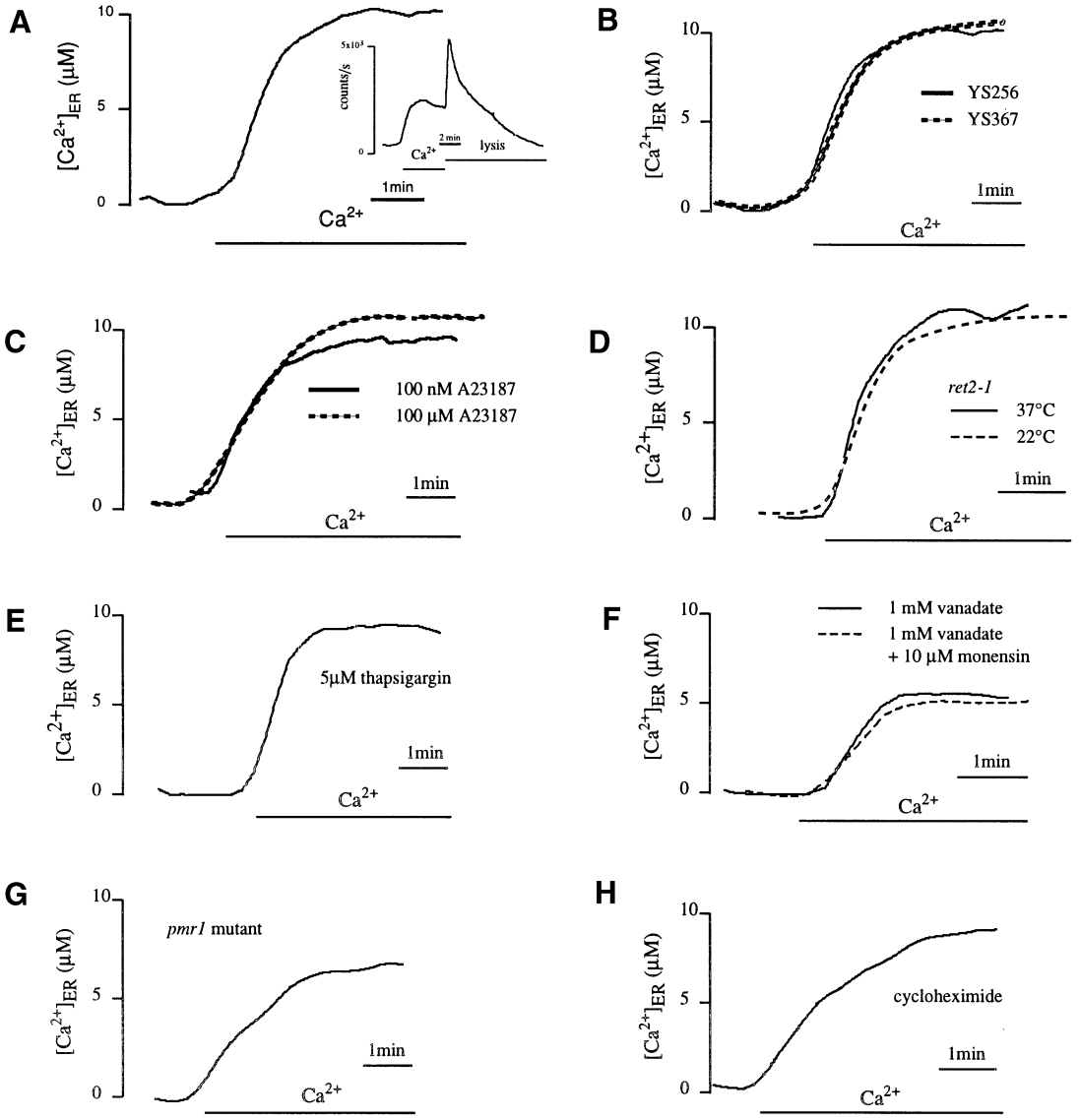

Fig. 5. Increase of luminal ER Ca^{2+} concentration upon re-addition of Ca^{2+} to Ca^{2+}-depleted cells. (A–H) Unless otherwise indicated [see (C)], spheroblasts were depleted of Ca^{2+} by incubation with 10 μM A23187 in the presence of 10 mM EGTA in normal synthetic yeast medium buffered to pH 7 with 10 mM HEPES. Aequorin reconstitution was carried out in the same medium for 1 h at 4°C with 5 μM coelenterazine. After extensive washing with medium containing 2% BSA and 5 mM EGTA (BSA/EGTA), the cells were resuspended in agarose and mounted on to coverslips. Finally, the coverslips were placed in the thermostated chamber of the luminometer and perfused with BSA/EGTA medium at 22°C. Where indicated, the BSA/EGTA medium was replaced with medium containing 1 mM $CaCl_2$. At the end of the experiment the cells were lysed in Ca^{2+}-rich buffer containing digitonin and the luminescence values were converted into Ca^{2+} concentrations. (**A**) ER Ca^{2+} sequestration in wild-type cells. Inset shows the primary photon counts during refilling and final lysis. (**B**) Ca^{2+} uptake in different strain backgrounds. (**C**) Effect of different ionophore concentrations during reconstitution. (**D**) Ca^{2+} influx in *ret2-1* mutant at permissive (22°C) and restrictive temperature (37°C). (**E**) Effect of 5 μM thapsigargin on ER refilling. (**F**) Reconstitution in the presence of 1 mM vanadate, and with 1 mM vanadate + 10 μM monensin. (**G**) ER Ca^{2+} sequestration in a *pmr1* null mutant. (**H**) ER Ca^{2+} uptake after treatment with cycloheximide.

to the catalytically important C-terminus of aequorin (Nomura *et al.*, 1991) and should evade Kar2(BiP)-dependent mechanisms for ER retention which may fail under Ca^{2+} limitation (Suzuki *et al.*, 1991). Accurate ER localization of the probe was thoroughly evaluated by (i) subcellular fractionation, (ii) immunofluorescence microscopy and (iii) examination of ER–Aeq for the presence of Golgi-specific carbohydrate modifications. By these criteria, ER–Aeq was strictly confined to the ER under all experimental conditions, even though the Ca^{2+}-depletion protocol required for holoaequorin reconstitution *in vivo* (see below) induced some restructuring (fragmentation) of the ER in *pmr1* cells. This phenomenon could either reflect a stress response of *pmr1* cells to some general experimental condition (fixation, perfusion) or result from specific, calcium-dependent mechanisms for ER restructuring in yeast. In animal cells, ER vesicularization was observed upon expression of BiP variants defective for ATP hydrolysis (Hendershot *et al.*, 1995) and in response to a persistent increase in cytosolic Ca^{2+} (Subramanian and Meyer, 1997). Ca^{2+} depletion also changed the distribution of Emp47, which is related to a lectin that binds mannose residues in a calcium-dependent manner (ERGIC-53; Itin *et al.*, 1996). Thus, the observed

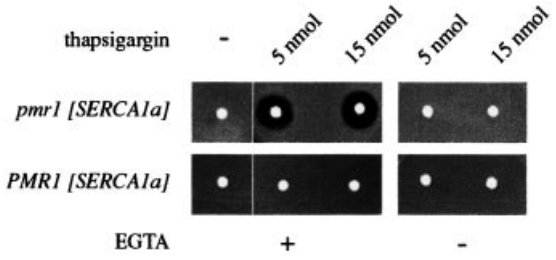

Fig. 6. Suppression of the EGTA-hypersensitivity of *pmr1* mutants by SERCA1a is sensitive to thapsigargin. Strains YR663 (*pmr1*) and YS380 (*PMR1*) expressing SERCA1a were spread onto synthetic media and supplemented with (+) or without (–) 15 mM EGTA. Ten microliters of thapsigargin solution (0.5 and 1.5 mM in DMSO) was applied to filter discs. The mock control contained only DMSO. Plates were photographed after 3 days' incubation at 25°C.

redistribution of Emp47 might reflect an ion requirement for proper localization of this Golgi protein. These ambiguities, however, did not impinge upon the intracellular localization of ER–Aeq, which was strictly associated with ER markers under all experimental conditions.

Efficient reconstitution of ER–Aeq *in vivo* required Ca^{2+} depletion to prevent premature aequorin consumption, a problem frequently encountered with this photoprotein in Ca^{2+}-rich organelles. For the same reason, we employed an aequorin variant in our fusion with reduced Ca^{2+}-affinity due to a single amino acid exchange (Montero *et al*., 1995). With these precautions, we used our probe and found that refilling of the ER with Ca^{2+} reached a steady-state level during a time period of ~2 min, after which 40–50% of total reconstituted ER–Aeq was still uncharged as demonstrated by immediate cell lysis in a Ca^{2+}-rich digitonin buffer. Such a ratio of uncharged aequorin allows for conversion of luminescence into Ca^{2+} values based on the method of Allen *et al*. (1977). Thus, ER–Aeq is a suitable sensor of ER Ca^{2+} in yeast.

Characteristics of ER Ca^{2+} uptake in wild-type cells

Using ER–AEQ in two different wild-type strain backgrounds, we found that yeast cells at steady-state maintain a free Ca^{2+} concentration of ~10 μM within the ER. Studies on free Ca^{2+} in the mammalian ER report a range from 1 to 400 μM. Pinton *et al*. (1998) found ~400 μM free Ca^{2+} using a soluble, ER-resident aequorin and a synthetic coelenterazine analog. Similar values (40–400 μM) were recently obtained with a novel system wherein two fluorescent proteins were linked via calmodulin to allow Ca^{2+}-dependent fluorescence energy transfer (Miyawaki *et al*., 1997). In contrast, Button and Eidsath (1996) employed a membrane-attached aequorin chimera to find a concentration of 1–2 μM, suggesting that at least a fraction of the ER membrane system might harbor a relatively low Ca^{2+} concentration. Despite the divergence in the data obained from animal cells (reviewed by Meldolesi and Pozzan, 1998), our measurements of the physiological steady-state level of free Ca^{2+} in the yeast ER indicate a substantial difference in ER Ca^{2+} handling between yeast and animal cells. It is most likely that the lack of a specialized SERCA-type Ca^{2+} pump from the yeast ER is the primary cause for this peculiarity, since Ca^{2+} uptake was completely resistant to thapsigargin. Moreover, the moderate level of free Ca^{2+} (10 μM) within the yeast ER might well limit the use of this compartment for intracellular Ca^{2+} signaling and thus implicate other organelles (Golgi, vacuole) as dynamic Ca^{2+} stores in this eukaryotic micro-organism. It should also be noted that yeast cells can survive prolonged Ca^{2+}-depletion if sufficient Mn^{2+} is available in the growth medium. Under such conditions, yeast cells retain only 3% of their normal cellular Ca^{2+} content without apparent effects on growth (Loukin and Kung, 1995; Dürr *et al*., 1998).

Ca^{2+} refilling into the ER was unaffected by monensin, an ionophore capable of collapsing transmembrane H^+ gradients which potentially energize a putative ER Ca^{2+}/H^+ antiport. Since Ca^{2+} uptake into purified yeast Golgi vesicles is markedly sensitive to the H^+ ionophore (Sorin *et al*., 1997), presumably due to the presence of the vacuolar Ca^{2+}/H^+ antiport Vcx1 in these membranes, the monensin insensitivity we observed would underscore the localization of ER–Aeq in a compartment distinctly different from Golgi. However, Ca^{2+} uptake into mammalian Golgi *in vivo*, is insensitive to H^+ dissipating agents (Pinton *et al*., 1998). Therefore, it remains to be seen whether Vcx1 is a genuine Ca^{2+} transporter of yeast Golgi, but under the conditions of this study we failed to detect such an activity in the ER membrane.

The role of Pmr1 in ER Ca^{2+} sequestration

A partial vanadate-sensitivity of ER Ca^{2+} uptake and the low ER Ca^{2+} level of *pmr1* cells together point to the P-type ATPase Pmr1 as the main ER Ca^{2+} transporter in yeast. Loss of Pmr1 activity lowered free Ca^{2+} in the ER from 10 to ~5 μM, providing direct biochemical support for our hypothesis that the ER-associated defects observed in *pmr1*, as discussed in our previous study (Dürr *et al*., 1998), result from an altered ion content of this compartment. Although our new data strongly insinuate a direct role of the Pmr1 ion pump in ER Ca^{2+} sequestration, we can not rigorously exclude another interpretation. Pmr1 could serve just to stimulate an 'elusive' and as yet unidentified ER Ca^{2+}-transporter that would operate with reduced activity in *pmr1* cells. ER fragmentation in the *pmr1* mutant could also seclude a fraction of the ER–Aeq probe into vesicles lacking this transporter, thereby causing an artificial underestimate of free ER Ca^{2+} in *pmr1* cells. It should be emphasized, however, that the ER-related defects of *pmr1* cells are already manifest in regular, Ca^{2+}-rich media, i.e. under conditions that do not cause ER fragmentation (Dürr *et al*., 1998).

Therefore, we conclude from the present study that yeast lacks a genuine ER Ca^{2+}-transporter specific to this compartment, but instead utilizes, at least in part, the secretory pathway pump Pmr1 for ER Ca^{2+} uptake. Undoubtedly, the main cellular pool of Pmr1 resides in Golgi compartments (Antebi and Fink, 1992; Schröder *et al*., 1995; Sorin *et al*., 1997). Nevertheless, subcellular fractionation experiments with cells grown in regular, Ca^{2+}-rich media consistently find a small fraction of Pmr1 ($<<$10%) associated with ER markers (Antebi and Fink, 1992; Sorin *et al*., 1997), and a non-functional Pmr1 mutant protein was shown to enrich in ER fractions (Sorin *et al*., 1997). Unfortunately, rigorous biochemical analysis of the presumed ER pool of Pmr1 is severely compromised by the failure of available fractionation procedures to separate ER from early Golgi compartments completely.

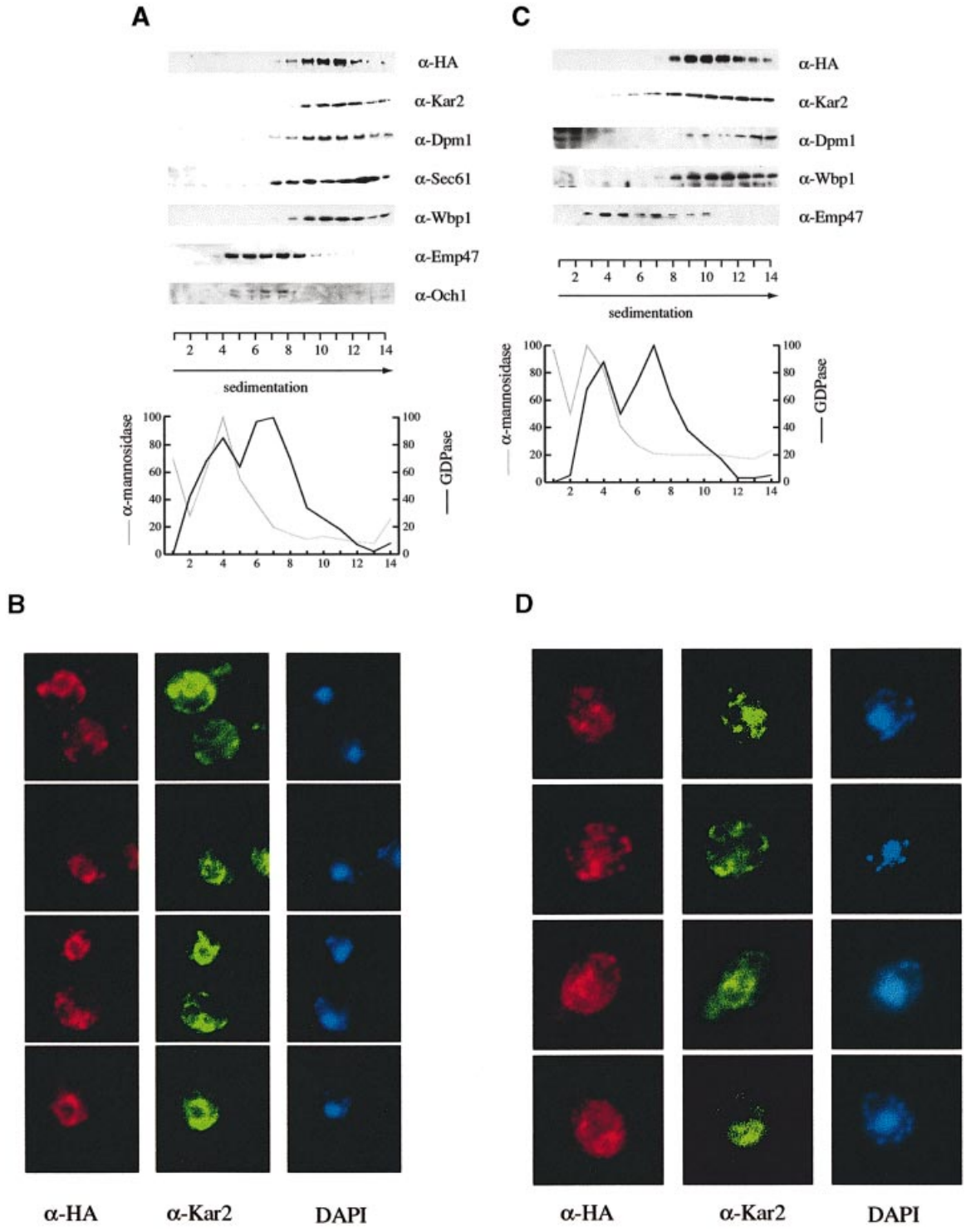

Fig. 7. Localization of ER–Aeq in *pmr1* mutants. (**A**) ER–Aeq cofractionated with ER markers. Subcellular fractionation was performed as described in Materials and methods. (**B**) Co-staining of Kar2 with ER–Aeq. Double labeling immunofluorescence with 12CA5 and anti-Kar2 antibody was performed as described in Materials and methods. 12CA5 was visualized with Cy3 and anti-Kar2 with FITC. Nuclei were stained with DAPI. (**C**) Ca^{2+} deprivation in *pmr1* mutants does not alter cofractionation of ER–Aeq with ER markers. *pmr1* cells expressing ER–Aeq were treated as for aequorin reconstitution. After spheroblasting, the homogenate was subjected to subcellular fractionation on a sucrose density gradient. Aliquots were analyzed by Western blotting and enzymatic assays as described previously. (**D**) In Ca^{2+}-depleted *pmr1* cells Kar2 colocalizes with ER–Aeq, despite vesicularization of ER structures. Cells were treated virtually the same as those used for Ca^{2+} measurements. Spheroblasts were incubated in the presence of 10 μM A23187 for 1 h at 4°C in the presence of 5 mM EGTA. Fixation was followed by simultaneous incubation with 12CA5 and polyclonal anti-Kar2 antibody. 12CA5 was visualized with Cy3-conjugated goat anti-mouse antibody (left panel) and anti-Kar2 antibody with FITC-conjugated goat anti-rabbit antibody (middle). DNA was stained with DAPI (right).

However, since we found that ER Ca^{2+} uptake proceeded unperturbed after cycloheximide treatment or a block in Golgi-to-ER retrograde transport, it seems likely that a fraction of Pmr1 is indeed retained in the ER to actively contribute to the ionic milieu of this compartment. In a similar fashion, putative ER pools of Pmc1, Vcx1 or both, could be responsible for residual Ca^{2+} uptake into the ER of *pmr1* cells, since genetic studies show that yeast cells remain viable with any one of the three Ca^{2+} transporters as the sole intracellular Ca^{2+} transport system (Cunningham and Fink, 1996).

Future studies should address how yeast cells balance activity and spatial distribution of their endo-membraneous Ca^{2+} transporters with the need for Ca^{2+} ions in the ER. As we have shown, ER–Aeq is a suitable probe to monitor free Ca^{2+} in this compartment. Together with probes targeted into other secretory organelles, this tool should permit the characterization of conditional transport mutants and thus ultimately allow the evaluation of individual components and their contributions to Ca^{2+} sequestration into the yeast ER.

Materials and methods

Yeast strains and growth conditions

Yeast strains used in this study were YS256 (*MAT*α *ade2 his3-*Δ*200 leu2-3,112 lys2-*Δ*201 ura3-52 STT3::HA::AEQ::LEU2*), YS258 (the same as YS256 except *pmr1-*Δ*2::HIS3*), YS368 (*MAT***a** *leu2 ura3 his4 lys2 bar1 STT3::HA::AEQ::LEU2*), YS371 (*MAT***a** *leu2 ura3 his4 lys2 suc2 ret2-1 STT3::HA::AEQ::LEU2*; the parental strain is PC130 from P.Cosson), YS380 (*MAT*α *ade2 his3-*Δ*200 leu2-3,112 lys2-*Δ*201 ura3-52/br434* [*CEN URA3 PMA1::SERCA1A::ADC1*]) and YR663 (the same as YS380 except *pmr1-*Δ*1::LEU2*) (Dürr *et al*., 1998). Standard yeast culture media were prepared as described by Sherman *et al*. (1986).

Construction of the STT3::aequorin fusion allele

The *STT3*::aequorin fusion construct, obtained in several cloning steps using different helper templates, can be summarized briefly as follows: a unique *Mlu*I site was introduced into the *STT3* gene (at nucleotide + 2155 from the *STT3* start codon) just before the *STT3* stop codon, to allow in-frame fusion of additional sequences onto the full-length *STT3* coding sequence. Using this site, a (3×HA)::aequorin cassette was inserted encoding three repeats of the nine amino acid HA-epitope (81 bp in total), fused in-frame with modified aequorin cDNA sequences (Montero *et al*., 1995) encompassing nucleotides from position +25 (eight codons downstream of the aequorin start) to +591 (after the aequorin stop codon). Thus the aequorin domain encoded by this cassette also lacks the first eight amino acids of aequorin, like the aequorin construct successfully used in yeast by Nakajima-Shimada *et al*. (1991). As a 3′ terminator region, the *STT3 Hpa*I–*Xba*I fragment (nucleotides from +2093 to +2509) was spliced behind the aequorin stop codon, thus duplicating a small segment of the *STT3* open reading frame (ORF) (nucleotides from +2093 to +2154). Into this duplicated segment, we engineered a *Xho*I site to allow addition of *LEU2* sequences (a 2.2 kb *Sal*I–*Xho*I fragment), used as a selectable marker during transplacement of the entire construct as a *Spe*I–*Xba*I fragment to the endogenous *STT3* locus by transformation. The correct integration of this allele at the *STT3* locus was verified by Southern blotting analysis.

Subcellular fractionation and indirect immunofluorescence

Subcellular fractionation was carried out based on the protocol of Schröder *et al*. (1995). GDPase was measured as described by Abeijon *et al*. (1989). α-mannosidase was detected according to the method of Opheim (1978). The activities of GDPase and α-mannosidase are given as a percentage activity of the highest values measured.

For indirect immunofluorescence, cells grown to early log phase were fixed by adding concentrated formaldehyde solution and 1 M potassium phosphate pH 6.5 directly to cells in growth medium to a final concentration of 3.7% (w/v) and 100 mM, respectively. After 1 h fixation at 25°C, the procedure was carried out as described in Schröder *et al*. (1996).

Radiolabeling, immunoprecipitation and protease protection assay

Yeast strain YS256 expressing ER–Aeq and HA epitope-tagged Och1 was grown in SC media lacking uracil. Metabolic labeling with [^{35}S]-methionine (Amersham), cell lysis and immunoprecipitation were carried out as described by Harris and Waters (1996). Labeling in the presence of 10 μg/ml tunicamycin was carried out for 20 min prior to incubation with [^{35}S]methionine for 20 min.

To determine membrane topology, cells were spheroblasted and lysed at 4°C in a tissue grinder. After clearing, lysate was incubated at 4°C and treated with trypsin (500 μg/ml), trypsin (500 μg/ml)/Triton X-100 (0.5%) or left untreated. After 20 min the reaction was stopped by addition of trichloroacetic acid. Following centrifugation and washes with acetone, the pellet was resuspended in sample buffer and subjected to SDS–PAGE.

Aequorin reconstitution in vitro

Cells were grown overnight in yeast extract/peptone/dextrose (YPD). Approximately 10 OD_{600} units of cells were harvested, followed by washing and resuspending in 400 μl TEP buffer (50 mM Tris–HCl, 5 mM EDTA, 1 mM PMSF pH 7.5). Cells were lysed by vortexing with glass beads. After a clearing spin, 300 μl of crude cell lysate was supplemented with 50 mM 2-ME and 5 μM coelenterazine and incubated for 4 h on ice in the dark. The Ca^{2+}-dependent light emission was measured using a Packard Picolite luminometer by adding 10 mM $CaCl_2$ to a 50 μl sample.

In vivo Ca^{2+} measurements

Cells were grown overnight to log phase and 20 OD_{600} units were harvested and washed with synthetic complete medium (Sherman *et al*., 1986) containing 1.2 M sorbitol buffered with 10 mM HEPES to pH 7 (SP). After resuspension in 1 ml of SP, 100 U of zymolyase 100T and 3 μl 2-ME was added and incubated at room temperature. Spheroblasts were washed with SP containing 10 mM EGTA (SPE) and resuspended in 500 μl SPE (4°C) containing 10 μM Ca^{2+} ionophore A23187 and 14 mM 2-ME. After addition of coelenterazine to a final concentration of 5 μM, the cell suspension was incubated for 1 h at 4°C in the dark. Cells were then harvested, washed twice with SPE containing 5% BSA (4°C) and incubated for 5 min in SPE (5% BSA) at 4°C. After centrifugation, cells were resuspended in 100 μl SPE (5% BSA), followed by addition of 100 μl low melting point agarose (1.5%, 37°C). Fifty microliters of this cell suspension was then placed onto 13-mm round coverslips and stored at 4°C until the agarose solidified. The coverslips were transferred into a perfused, thermostated chamber (22°C; except *ret2-1* mutant at restrictive temperature), and placed in close proximity to a cooled, low-noise photomultiplier with a built-in amplifier–discriminator (EMI 9789, as described in Rizzuto *et al*., 1994). For Ca^{2+} refilling, the perfusion with SPE (5% BSA) was followed by perfusion with SP supplemented with 1 mM $CaCl_2$. Permeabilization with 100 μM digitonin in the presence of 10 mM $CaCl_2$ finished the experiment by discharge of residual aequorin. The aequorin luminescence data were captured by an EMI C660 photon counting board and stored in a personal computer.

For use of aequorin luminescence data in calculations of luminal Ca^{2+} concentrations during refilling, it is imperative that the entire refilling process consumes only a fraction of the total cellular pool of reconstituted holoaequorin. To meet this requirement in a potentially Ca^{2+}-rich environment (the ER), we employed an aequorin variant with reduced Ca^{2+} affinity due to a single amino acid exchange (Montero *et al*., 1995). Synthetic coelenterazine analogs, such as coelenterazine n (Shimomura *et al*., 1989), also allow modulation of the Ca^{2+}-sensitivity of aequorin sensors, but were not commercially available during the course of our experiments. In any case, the fraction of charged aequorin remaining after refilling can be determined by cell lysis in the presence of excess Ca^{2+}. The total number of photons counted during refilling and subsequent cell lysis in high Ca^{2+} is then set to represent the total amount of reconstituted aequorin. To calculate Ca^{2+} concentrations from our light emission data, we used the method described by Allen and Blinks (1978) which correlates a given Ca^{2+} concentration to the ratio between initial light emission at this Ca^{2+} concentration and the amount of reconstituted aequorin initially present. Since holoaequorin is continuously consumed during a refilling experiment, the remaining pool of charged aequorin must be recalculated for each timepoint as the difference between the total amount of holoaequorin, as determined by cell lysis in saturating Ca^{2+}, and the amount of consumed aequorin represented in the photons captured up to that timepoint. The mathematical algorithm used for these calculations is described in more detail

in Brini *et al*. (1995) and we used the Ca^{2+} affinity constants of mutant aequorin as determined by Montero *et al*. (1995).

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