Green fluorescent protein-tagged sarco(endo)plasmic reticulum Ca^{2+} -ATPase overexpression in *Paramecium* cells: isoforms, subcellular localization, biogenesis of cortical calcium stores and functional aspects

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Summary

We have followed the time-dependent transfection of *Paramecium* cells with a vector containing the gene of green fluorescent protein (GFP) attached to the C-terminus of the PtSERCA1 gene. The outlines of alveolar sacs (ASs) are labelled, as is the endoplasmic reticulum (ER) throughout the cell. When GFP fluorescence is compared with previous anti-PtSERCA1 antibody labelling, the much wider distribution of GFP (ER+ASs) indicates that only a small amount of SERCA molecules is normally retained in the ER. A second isoform, PtSERCA2, also occurs and its C-terminal GFP-tagging results in the same distribution pattern. However, when GFP is inserted in the major cytoplasmic loop, PtSERCA1 and two fusion proteins are mostly retained in the ER, probably because of the presence of the overt C-terminal KKXX ER-retention signal and/or masking of a signal for transfer into ASs. On the overall cell surface, new SERCA molecules seem to be permanently delivered from the ER to ASs by vesicle transport, whereas in the fission zone of dividing cells ASs may form anew. In cells overexpressing PtSERCA1 (with C-terminal GFP) in ASs, $[\text{Ca}^{2+}]$, regulation during exocytosis is not significantly different from controls, probably because their Ca^{2+} pump has to mediate only slow reuptake.

Introduction

Subplasmalemmal calcium stores are widely distributed, be it in the form of sarcoplasmic reticulum (SR) of striated muscle cells (Franzini-Armstrong and Protasi, 1997) or as cortical stores in neuronal (Henkart *et al.*, 1976; Buchanan

et al., 1993; Metuzals *et al.*, 1997), neurosecretory (Tse *et al.*, 1997) and some other systems (Golovina and Blaustein, 1997; Hussain and Inesi, 1999). Like the SR, such stores, generally considered special regions of the endoplasmic reticulum (ER), are physically linked to the cell membrane. Although selective functional analysis is difficult to achieve, such stores are frequently implied to be engaged in generating a rapid exocytotic response (Berridge, 1997, 1998; Mackrill, 1999).

In a *Paramecium* cell, almost the entire cell membrane, except for the origin of cilia and for docking sites of dense core secretory organelles ('trichocysts'), is lined with flat 'alveolar sacs', ASs (Allen, 1988; Plattner *et al.*, 1999). These are also linked to the cell membrane, with a ~15 nm broad subplasmalemmal space in between (Plattner *et al.*, 1991). ASs have been identified as calcium stores (Stelly *et al.*, 1991). They have been isolated and shown to pump $^{45}\text{Ca}^{2+}$ independent of calmodulin, CaM (Länge *et al.*, 1995; Kissmehl *et al.*, 1998). The pump contained in ASs has been cloned (Hauser *et al.*, 1998) and characterized biochemically and pharmacologically (Kissmehl *et al.*, 1998) as a 106 kDa sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA). It shares the overall molecular structure with SERCA isoforms of higher eukaryotes described by Ogawa *et al.* (1998). Using antibodies (ABs) against a cytoplasmic loop [not shared by the calmodulin-activated 130 kDa plasmalemmal Ca^{2+} -ATPase (Wright and Van Houten, 1990; Elwess and Van Houten, 1997)], we have localized this pump specifically to the ASs, although it was not detectable in microsomes enriched in ER (Hauser *et al.*, 1998; Kissmehl *et al.*, 1998). On the electron microscope (EM) level, this pump localizes to the 'inner' part of the AS membrane which faces the cell centre (Plattner *et al.*, 1999). As high-capacity low-affinity Ca^{2+} -binding proteins (CaBP), ASs contain a calsequestrin-like protein, whereas the ER contains a calreticulin-like protein (Plattner *et al.*, 1997b). Several of these aspects of ASs, in addition to activation of Ca^{2+} release by the ryanodine receptor agonist, 4-chloro-meta-cresol (Klauke *et al.* 2000), are strikingly similar to SR of skeletal muscle cells (Länge *et al.*, 1995). The biogenesis is largely unknown for any of the subplasmalemmal calcium stores of the secretory

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systems mentioned, as it is, to a considerable extent, for the SR (Franzini-Armstrong and Jorgensen, 1994; Protasi *et al.*, 1997). We now try to gather more detailed information for the ASs in *Paramecium*.

Our approach is based on previous cloning of the first isoform of the SERCA gene in *Paramecium tetraurelia*, PtSERCA1 (Hauser *et al.*, 1998), on the availability of a strong promoter and on the optimization of green fluorescent protein (GFP) expression in this organism after transfection by macronuclear injection (Hauser *et al.*, 2000). Time-dependent expression is monitored by confocal laser scanning microscopy (CLSM) to analyse the biogenesis of ASs. When such data are compared with previous immunolocalization of PtSERCA1 (Hauser *et al.*, 1998; Plattner *et al.*, 1999), this reveals a considerable discrepancy as only the overexpressed SERCA–GFP fusion protein is able to label the ER significantly, whereas ABs against PtSERCA1 significantly label ASs quite selectively in non-transfected cells. As we now find, the extent of delivery of the GFP–PtSERCA1 fusion protein to ASs depends on the fusion site. ER→AS transit occurs when the GFP gene is added to the C-terminus, but not when inserted between K542 and A543 of the major cytoplasmic loop of the SERCA molecule. We conclude that, normally, transit through different biosynthetic steps must be quite rapid and/or copy numbers in some structures, for example in the ER (though involved in biogenesis), must be low and that final delivery and enrichment of PtSERCA1 molecules normally aims at ASs.

We now describe a second isoform, PtSERCA2, which we find. It has 98% similarity to PtSERCA1. Results achieved with the two types of GFP fusion proteins are similar with PtSERCA1, depending on the fusion site.

Using previous fluorochrome imaging methods (Klaue and Plattner, 1997), we also analysed any effect of PtSERCA1–GFP overexpression on the regulation of internal Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, upon stimulation of synchronous trichocyst exocytosis.

Results

Identification of a second SERCA isoform

We discovered the occurrence of a second isoform, PtSERCA2. As it is found in a cDNA library, this suggests that it normally may also be expressed. Because the sequences of PtSERCA1 and 2 are 97% identical on the nucleotide level and 98% on an amino acid level, we can perform neither Northern blots to check expression of the two isoforms nor differential immunolocalization. In detail, there are only 19 differences between the two sequences within 1037 amino acids overall, and seven of these exchanges are conservative (Table 1). The peptide sequence, SSQNEKGNVLFIKG (506–519), previously

Table 1. Amino acid exchanges between the two cDNA sequences of PtSERCA1 and PtSERCA2.

Amino acid position	PtSERCA1	PtSERCA2	Conservative exchange
11	H	Y	–
12	A	G	c
15	L	V	–
20	G	V	–
36	E	S	–
58	K	N	c
156	I	V	c
172	V	A	–
173	E	D	c
196	V	I	c
228	V	I	c
253	S	Q	–
391	V	I	c
410	L	F	–
436	M	V	–
487	A	T	–
587	N	S	–
588	L	Q	–
600	S	N	–

c, conservative exchange.

used to prepare specific ABs (Hauser *et al.*, 1998), is identical in the two isoforms.

Nomenclature of PtSERCA–GFP fusion plasmids and proteins

We have constructed two fusion proteins between PtSERCA and GFPmut1. The first one contains GFPmut1 fused to the C-terminus of PtSERCA and is called PtSERCA–SSG1 for isoform PtSERCA1 and PtSERCA–SSG2 for isoform PtSERCA2. The abbreviation SSG is created from SERCA–SERCA–GFP, indicating that GFPmut1 is located at the C-terminal end of PtSERCA. The expression plasmids carrying the genes for the fusion proteins are called pPXV–SSG1 and pPXV–SSG2 respectively. The second fusion construct contains the GFPmut1 between amino acids K542 and A543 in the cytoplasmic loop of PtSERCA, as specified in the SERCA model by Ogawa *et al.* (1998), considering identical basic structure of PtSERCA (Hauser *et al.*, 1998). It is called PtSERCA–SGS1 for isoform PtSERCA1 and PtSERCA–SGS2 for isoform PtSERCA2. The abbreviation comes from SERCA–GFP–SERCA, indicating that GFPmut1 is located in the middle of the PtSERCA molecule. Accordingly, the expression plasmids again carry the name pPXV–SGS1 and pPXV–SGS2 respectively.

Construction and use of pPXV–SSG and pPXV–SGS

The first fusion protein construct is PtSERCA fused to the N-terminus of GFPmut1 (PtSERCA–SSG). In this construct, the C-terminal four amino acids KKIQ of PtSERCA are changed into KKPV and two amino acids

A

- (1) ...AAG AAA ACT TAA TGA GAA ATC ... original PtSERCA C-terminus
... K₁₀₃₄ K I Q *
- (2) ...AAG AAA CCG GTC TGA GAA ATC ... mutated PtSERCA C-terminus (underlined:
... K₁₀₃₄ K P V * Agel restriction site for joining)
- (3) ...CTA CCG GTC GCC ACC **ATG** GGT... 5' untranslated sequence of GFPmut1
... L P V A T M G ... (**ATG**: start of GFPmut1)
- (4) ...AAG AAA CCG GTC GCC ACC **ATG** GGT... Sequence of the fusion between PtSERCA and
... K₁₀₃₄ K P V A T M G GFPmut1 at the point of junction.

B

- (1) ...CCA TTG AAA GCT TTA **ATG** GGT AAA... Junction of PtSERCA phosphorylation domain and
... P L K₅₄₂ A₅₄₃ L M G K ... GFPmut1 N-terminus (underlined: *Hind*III restriction site)
(**ATG**: start of GFPmut1)
- (2) ...CGA GCT CAA GCT TAA GAC AAG AAT... Junction of GFPmut1 C-terminus and PtSERCA
... R A Q A₅₄₃ Q₅₄₄ D K N ... nucleotide
binding domain (underlined *Hind*III
restriction site)
(**CAA**: last codon of GFPmut1)

Fig. 1. Sequence details of junctions between PtSERCA and GFPmut1.

A. Sequence junction between C-terminus of PtSERCA and N-terminus of GFPmut1 in pPXV-SSG. The original PtSERCA C-terminus (1) was mutated by PCR to generate an *Agel* restriction site (underlined and italic) (2). GFPmut1 (3) and PtSERCA were joined via the indicated *Agel* restriction site to create the fusion site shown in (4). Numbers refer to amino acid positions.

B. Sequence junctions between PtSERCA and GFPmut1 in pPXV-SGS. (1) The N-terminus of GFPmut1 linked to PtSERCA via a *Hind*III restriction site (underlined and italic). (2) The C-terminus of GFPmut1 joined to PtSERCA via the same *Hind*III restriction site. Numbers indicate amino acid positions (see also *Experimental procedures*).

A and T are added to link the protein to GFPmut1 (Fig. 1A). KKXX is discussed as an ER retrieval signal in animal cells (Nilsson *et al.*, 1989; Jackson *et al.*, 1990, 1993). Perhaps it functions as an ER retention signal or as a target signal for location of the PtSERCAs to the ASs.

As the KKXX targeting signal is masked in the fusion proteins, there could be mistargeting of the overexpressed PtSERCA-SSG to other compartments and even to the cell membrane. Therefore, we constructed a second fusion protein which contains the GFPmut1 domain in the major cytoplasmatic loop between membrane-spanning domains 4 and 5. Here, GFPmut1 is inserted between K542 and A543 of PtSERCA and thus separates the phosphorylation domain and the nucleotide-binding domain from each other. This fusion protein contains the original KKIQ sequence at the C-terminus (Fig. 1B).

We expressed both SERCA isoforms, with GFPmut1 inserted inside or attached to the C-terminus. Cells overexpressing PtSERCA-SSG1 have been further evaluated by Northern blot analysis. Quantification of the signal obtained is difficult because that of non-transfected cells is almost absent. Because our system allows much stronger overexpression than previously

achieved with any other system, we considered it feasible to analyse its effect on $[Ca^{2+}]_i$ homeostasis (below). Results achieved with the different fusion proteins by CLSM analysis vary widely, as described below.

Identification of two distinct calcium stores ER and ASs

In the subsequent context, it is important to differentiate between the two extended calcium stores of a *Paramecium* cell, i.e. ER and ASs. Based on previous work and using the same ABs (Plattner *et al.*, 1997b), we identified, by immunofluorescence, ER and ASs by their selective content of calreticulin- and calsequestrin-like protein (Fig. 2A and B). ABs against calreticulin show a patchy distribution throughout the cell, whereas those against calsequestrin are enriched at sites where ASs are known to be localized, i.e. in a thin cortical layer and in the oral region. We then applied the ER-specific affinity stain DiOC₁₈ for short time periods (10 min) to visualize by CLSM (Fig. 3) the patchy distribution of the ER throughout the cell (Fig. 3B) and the absence of label from ASs (Fig. 3B and C), even when the stain has reached the most peripheral ER extensions. This strongly suggests

Fig. 2. Immuno-FITC localization of calreticulin-like (A) and of calsequestrin-like (B) protein, using monospecific antibodies as described in the *Experimental procedures*. Note the occurrence of label in a patchy arrangement in A and restriction of label to a thin cortical layer and to the oral region in B. Bars = 10 μ m.

the absence of a patent physical connection between the two organelles – in full compatibility with our view of AS biogenesis derived from PtSERCA–GFP transfection studies, as outlined below and summarized in Table 2.

CLSM and EM analysis of cells transfected with PtSERCA–GFP fusion protein with GFPmut1 attached to the C-terminus, PtSERCA–SSG

Transfected cells were analysed in non-synchronous cultures after numerous cell divisions. PtSERCA–SSG1 becomes concentrated on the cell periphery, where ER-type structures (identified by DiOC₁₈ staining; Plattner *et al.*, 1997b) with numerous perforations approach ASs (Fig. 4). ASs themselves are intensely labelled (further identified below). In median optical sections, this becomes evident by continuous fluorescent cell contours corresponding to the extension of ASs over almost the entire cell surface and their flat shape, with a luminal width of only 100 nm on average (Hardt and Plattner, 1999). Surface views allow one to recognize plaque-like structures corresponding in size and arrangement to individual

ASs, with a dark dot at the emergence of a cilium. This is precisely the appearance obtained by staining of permeabilized cells with ABs against the PtSERCA1 molecule (Hauser *et al.*, 1998). Concomitantly, after expression of the PtSERCA–SSG1 fusion protein, immunogold EM analysis with ABs against GFP shows labelling of the inner part of ASs (Fig. 5), just as with anti-SERCA ABs (Plattner *et al.*, 1999). In addition, gold granules also occur to some extent on the complex formed by the closely apposed outer region of ASs and the cell membrane (data not shown). Labelling cannot be resolved between these two membranes because they are only ~15 nm apart (Plattner *et al.*, 1991), whereas indirect immunogold labelling allows for a resolution of only ~20–30 nm. Yet overexpression of PtSERCA–SSG1 clearly causes some drift of the fusion protein into outer regions of ASs (not shown) where SERCA molecules are not normally positioned (Plattner *et al.*, 1999).

Other structures labelled after transfection with PtSERCA–SSG1 are the nuclear envelope, which is an established calcium store (Rogue and Malviya, 1999),

Table 2. Structures labelled with the different procedures.

Structure	DiOC ₁₈	PtSERCA–SSG1	PtSERCA–SGS1
Endoplasmic reticulum	+++	(+++)	+++
Nuclear envelope	+	++	++
Cell cortex (alveolar sacs)	–	+++	(+)
Oral region	–	+++	+++
Mitochondria	–	–	–
Secretory organelles (trichocysts)	–	–	–
Cilia	–	(+)	–
Calcium crystal vacuoles	–	–	–
Food vacuoles			
Membranes	–	–	–
Contents	–	–	(+)
Osmoregulatory system			
Contractile vacuole	–	–	–
Radial canals + spongione	–	+++	++

a. + to +++, weak to strong labelling. (+), occasional weak labelling; (+++), transient labelling in ER before label reaches ASs.

Fig. 3. Affinity labelling of the ER, 10 min after injection of DiOC₁₈ dissolved in an oil droplet (labelled by +), from where the stain diffuses into a halo and then into the ER (labelled 'er'). Note in B and C selective staining of the ER, whereas ASs remain unstained (arrows), even when closely approached by heavily stained ER cisternae. The membrane of a crystal vacuole (c), identified in the transmitted light image (A), is also unstained. Bars = 10 μ m.

parts of the osmoregulatory system, i.e. radial canals, presumably with the attached spongiome, and the oral region (Table 2). Here, the label again coincides with the occurrence of alveolar sacs (Allen, 1988).

Most important for aspects of biogenesis is the consistent observation that in dividing cells both of the

two oral regions are labelled, although one with higher intensity (Fig. 6). Whereas the old cell surface is intensely labelled, this is not the case with the new cell boundary formed in the fission zone. This indicates *de novo* formation of ASs in this region. The intense labelling of one of the two oral regions may reflect its established *de novo* formation, whereas occurrence of some labelling in the second oral region may reflect a steady-state situation with ongoing replacement.

After transfection with PtSERCA–SSG2, essentially the same labelling pattern appears. Figure 7 shows an example with labelled ER seen as specks, ASs seen as fluorescent contours and parts of the osmoregulatory system. Although the contractile vacuole remains unlabelled, in all cases analysed labelling of radial canals is enforced in distal regions, again most probably by the adherent spongiome (Allen, 1988).

CLSM analysis of cells transfected with PtSERCA–GFP fusion protein with GFPmut1 inserted in the major cytoplasmic loop, PtSERCA–SGS

An example of ER labelling is presented in Fig. 8. Even where the ER closely approaches the cell surface, no evident labelling of ASs occurs. This reflects our general observation that this fusion protein causes only little labelling, if any, of ASs. The label resides predominantly in ER deep inside the cell, but again radial canals are also labelled, as are old and new oral regions in dividing cells (data not shown).

Results obtained with this fusion protein in comparison with that described above are compiled in Table 2. With this fusion protein, we observe occasional delivery into food vacuoles (Fig. 9).

General observations with the fusion proteins

On no occasion could we observe labelling of mitochondria, trichocysts or, quite remarkably, of 'calcium crystal vacuoles', e.g. in Fig. 6. Also remarkably, with the C-terminally tagged fusion protein, we clearly recognized labelling of some individual cilia, in widely distant positions (e.g. in Fig. 4). As their number was < 1% in all cells analysed, this may indicate occurrence of some mis-targeting, although to a limited extent, under conditions of overexpression.

[Ca²⁺]_i transients during aminoethyl-dextran (AED)-stimulated exocytosis in cells transfected with PtSERCA–SSG1

Within statistical fluctuations, this does not result in any alteration of [Ca²⁺]_i responses during AED-stimulated exocytosis (Table 3) compared with previous experiments

Fig. 4. PtSERCA-SSG1. Median (A), superficial (B) and intermediate (D) focal steps show strong labelling of the ER (lower left in A, B and D) and of the outermost cortex layer (arrowheads), whose ASs become visible as distinct entities in B. C. Transmitted light image; as, alveolar sacs; er, ER; fv, food vacuoles; t, trichocysts. Bar = 10 μ m.

using non-transfected cells. We analysed the following parameters with transfected and Fluo-3-injected cells: f/f_0 ratios for determining changes in intracellular free calcium concentrations, $[Ca^{2+}]_i$; during exocytosis, stimulation by

aminoethylidextran (AED), as well as $t_{1/2}^{up}$ and $t_{1/2}^{down}$, i.e. the increase and decrease of $[Ca^{2+}]_i$ respectively. Although we could not exclude some impairment of pumping activity by coupling GFP to the C-terminal domain of the

Fig. 5. Immunogold EM labelling of AS inner membrane regions (arrowheads) with antibodies against GFP, 12 days after transfection with PtSERCA-SSG1. Note scarcity of label on the outer membrane complex (C) formed by the tightly apposed outer AS membrane and the cell membrane. Bar = 0.1 μ m.

Fig. 6. Dividing cell overexpressing PtSERCA-SSG1.

A. GFP fluorescence.

B. Transmitted light. Typically, both cytosomes (cs) of a dividing cell are labelled, frequently with more intensity in one and with more intense labelling on one side (arrowheads). In addition, cell contours (arrows) with old ASs are intensely labelled, in contrast to the newly forming cell boundary of the fission zone (double arrowheads). Crystals (c) contained in vacuoles display some autofluorescence, yet their membranes do not fluoresce. Bars = 10 μ m.

SERCA molecule, this is unlikely as the established arrangement of functional domains (MacLennan *et al.*, 1997; Ogawa *et al.*, 1998) remained untouched.

Discussion

Specific problems inherent to ciliate molecular biology and use of GFP as a tag

As previously described (Hauser *et al.*, 2000), the GFP gene from *Aequorea victoria* had to be modified. (i) Considering codon usage and fluorescence quantum

yield, in wild-type GFP two amino acid substitutions were carried out, i.e. F64L and S65T to obtain the previously described GFPmut1, the fluorescence of which is 35-fold higher than with wild-type GFP (Cormack *et al.*, 1996). (ii) The *Paramecium*-specific stop codon TGA was substituted for the original TAA stop codon, which in *Paramecium* would be read as glutamine.

Although GFP is frequently used as an *in vivo* tag (Conn, 1999), it may entail some problems to be checked in every system. In *Paramecium*, a large copy number is necessary to yield sufficient fluorescent signal with the free cytosolic protein (Hauser *et al.*, 2000). In the present context, enrichment in specific membranes will facilitate tracing of a biogenetic pathway. Overexpression may help to visualize a protein in compartments which normally contain only a low copy number. This may explain that, by immunofluorescence, PtSERCA1 has been detected only in ASs (Hauser *et al.*, 1998), whereas now the ER and some additional structures are clearly labelled. However, this may entail a block in the early steps or mistargeting in later steps. There is no general drift of a GFP fusion protein within a membrane system, as we derive from selective labelling of specific regions of the osmoregulatory system.

The size of a spacer between a fusion gene coding for an intrinsic protein and a GFP molecule may have a variable effect on expression (García-Mata *et al.*, 1999;

Fig. 7. Transfection with PtSERCA-SSG2 results in an identical labelling pattern as with PtSERCA-SSG1, including labelling of the ER, the outermost cortical layer (with ASs; labelled by arrows) and radial canals (arrowheads) with attached structures, whereas the contractile vacuole (asterisk) remains unlabelled. Bar = 10 μ m.

Table 3. Effects of PtSERCA-SSG1 overexpression on $(Ca^{2+})_i$ dynamics upon AED-stimulated exocytosis.

	$t_{1/2}^{up}$	f/f_0 ratio	$t_{1/2}^{down}$
Transfected cells			
This paper	≤ 1 s	3.6 ± 1.4	5.0 ± 2.0 s
Non-transfected cells			
Klauke and Plattner (1997)	≤ 1 s	2.9 ± 0.9	n.d.
Klauke <i>et al.</i> (2000)	≤ 1 s	5.4 ± 0.5	2.0 ± 1.0 s

Values $\pm$ SD. n.d., not done.

Fig. 8. PtSERCA–SGS1 is not exported from the intensely labelled ER to ASs, as recognized by the absence of a fluorescent cortical rim (compare with Fig. 4). Bars = 10 μ m.

Prescott *et al.*, 1999), so it always has to be tested anew. By qualitative evaluation, we established the following details. (i) Transcription and targeting do not differ between the two PtSERCA isoforms analysed. Over-expression of PtSERCA–SSG1 causes only limited mistargeting, considering that only $<1\%$ of cilia are labelled. (ii) GFP tagging at the C-terminus allows for transfer into ASs, whereas GFP insertion into the major cytoplasmic loop causes retention in ER. (iii) In the last case, some label goes into phagolysosomal compartments. (iv) Irrespective of the construct used, the radial canals (with attached structures) of the osmoregulatory system are labelled.

Biogenesis of cortical stores in relation to ER

Previous anti-PtSERCA1 (peptide) AB labelling was restricted to ASs (Hauser *et al.*, 1998), DiOC₁₈ stains the ER and different luminal Ca-binding proteins are found in the two organelles (Figs 2 and 3). On this basis, ER and ASs can be clearly differentiated. In *Paramecium*, Golgi fields are so small and widely scattered (Allen, 1988) that their implication can hardly be discussed.

A C-terminal KKXX (KKIQ) sequence occurs in PtSERCA isoform 1 (Hauser *et al.*, 1998) and 2 (this study). In mammalian cells, this is considered a signal for retention in the ER or for recycling to the ER (Jackson

Fig. 9. PtSERCA–SGS1 is occasionally sequestered into food vacuoles (fv). Bar = 10 μ m.

et al., 1993). A larger signal within the N-terminal half of the SERCA molecule has been implied for delivery into the SR (Black *et al.*, 1998). This may be disturbed in our case when GFP is inserted into the major cytoplasmic loop because we scarcely see PtSERCA–SGS in ASs. Alternatively, some membrane-associated proteins may have to interact with ER components to induce their exit (Herrmann *et al.*, 1999). A more detailed analysis on targeting mechanisms would be beyond the frame of this work as it would have to include systematic amino acid exchange studies as well as analysis of auxiliary proteins (see below).

Theoretically, in skeletal muscle cells, SR may form from ER either by gradual differentiation or by outgrowth of distinct regions, with the implication of selective delivery of calsequestrin to the SR and selective retention of some ER-specific components (Flucher, 1992; Volpe *et al.*, 1992). It is difficult to derive transport routes from staining experiments because labelling intensity will depend on relative binding intensity, on transit time and on the number of copies of a protein present in a certain structure. Overexpression of PtSERCA–SSG fusion protein in our study may indicate a genuine biogenetic pathway. Generally, negative results with immunostaining may also be attributed to the highly variable sensitivity of different regions of a SERCA molecule (Moller *et al.*, 1997). This could call into question some of the conclusions one may derive from immunolocalization studies on biogenetic pathways. During overexpression in *Paramecium*, SERCA molecules may in part be forced beyond their usual location, i.e. the inner region of ASs (Plattner *et al.*, 1999), because we now see some gold-labelled GFP also in the outer membrane region (data not shown).

GFP fluorescence occurs in ASs at two independent sites, i.e. not only at the cell periphery but also in the oral region. Considering the unknown biogenesis of alveolar sacs (Capdeville *et al.*, 1993), how do our findings compare with previous morphogenetic studies? In fact,

structural analysis as well as analysis of underlying protein phosphorylation events indicate a dual origin of cortical morphogenesis in *Paramecium* (Iftode *et al.*, 1989; Sperling *et al.*, 1991) as in *Tetrahymena* (Kaczanowska *et al.*, 1999), although details could not be specified up to now. Both the oral region and the general cell surface are considered to represent two independent morphogenetic regions during cell division (Jerka-Dziedzic and Beisson, 1990). We now consider this applicable also to the biogenesis of ASs. As during cell division only one oral region is formed anew in *Paramecium* (Jones, 1976), labelling of both structures (Fig. 6) may indicate rapid material turnover even in the inherited oral region. In the rest of the cell surface, no selective deposition of GFP fusion protein in 'hot-spots' is observed, thus indicating steady diffuse formation and expansion during normal cell growth. Our results suggest formation from ER which closely approaches ASs. Even if one might have the impression, in some CLSM images, that some rare connections might occur between ER and ASs, this cannot be ascertained because of limited z-resolution. In fact, our DiOC₁₈ labelling studies show no such physical connections. SERCA molecules may be transported from ER to ASs over the last small distance by vesicles, rather than by local differentiation within a membrane continuum.

Generally, information on retention/targeting signals for SERCA molecules is sparse. Some unidentified cytosolic regions may bind to ankyrin (Zhou *et al.*, 1997; Kordeli *et al.*, 1998; Tuvia *et al.*, 1999) or to mitsugumin, a member of the synaptophysin family (Nishi *et al.*, 1999). So far, we can contribute new aspects to biogenesis only on the organellar level, and we suggest the occurrence of transport signals in the SERCA molecule itself.

In the ciliate *Euplotes*, EM morphological studies indicate that ASs after conjugation are reformed by fusion of some smooth tubules and vesicles whose structure is different from regular ER (Geyer and Kloetzel, 1987). This is compatible with our Fig. 6, suggesting *de novo* formation of ASs specifically in the fission zone.

Label outside ER and alveolar sacs

Components of the phagolysosomal apparatus may not be considered a regular degradative pathway. Only overexpression of the fusion proteins, PtSERCA–SGS, not amenable to ASs may be fed into these compartments (Fig. 9) specified by Fok and Allen (1993).

As PtSERCA–GFP fluorescence is absent in most cases from the membranes of vacuoles containing calcium crystals, as specified by (Grover *et al.*, 1997), they may take up Ca²⁺ by an alternative transport mechanism still to be elucidated. As we show in Fig. 3, these membranes are not readily stained with DiOC₁₈.

The biogenesis of the osmoregulatory system is unknown. We now see its radial canals, with attached structures (bona fide spongiome tubules), consistently intensely labelled by PtSERCA–GFP fusion protein. This holds for either SERCA isoform and for either type of GFP tagging, thus indicating independence from targeting domains relevant for ER→AS transfer. In contrast, the contractile vacuole membrane and the excretory porus always remain unlabelled. In *Paramecium*, CaM has been localized selectively to the contractile vacuoles (Momayezi *et al.*, 1986), just as in *Dictyostelium* (Zhu *et al.*, 1993). Also in the vacuole, a plasmalemmal type CaM-activated Ca^{2+} -ATPase has been demonstrated to occur by recent molecular studies in *Dictyostelium* (Moniakis *et al.*, 1999), whereas in *Paramecium* occurrence of a CaM-activated Ca^{2+} pump is known only for the somatic (non-ciliary) cell membrane (Elwess and Van Houten, 1997) but not yet for the vacuole. This may be expected from results with *Dictyostelium* and from our present finding of the absence of a SERCA-type pump from the contractile vacuole. On the other hand, its presence in radial canals (with adjacent spongiome) now suggests biogenesis as an ER derivative and its SERCA-type pump may now be considered a genuine transport-active component contributing to latent Ca^{2+} homeostasis.

Expression of PtSERCA isoforms

The high similarity of PtSERCA1 and 2 (Table 1) impeded differentiation by Western or Northern blot analysis. Because the overexpressed GFP fusion proteins of PtSERCA1 and PtSERCA2 show the same staining pattern, we assume that both isoforms may co-exist in the same target organelles, notably the alveolar sacs. Co-expression of different isoforms in calcium stores of mammalian secretory cells (Burgoyne *et al.*, 1989; Webb and Dormer, 1995; Vanlingen *et al.*, 1997), in vascular endothelial cells (Mountian *et al.*, 1999) or in the SR of chronically stimulated muscle fibres (Zhang *et al.*, 1997) has been suggested to serve specific requirements of different subtypes of stores. In the two isoforms that we found, none of the relevant regions, when compared with established SERCA structures in the SR (Thomas and Hanley, 1994; MacLennan *et al.*, 1997; Ogawa *et al.*, 1998), appears to us to be of sufficient significance to entail any such functional variation. The two PtSERCA isoforms may therefore reflect a general tendency of the ciliate genome to accumulate minor mutations, as reported for phosphoglucosyltransferase (Hauser *et al.*, 1997), or major mutations, as found with histone H3 (Bernhard, 1999). The reason may be lower selection pressure in the replication-independent variants of the macronucleus (Bernhard and Schlegel, 1998). Alternatively, occurrence

of more than one SERCA gene could facilitate over-expression upon demand, as occurring during permanent stimulation of muscle cells (Peters *et al.*, 1999)

Effects on $[\text{Ca}^{2+}]_i$ homeostasis

In different cell types, overexpression of components of calcium stores or of the plasmalemmal Ca^{2+} pump generally causes only limited effects (Misquitta *et al.*, 1999). In HeLa cells, $[\text{Ca}^{2+}]_i$ dissolved in the ER slightly decreases after calreticulin overexpression (Roderick *et al.*, 1998). Yet upon stimulation, in such cells cytosolic $[\text{Ca}^{2+}]_i$ changes differ only slightly from non-transfected cells (Bastianutto *et al.*, 1995). Expression of heterologous SERCA1 in oocytes causes increased $[\text{Ca}^{2+}]_i$ amplitudes and frequency of Ca^{2+} waves (Camacho and Lechleitner, 1993). The effect seen after SERCA overexpression in hearts of transgenic rats varies from none (Hammes *et al.*, 1998) to prolonged transients and accelerated relaxation (Giordano *et al.*, 1997; He *et al.*, 1997).

We achieved very strong overexpression of the fusion protein from PtSERCA–SSG1, where functional sites, as defined in the established models for SERCA molecules (Thomas and Hanley, 1994; Mintz and Guillain, 1997; Ogawa *et al.*, 1998), remain untouched. Nevertheless, we see no significant effect on $[\text{Ca}^{2+}]_i$ transients after exocytosis stimulation (Table 3). This may probably be explained by the overall kinetics that involve very rapid Ca^{2+} release from alveolar sacs and superimposed Ca^{2+} influx (Plattner *et al.*, 1997a; Hardt and Plattner, 1999), the spread of the $[\text{Ca}^{2+}]_i$ signal during stimulation (Klauke and Plattner, 1997), but only very slow reuptake (Länge *et al.*, 1995, 1996). Ca^{2+} totally stored in the sacs does not vary even when cells are kept at 10 times higher $[\text{Ca}^{2+}]_o$ (Hardt and Plattner, 1999), although this entails rapid Ca^{2+} uptake into the cytosol (Erxleben *et al.*, 1997). Storage capacity may be rather limited by the binding capacity of luminal proteins. In *Paramecium*, PtSERCA-type pumps are evidently designed for long-term regulation via steady store refilling. Their influence during the 20–30 s required for recovery of $[\text{Ca}^{2+}]_i$ (Klauke and Plattner, 1997) must be negligible according to balance calculations, whereas the effects of cytosolic Ca^{2+} -binding proteins may by far predominate (Plattner and Klauke, 2000).

Does the theoretical possibility exist that PtSERCA–SSG1 molecules could be non-functional? This would be difficult to disprove as in our system overexpression due to transfection cannot easily be controlled with individual cells (as used for fluorochrome imaging) without GFP tagging. However, this aspect seems unlikely because GFP is attached remote from any functional domains relevant for pumping activity (see also *Results*).

Experimental procedures

Materials

Restriction enzymes come from New England Biolabs. ABs against GFP are from Clontech Laboratories. Gold conjugates used are as specified by Momayezi *et al.* (2000). All reagents and solvents used are of analytical grade.

Cell cultures and autogamy induction

Wild-type 7S cells are cultivated either in sterile organic medium or in bacterized medium with *Enterobacter aerogenes* added as a food supply. Cells are used either after spontaneous autogamy or autogamy is induced by starving. Autogamy is registered by macronuclear fragmentation applying <50 µM Hoechst stain 33342 (S. Zitzmann, K. Hauser and H. Plattner, unpublished observation). Appropriate cells are transfected after 3–4 fissions after autogamy by microinjection of constructs into the macronucleus which enables clones to be obtained from over 20 cell fissions (Berger and Rahemtullah, 1990).

Isolation of the *PtSERCA2* gene

The coding sequence for *PtSERCA2* is obtained in the same way as already described for *PtSERCA1* (Hauser *et al.*, 1998). Accession numbers (EMBL) are as follows: Y17469 for *PtSERCA1* and AJ272209 for *PtSERCA2*.

DNA sequencing

Plasmid DNA is purified by the Wizard Plasmid Miniprep (Promega) and used as template for sequencing with the ¹⁷Sequencing Kit (Pharmacia) and multiple primers (synthesized by MWG-Biotech). Sequencing is carried out by the dideoxy method (Sanger *et al.*, 1977).

Construction of plasmids

Construction of pPXV–SSG1 and pPXV–SSG2. The nomenclature we use is explained in the *Results* section. The two open reading frames (ORFs) of *PtSERCA1* and *PtSERCA2* are amplified by PCR using the primers specified below and the cDNA containing plasmids C6 (for *PtSERCA1*) and C2 (for *PtSERCA2*) as templates.

Because in both plasmids C6 and C2 the first 21 nucleotides of the corresponding 5' end of the coding sequence are missing, sense primers for PCR are designed to insert the missing nucleotides according to the isolated genomic sequences together with a *SpeI* restriction site in front of the start ATG for facilitated cloning. Antisense primers introduce an *AgeI* restriction site immediately in front of the stop codon ATG at the 3' end of the ORFs. This restriction site is used to fuse the GFP ORF in frame to the C-terminal end of *PtSERCA*. For both isoforms, the same primers are used for PCR amplification because there is no difference between the two sequences in the regions concerned. Primer sequences are as follows: sense primer,

5'-GAATTCGGCAGGAGACTAGTATGGCAGAAATTGACT-TGAATTAGCCATTTTCATGCATATCCCC-3' (bold, *SpeI* restriction site; underlined, inserted nucleotides for the missing 5' end); antisense primer 5'-GATTTCTCA-TACCGGTTTCTTCCTCTCCT-3' (underlined, stop codon complementary sequence; bold, *AgeI* restriction site). After amplification, fragments are purified and cleaved with the restriction endonucleases *SpeI* and *AgeI*.

GFP ORF is amplified from pGFP-C1 (Clontech Laboratories; accession no. U19280), which contains wild-type GFP. In a two-step PCR, the ORF is amplified and modified into the GFPmut1 form (Cormack *et al.*, 1996), as described by Hauser *et al.* (2000). This amplified GFPmut1 form contains an *AgeI* restriction site 12 nucleotides upstream of the start codon ATG, a newly introduced TGA stop codon at the 3' end of the ORF and a *XhoI* restriction site 11 nucleotides downstream from the stop codon. After amplification, the fragment is purified and cleaved with *AgeI* and *XhoI*.

The two fragments (*PtSERCA* and GFPmut1) are ligated in one step into the expression vector pPXV (Haynes *et al.*, 1995), which is cut with *SpeI* and *XhoI*. The resulting vectors are called pPXV–SSG1 and pPXV–SSG2. They contain the ORFs of *PtSERCA1* and *PtSERCA2*, respectively, fused in frame to the 5' end of GFPmut1.

Construction of pPXV–SGS1 and pPXV–SGS2. The open reading frames of *PtSERCA1* and *PtSERCA2* contain a *HindIII* restriction site at position 1625 (referring to A of ATG start codon as 1). This site is used for insertion of the GFPmut1 ORF.

The GFPmut1 ORF is amplified by PCR from a modified plasmid pGFP-C1 which contains the GFPmut1 form (see above). Primer sequences are as follows: sense primer, 5'-GATCCGCTAGCGCACC GGTTAAGCTTTAATGGGTAAAGG 3' (underlined is a *NheI* restriction site used for cloning, bold are nucleotides different from the template, bold and italic is a newly introduced *HindIII* restriction site, italic is start codon ATG from the GFPmut1 ORF); antisense primer covers nucleotides 1348–1318 of pGFP-C1 which represent the 3' end of GFPmut1 ORF without a stop codon. The amplification product is purified, digested with *NheI* and *XhoI* and cloned into pGFP-C1, which has before been cleaved with *NheI* and *XhoI* to remove the wild-type GFP ORF. This plasmid is called pGFP–*HindIII*. From this plasmid, the GFPmut1 ORF is released by cutting with *HindIII*.

The plasmids pPXV–SSG1 and pPXV–SSG2 are cut with *HindIII* and *XhoI* to remove the 3' end and the GFPmut1 ORF. To recover the original 3' ends of the *PtSERCA* ORFs, the cDNA clones C6 (Hauser *et al.*, 1998) and C2 are cut with *HindIII* and *XhoI* and the obtained fragments are ligated into the *HindIII*–*XhoI* cut pPXV–SSGs. The resulting plasmids pPXV–*SERCA1* and pPXV–*SERCA2* are cut with *HindIII* and the GFPmut1 fragment is inserted. The obtained plasmids are called pPXV–SGS1 and pPXV–SGS2. Correctness of any sequence obtained by mutation, cloning or PCR is confirmed by sequencing.

Transfection by microinjection and CLSM analysis

Cells are injected with 5–10 pl of a solution with ~10⁵ copies pl⁻¹ of the respective genes, contained in

10 mM tris-HCl buffer, pH 7.2, into the macronucleus (which may fragment, but then reassembles) of cells after autogamy performance to maintain the macronuclear genome. Each cell is then transferred in 200 μ l fresh medium and the resulting clones are followed for up to 15 days by CLSM (type Odyssey from Noran), attached to an inverted microscope, type axiovert 100TV (Zeiss), equipped with the same filter set as used below for Fluo-3.

Analysis of $[Ca^{2+}]$ transients during stimulated exocytosis

Cells strongly expressing PtSERCA-SSG1 (see *Results* for nomenclature) in the cortical calcium stores, ASs, are injected with Fluo-3, stimulated by aminoethyl-dextran (AED; Plattner *et al.*, 1985) at $[Ca^{2+}]_o \sim 50 \mu M$ and analysed in the CLSM by the f/f_o ratio method ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 520$ nm) as specified by Erxleben *et al.* (1997) and Klauke and Plattner (1997).

Affinity- and immunofluorescence staining

Non-transfected cells were loaded with DiOC₁₈ by injection in an oil droplet as described previously (Plattner *et al.*, 1997b). Immunostaining with ABs against calsequestrin and calreticulin was also carried out as reported previously (Plattner *et al.*, 1997b). DiOC₁₈ was analysed by CLSM and immunostaining by conventional fluorescein isothiocyanate (FITC) fluorescence.

EM immunogold labelling

Individual transfected cells were fixed for 1 h at 0°C in 8% formaldehyde ($\pm 0.1\%$ glutaraldehyde) + 1 mM CaCl₂ in 100 mM PIPES buffer, pH 7.2, followed by ethanol dehydration series and embedding in Unicryl for 48 h UV polymerization at -35°C. Ultrathin sections are labelled with anti-GFP ABs, followed by goat anti-rabbit AB- or protein A-gold conjugates and EM evaluation as described by Momayez *et al.* (2000).

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