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Molecular characterisation of the smooth endoplasmic reticulum Ca^{2+}-ATPase of *Porcellio scaber* and its expression in sternal epithelia during the moult cycle

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Summary

The anterior sternal epithelial cells of the terrestrial isopod *Porcellio scaber* transport large amounts of calcium during the formation and resorption of intermittent calcium carbonate deposits. Recent investigations on epithelia involved in mineralisation processes suggest a role of the smooth endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in transcellular calcium transport. We present the first molecular characterisation of a SERCA within a crustacean mineralising epithelium. We cloned the SERCA from a cDNA library of the anterior sternal epithelium and used *in situ* hybridisation to compare the expression of the SERCA mRNA between three different moulting stages. The full-length SERCA cDNA has an open reading frame of 3006 nucleotides. The deduced 1002 amino-acid polypeptide has a predicted molecular mass of 109.7 kDa and 87% identity to the SERCA of *Procambarus clarkii* axial muscle isoform. *In situ* hybridisation confirmed expression within the anterior sternal epithelium and revealed an increase in SERCA mRNA abundance from the non-transporting, early premoult stage to the calcium transporting, late premoult and intramoult stage. The results support previous indications of a contribution by the smooth endoplasmic reticulum to transcellular calcium transport and suggest a transcriptional regulation of SERCA activity.

Key words: *Porcellio scaber*, smooth endoplasmic reticulum, Ca^{2+}-ATPase, SERCA, biomineralisation, Isopoda, Crustacea, epithelial calcium transport.

Introduction

Recent investigations on specialized epithelia suggest a role of the smooth endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in epithelial Ca^{2+}-transport (Franklin et al., 2001; Hagedorn and Ziegler, 2002). These epithelia are involved in quick mineralisation processes in which epithelial Ca^{2+}-transport predominantly follows a transcellular route (transport through the cells) (Hubbard, 2000; Roer, 1980; Wheatly, 1997; Ziegler, 2002), rather than a paracellular pathway in which Ca^{2+} would move extracellularly across the apical cell contacts and between the epithelial cells along its electrochemical gradient (Bronner, 1991). In the transcellular pathway, Ca^{2+} enters the cells passively across the plasma membrane at one side of the cells and is actively extruded on the other side (Ahearn and Franco, 1993; Ahearn and Zhuang, 1996; Roer, 1980). The most critical step in transcellular epithelial Ca^{2+}-transport, however, is the Ca^{2+} transport within the cells, through the cytoplasm from one side to the plasma membrane on the opposite side. Sustained elevated concentrations of Ca^{2+} within the cytoplasm would interfere with the multiple regulatory functions of cytosolic free Ca^{2+} signals. In addition, such elevated concentrations of ionized calcium can lead to cell damage and even cell death (Berridge, 1993). How such a toxic rise is prevented during epithelial Ca^{2+}-transport is still unknown. Simkiss (1996) proposed a model in which organelles, e.g. the smooth endoplasmic reticulum (SER), function as a transient calcium store allowing vectorial bulk flow through epithelial cells without toxic effects. Within cells the SER generally functions as source and sink for cytosolic Ca^{2+} signals, with the inositol 1,4,5 trisphosphate (IP_3)-receptors and/or ryanodine-receptors releasing Ca^{2+} and the SERCA pumping Ca^{2+} back in a regulated fashion (Hussain and Inesi, 1999). Previous investigations on the anterior sternal epithelium (ASE) of *Porcellio scaber* (Hagedorn and Ziegler, 2002; Ziegler, 2002) suggest that the SER contributes to transcellular calcium transport.

Like most crustaceans, *P. scaber* has a calcified cuticle, which is moulted regularly to allow for growth of the animal. During premoult the ASE transports Ca^{2+}, originating from the posterior cuticle to form large $CaCO_3$ deposits located within the ecdysial gap of the first four anterior sternites (Messner, 1965). Since isopods moult the posterior half of the body first, followed by the anterior half, these deposits are degraded by the ASE within 24 h after posterior moult and used for the

mineralisation of the new posterior cuticle (Steel, 1993). Ultrastructural investigations have shown that during the formation and degradation of the $CaCO_3$ deposits the ASE is differentiated for ion transport (Glötzner and Ziegler, 2000; Ziegler, 1996). Electron microprobe analysis of shock-frozen and freeze-dried cryosections of the ASE revealed that the increase in cytoplasmic calcium is not uniform across the cell, but occurs by increasing the number of areas having high calcium concentrations, which are presumably the SER (Ziegler, 2002). This hypothesis was supported by an *in situ* assay indicating an increase in SERCA activity during epithelial Ca^{2+}-transport (Hagedorn and Ziegler, 2002). These results are in agreement with previous findings of a correlation between enamel mineralisation and activity of a SERCA in vertebrate dental enamel cells (Franklin et al., 2001).

Several SERCA isoforms have been identified in the crustaceans *Artemia franciscana* (Escalante and Sastre, 1993) and *Procambarus clarkii* (Chen et al., 2002; Zhang et al., 2000a), but the SERCA isoform involved in epithelial Ca^{2+}-transport has not yet been characterised, nor is it known if the rise in SERCA activity observed in the ASE is accompanied by an increase in mRNA abundance. In order to increase our knowledge about SERCA expression in crustacean Ca^{2+} transporting epithelia, we identified the SERCA cDNA of the ASE of *P. scaber* and tested whether the SERCA mRNA level is upregulated during epithelial Ca^{2+}-transport. Our results show that the SERCA of the ASE has a very high similarity to the axial muscle isoform of *Procambarus clarkii* and *Artemia franciscana*. In contrast to the finding in dental enamel epithelium, we observed an increase in SERCA mRNA abundance from the non-transporting to the calcium-transporting stages.

Materials and methods

Animals

Porcellio scaber (Latrielle) were kept and bred as described earlier (Ziegler, 1996). We used animals from three different moulting stages. Animals during the late premoult stage were defined by their well-developed sternal deposits. Animals in intramoult (the period between posterior and anterior moult) were used after the $CaCO_3$ deposits had been partly degraded to ensure that resorption of the deposits was taking place. *P. scaber* moults every 6 weeks (Drobne and Štrus, 1996) and therefore has no defined intermoult stage. Instead we used animals 6–8 days after the anterior moult as a control stage. During this early premoult stage, apolysis of the old cuticle has already occurred in some animals (Ziegler, 1996) and the lack of $CaCO_3$ deposits indicates that no net calcium transport occurs across the sternal epithelium.

Isolation of a partial SERCA cDNA sequence from anterior sternal epithelium total RNA

Total RNA was extracted from anterior sternal epithelium (ASE) tissue under RNAse-free conditions (Chomczynski and Sacchi, 1987) using reagents obtained from Promega Corporation (Madison, USA). ASE of 20 animals in the late premoult stage were carefully dissected and pooled in *RNA*later (Ambion, Houston, USA). Reverse transcription of poly$(A)^+$ mRNA was performed with oligo-dT primer and Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany). On the basis of published SERCA sequences we designed the degenerate forward SERCA-F1 (5′-TGG GCN AT(A/C/T) AA(C/T) AT(A/C/T) GGN CA(C/T) TT-3′) and reverse SERCA-R1 (5′-ATN GC(G/A) TT(T/C) TT(T/C) TTN GCC AT-3′) primers. Using the polymerase chain reaction (PCR) we amplified a putative SERCA cDNA fragment. Following separation on agarose gels (Fig. 1A), this PCR product was extracted from the gel slices (Qiagen QiaQuick, Valencia, USA) and sequenced using SERCA-F1 and SERCA-R1 by the dideoxynucleotide method (Sanger et al., 1997) at the Marine DNA Sequencing Center of Mount Desert Island Biological Laboratory. A BLAST search (Altschul et al., 1997) of GenBank confirmed close matches to published SERCA sequences of other species.

Amplification of full-length SERCA cDNA

An amplified, adapter-ligated cDNA pool (‘library’) was constructed from ASE total RNA, as described by Matz (2002). Using the primer 5′-CGC AGT CGG TAC $(T)_{13}$-3′, reverse transcription of 1 μg total RNA from ASE tissue was done with Powerscript reverse transcriptase (Clontech, Palo Alto, USA). Second-strand synthesis was performed with DNA polymerase I (0.3 U/μl), RNase H (0.01 U/μl) and *E. coli* DNA ligase (0.06 U/μl). An adaptor consisting of the 5′-CGA CGT GGA

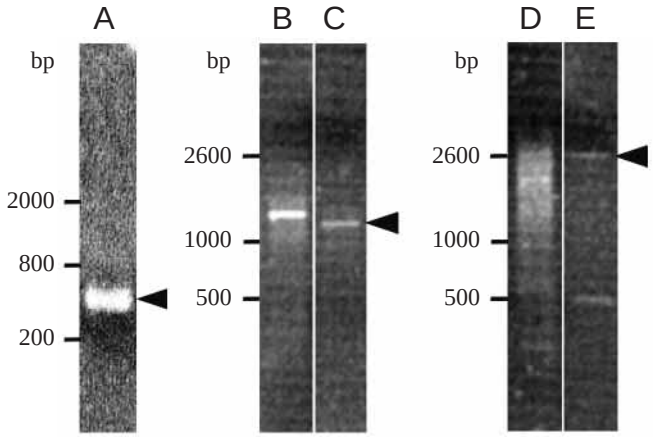

Fig. 1. Polymerase chain reaction amplification of the epithelial SERCA cDNA fragment. (A) Amplification of a 513 bp band from total anterior sternal epithelium RNA using the degenerate primers SERCA-F1 and SERCA-R1; 5′-RACE (B,C) and 3′-RACE (D,E) were performed with the constructed adaptor-ligated cDNA pool to clone the full-length SERCA cDNA. The specific primers GSP1 and DAP-st11 (B) and the nested primers GSP2 and DAP (C) were used to amplify the 5′-end. GSP3 and DAP-TRsa (D) and the nested primers GSP4 and DAP (E) were used to amplify the 3′-end. The indicated bands at approx. 500 bp, 1.2 kbp and 2.6 kbp were extracted, purified, reamplified and sequenced. See Materials and methods for details.

CTA TCC ATG AAC GCA ACT CTC CGA CCT CTC ACC GAG TAC G-3′ and the complementary 5′-C GTA CTC GGT-3′ oligonucleotides was ligated to the double-stranded cDNA. The double-stranded cDNA pool was amplified using the Advantage 2 PCR-kit (Clontech) with the adaptor primer (TRsa: 5′-CGC AGT CGG TAC $(T)_{13}$-3′) and the distal adaptor primer (DAP: 5′-CGA CGT GGA CTA TCC ATG AAC GCA-3′). PCR conditions were: 94°C for 40 s, 63°C for 1 min, 72°C for 2.5 min for 13 cycles. A sample was separated in a 1% agarose gel electrophoresis to confirm the presence of cDNA.

The method described by Matz (2002) was used to amplify full-length cDNAs, with two rounds of step-out and nested PCR. Primers were used at a concentration of 100 nmol l^{-1}. The gene-specific primers were synthesized on the basis of the previously obtained sequence of the *Porcellio scaber* SERCA (GenBank accession no. AF375959). For amplification, Advantage 2 Polymerase (Clontech) was used with the supplied amplification buffer and dNTPs were added at a concentration of 0.75 μmol l^{-1}. As a template, 1 μl of the cDNA library (diluted 1:50) was used for a 20 μl PCR mixture. Agarose gels of the PCR products are shown in Fig. 1B–E and the main strategy of the procedure is outlined in Fig. 2. For the first PCR round, the antisense gene-specific primer for 5′-RACE was GSP1 (5′-AAT CGT TTC CTT CTG CTT TGT CT-3′) and the adaptor-specific sense primer DAP-st11 (5′-CGA CGT GGA CTA TCC ATG AAC GCA ACT CTC CGA CCT CTC ACC GA-3′). The sense specific primer for 3′-RACE was GSP3 (5′-CTT GCT GTA GCC GCT ATT CCT-3′) and the adaptor-specific reverse primer DAP-TRsa (5′-CGA CGT GGA CTA TCC ATG AAC GCA CGC AGT CGG TAC T_{13}). We used 28 cycles under the following conditions: 94°C for 40 s, 65°C for 1 min and 72°C for 2.5 min.

For the second round of PCR, the preceding 5′- and 3′-RACE reactions were diluted 1:50 with H_2O and 1 μl of the diluted mix was used in a total 20 μl mixture. The adaptor-specific primer DAP was added to both 5′- and 3′-RACE products. The nested, reverse gene-specific primer GSP2 (5′-GGA AGA GAA CGC ACA ATA GCA T-3′) was used for 5′-RACE and the nested, forward gene-specific primer GSP4 (5′-ATG CTA TTG TGC GTT CTC TTC C-3′) for 3′-RACE. PCR conditions were as described above and amplification was done for 16 cycles. A sample (15 μl) of each PCR round was electrophoretically separated on a 1% agarose gel. The bands detected in the 5′- and 3′-RACE reaction were cut out of the gel, the DNA cleaned using the DNA-extraction kit (Qiagen) and sequenced by SequiServe (www.sequiserve.de). More SERCA-specific primers were designed to sequence the open reading frames of the fragments. The cDNA sequence was analysed using BioEdit (Hall, 1999) and compared with published sequences in GenBank using the BLAST algorithm (Altschul et al., 1997).

Phylogenetic analysis

ClustalW (Thompson et al., 1994), as implemented in the Biological Workbench (http://workbench.sdsc.edu), was used to align full-length SERCA sequences. Phylogenetic and molecular evolutionary analyses were conducted with MEGA version 2.1 (Kumar et al., 2001) using the neighbour joining algorithm (Saitou and Nei, 1987). Species and their GenBank accession numbers are given in Fig. 7.

In situ *hybridisation of SERCA mRNA*

The probe was a biotin-labeled 23-mer oligonucleotide (GA GCT GGA TCA TTG AAG TGA CCA) complementary to a fragment of the mRNA encoding the *Porcellio* SERCA (Fig. 3, nucleotides 977–1000). An oligonucleotide with the antisense sequence of the Na^+/Ca^{2+}-exchanger (AGA GGT CGT GTC ATG GTT CCG) was used for control experiments (Ziegler et al., 2002). All probes were labeled with two biotins and were synthesized by Dr M. Hinz (University of Ulm). A total of nine sternites from the early premoult, late premoult and intramoult stages (3 animals at each moulting stage), including the ASE and, as a control, the ganglial nervous tissue, were fixed for 1 h

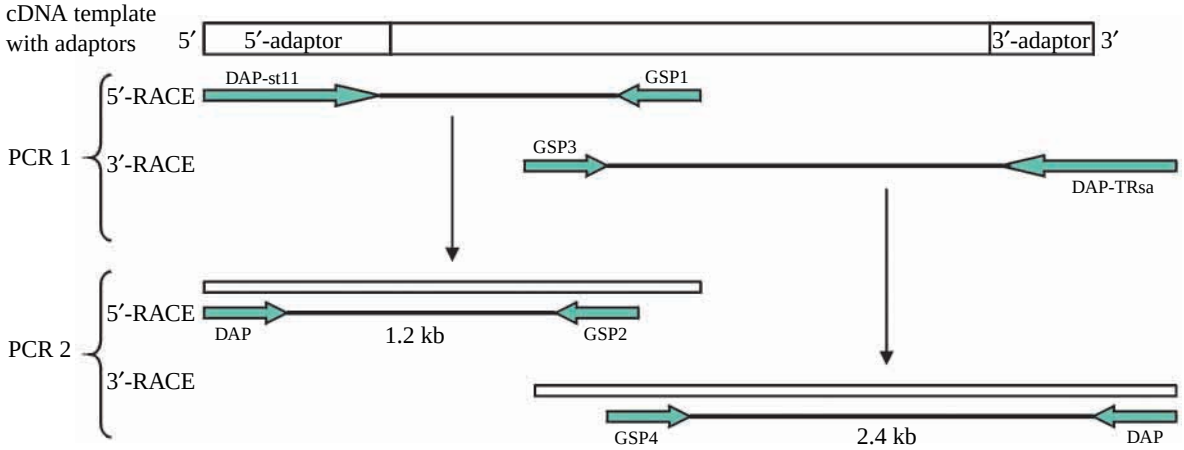

Fig. 2. Main strategy of the 5′ and 3′-RACE reactions according to the procedure of Matz (2002). Two sequential rounds of polymerase chain reaction (PCR) were performed with adaptor- and sequence-specific primer. Primer sequences and reaction conditions are described in Materials and methods.

in a mixture of 4% paraformaldehyde, 0.25% glutaraldehyde in 0.1 mol l^{-1} cacodylate buffer, pH 7.3. The sternal epithelia were immersed in 2.3 mol l^{-1} sucrose in 0.1 mol l^{-1} sodium cacodylate buffer (SCB) for 90 min, mounted on aluminium rods and frozen in liquid nitrogen. Semithin (0.7 μm) sections were cut sagittally with an Ultracut S microtome equipped with a FCS cryochamber (Leica, Wien Austria), using glass knives and an antistatic device (Diatome, Biel, Switzerland) at temperatures of –70°C for the specimen holder, knife and cryochamber. Sections were transferred to lysine-covered glass slides (Polyprep, Sigma), thawed and washed with Tris-buffered saline (TBS). The hybridisation procedure was done as described in detail previously (Ziegler et al., 2002). The labelled probes were applied at a concentration of 1 ng μl^{-1} and the sections hybridized overnight at 50°C. Detection was done using the TSA™ Cyanin 3 System (NEN™ Life Science Products, Boston, USA) employing horseradish peroxidase (HRP)-conjugated streptavidin and the fluorescent HRP-substrate Cy3-Tyramide, diluted 1:50 in 1× amplification buffer, for 10 min in the dark. After washing, the sections were mounted in 20% glycerol in 80% TBS and examined with a fluorescence microscope (Axiophot, Zeiss, Jena, Germany). All solutions were prepared with diethylpyrocarbonate (DEPC)-treated water and all glassware and instruments had been sterilized.

Results

Using degenerate primers, we amplified a cDNA fragment of 513 base pairs (bp) from total ASE RNA. The corresponding protein sequence had 90% identity to the axial muscle SERCA isoform of *Procambarus clarkii* (GenBank accession no. AAB82291). Using specific primers deduced from this fragment and the amplified cDNA pools from the ASE as a template (see Materials and methods) we amplified 1.2 kb and 2.6 kb putative SERCA cDNA-fragments (Fig. 1B–E). Sequencing and alignment of overlapping sequences revealed a 3333 nucleotides (nt) long cDNA with an open reading frame of 3006 nt (GenBank accession no. AY158663) encoding a putative 1002 amino acid polypeptide with a predicted molecular mass of 109.7 kDa and a predicted isoelectric point of 5.05. The cDNA sequence with parts of the 5′ and 3′ flanking untranslated region and the putative amino acid sequence are shown in Fig. 3. The putative start codon is part of a Kozak consensus sequence (CCATCATGG) with a T at position –2 instead of a C. The sequence shows a purine at position –3 and a G at +4, necessary for an efficient translation initiation (Kozak, 1991). Several in-frame stop codons are found prior the putative start site and after the initial stop codon. No evidence was found for additional isoforms or alternative splicing products.

The deduced full-length amino acid sequence showed high identity with invertebrate SERCAs, e.g. 87% with the SERCA-sequence of *Procambarus clarkii* (GenBank accession no. AAB82291), 80% with the SERCA of *Drosophila melanogaster* (AAB00735) and 79% with the SERCA of *Artemia franciscana* (CAA35980) (not shown). The submitted sequence also had high identity to many vertebrate SERCAs, e.g. 71% to human SERCA1 (O14983), 71% to SERCA2 from chicken (QO3669) and 67% to human SERCA3 (Q93084). Putative functional residues are shown in Fig. 3. Hydrophobicity analysis of the amino acid sequence (Fig. 4) predicts ten transmembrane domains and a high similarity with other vertebrate and invertebrate SERCAs (for comparison, see Chen et al., 2002; Zhang et al., 2000a). *In situ* hybridisation revealed that the amount of SERCA mRNA in the anterior sternal epithelial cells of the early premoult stage was below the detection limit (Fig. 5A–D). A large increase in the SERCA mRNA signal occurs from the early premoult to the late premoult stage. The magnitude of the signal in the intramoult stage was similar to that in the late premoult stage (Fig. 5E–H). Varying amounts of unspecific binding occurred in the thick cuticle of early premoult animals. In the ganglial nervous tissue no change in the SERCA mRNA abundance was detected between moulting stages (Fig. 6A–F). Sections treated with the control probe were virtually devoid of any signal.

Discussion

To our knowledge, we present for the first time the molecular characterisation of a SERCA in a crustacean mineralising epithelium and changes in its mRNA abundance in Ca-transporting and non-transporting moulting stages. These results extend previous work on SERCA activity in the anterior sternal epithelium (ASE) of the terrestrial isopod *P. scaber* (Hagedorn and Ziegler, 2002), molecular characterisation and moult-related changes in SERCA mRNA expression in muscle tissues of the crayfish *Procambarus clarkii* (Chen et al., 2002; Zhang et al., 2000a), and on SERCA activity and mRNA expression in a vertebrate mineralising epithelium (Franklin et al., 2001).

Amplification of a full-length SERCA cDNA along with the *in situ* hybridisation results indicate that the sternal epithelium of *P. scaber* expresses a SERCA. The SERCA of the ASE of *P. scaber* is most similar to the axial muscle SERCA isoform of *Procambarus clarkii* (Zhang et al., 2000a), which shares 87% identical amino acids. Both are the same length, 1002 amino acids, and have identical putative functional domains, e.g. the phosphorylation site (Lee and East, 2001), the 63-67 KILLL residues (Fig. 3) known as the putative retention-signal for the ER/SR (Magyar and Varadi, 1990) and the residues that are thought to be involved in the formation of two Ca^{2+}-binding sites (Zhang et al., 2000b). However, the C and N termini of these proteins are less highly conserved. For example, within the first 27 amino acids of the *Porcellio* SERCA C terminus, 18 amino acids deviate from the corresponding *Procambarus* sequence, four of them from hydrophilic to hydrophobic residues ($S_4 \rightarrow A$, $Q_{10} \rightarrow A$, $E_{20} \rightarrow I$, $D_{26} \rightarrow A$). In the last 15 amino acids of the N terminus of the *Porcellio* sequence, 7 amino acids differ from the axial muscle SERCA sequence, including two hydrophobic residues becoming hydrophilic ($A_{997} \rightarrow E$, $I_{999} \rightarrow K$).

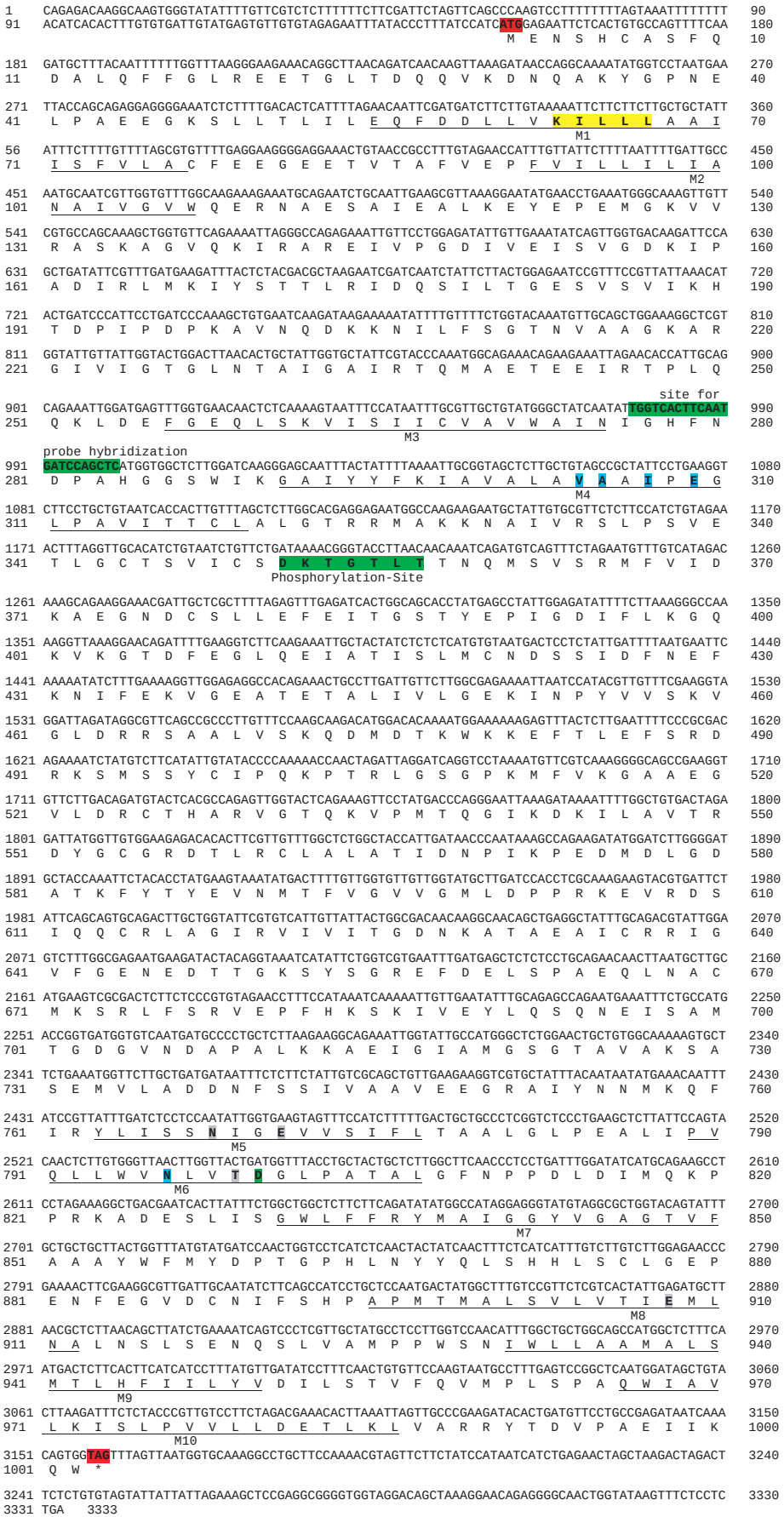

Fig. 3. Nucleotide (top) and deduced amino acid (bottom) sequences of the *Porcellio scaber* smooth endoplasmic reticulum Ca^{2+}-ATPase (SERCA) cDNA. Nucleotides and amino acids are numbered at the sides of the sequence. Start and stop (asterisk) codons are labelled in red. The KILLL-motif (yellow), phosphorylation site and site for probe hybridisation (green), and residues forming calcium-binding site I (grey) and binding site II (blue), and residue D_{800} (green) bridging sites I and II are labelled. Putative transmembrane regions (M1–M10) are underlined.

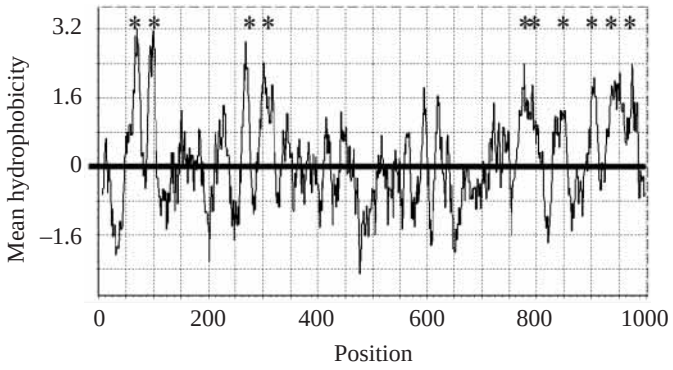

Fig. 4. Hydrophobicity plot of *Porcellio scaber* anterior sternal epithelium SERCA. Hydrophobicity analysis was done by the method of Kyte and Doolittle (1982) with a window of 12 residues using BioEdit. Asterisks indicate putative transmembrane regions.

The lack of any indication of other SERCA isoforms in the sternal tissue does not exclude their existence, particularly in other tissues of *P. scaber*. In addition to the axial muscle isoform already mentioned (Chen et al., 2002), a second cardiac-muscle tissue-specific SERCA isoform (AAB82290) has been found in *Procambarus clarkii*. The *Porcellio* SERCA is also highly homologous to the cardiac-muscle isoform, which has a hydrophobic N terminus containing an additional 18 amino acids, possibly forming an additional 11th transmembrane spanning region (Chen et al., 2002). Two isoforms have also been found in *Artemia franciscana* embryonic and adult tissue, where the 6-amino-acid N terminus in one isoform is substituted by 30 amino acids in the other. Escalante and Sastre (1995) showed that these isoforms arise from regulated expression by alternative tissue-specific promotors rather than expression from different genes.

Even though the arthropod SERCAs have a high similarity to vertebrate SERCAs, they generally cannot be classified as any of the vertebrate SERCA sequences. Vertebrate SERCAs are encoded by three different genes that are mainly expressed in a tissue-specific manner. The SERCA1 isoforms are mostly found in fast twitch muscle, SERCA2a in cardiac and slow twitch skeletal muscle, and the alternative splicing product SERCA2b is distributed ubiquitously. SERCA3, which occurs in epithelia and endothelia, has been found in three alternatively spliced products. As far as it is known, the arthropod SERCAs, including the new *Porcellio* SERCA sequence, are encoded by only one gene, giving rise to the hypothesis that one arthropod and the three vertebrate genes derived from a common ancestral gene (Escalante and Sastre, 1996). The phylogenetic tree in Fig. 7, showing the relationship between full-length arthropod and some vertebrate SERCAs, supports this hypothesis. It is of interest that the sequences of the Malacostraca *Porcellio* and *Procambarus* are more closely related to insects than to the branchiopod crustacean *Artemia*. The *Artemia* SERCA sequence differs from the Malacostraca and insect sequence by an insertion of three amino acids, $D_{83}E_{84}A_{85}$, and one amino acid at P_{381}. At present it is unknown whether this is specific to *Artemia franciscana*, or is an apomorph character of the Branchiopoda; however, it is worth noting that there is increasing controversy

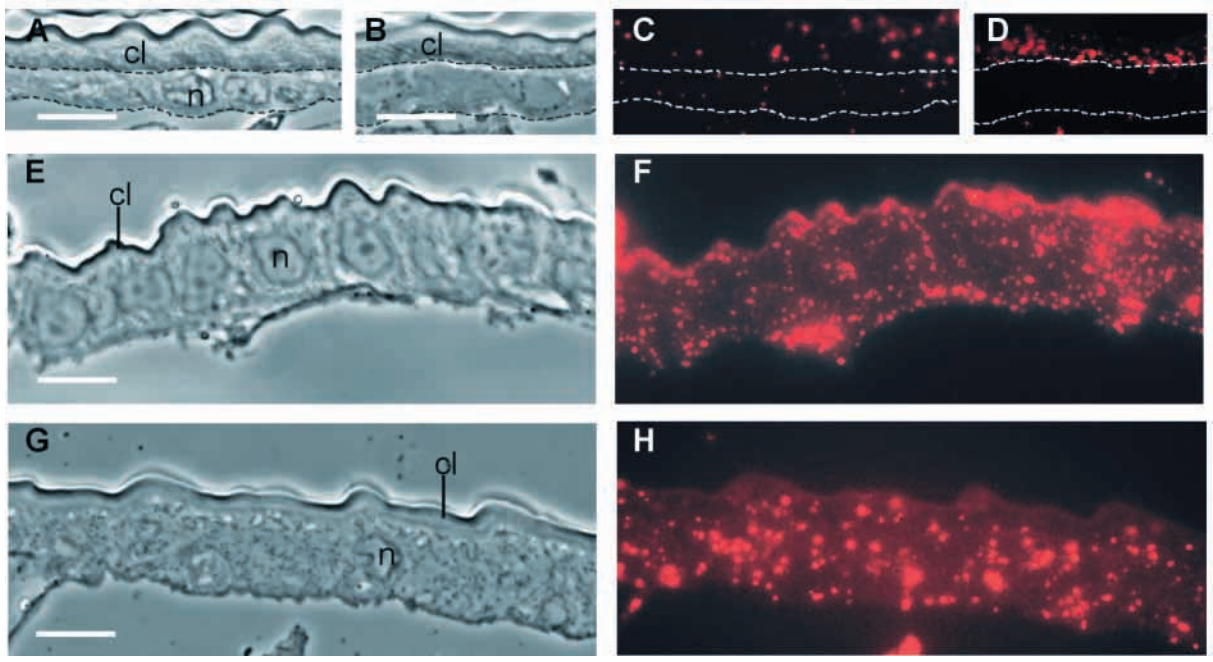

Fig. 5. *In situ* hybridisation of cryosections (0.7 μm thick) of the anterior sternal epithelium (ASE) of *Porcellio scaber* in three different moulting stages (A–D, early premoult; E,F, late premoult; G,H, intramoult) with specific biotinylated probes for SERCA mRNA. (A,B,E,G) Phase contrast micrographs; (C,D,F,H) corresponding fluorescence micrographs. cl, cuticle, n, nucleus. Scale bars, 10 μm. Broken lines in A–D outline the ASE.

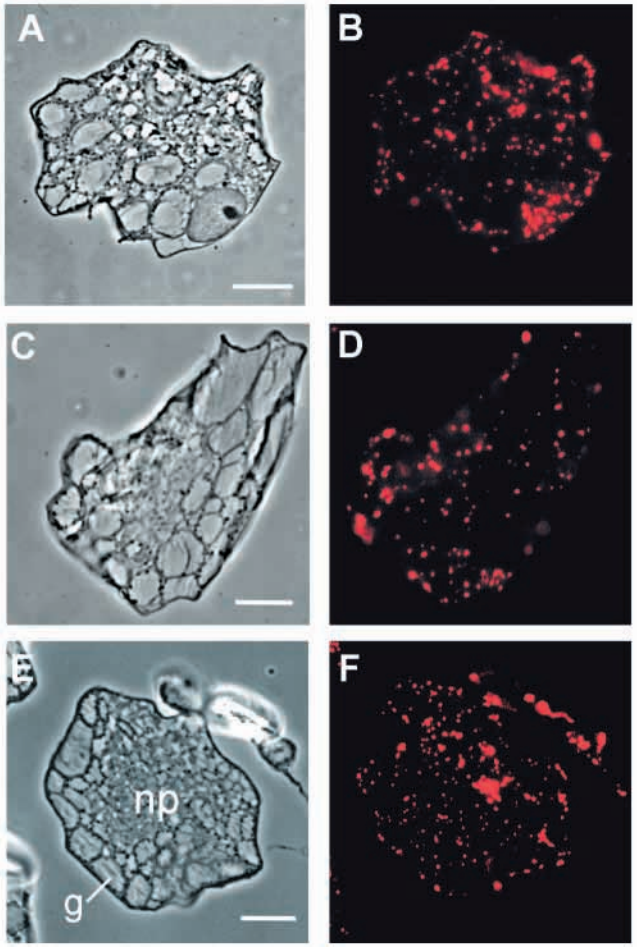

Fig. 6. *In situ* hybridisation of cryosections (0.7 μm thick) of ganglial nervous tissue from *Porcellio scaber* in three different moulting stages (A,B, early premoult; C,D, late premoult; E,F, intramoult) with specific biotinylated probes for SERCA mRNA. (A,C,E) Phase contrast micrographs; (B,D,F) corresponding fluorescence micrographs. np, neuropil; g, glia. Scale bars, 10 μm.

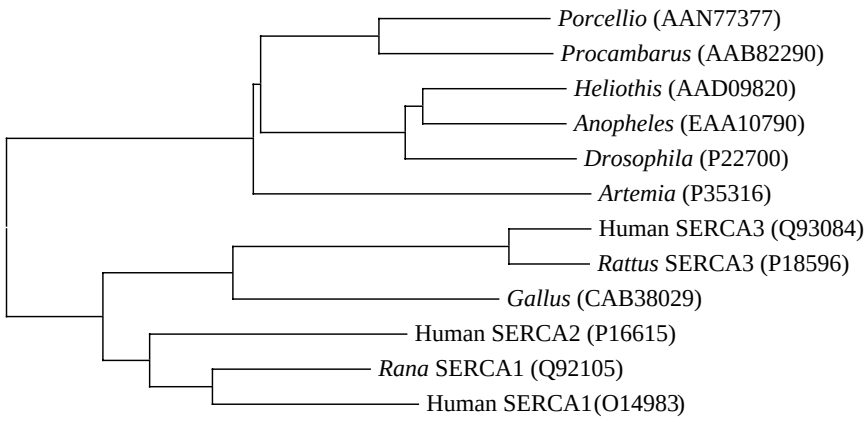

Fig. 7. Dendrogram of full-length arthropod and vertebrate SERCAs. Multiple alignment was completed using ClustalW and the neighbor-joining algorithm was then used to construct the tree with Mega2. Comparable results were obtained using related algorithms. GenBank accession numbers are given in parentheses.

about the monophyletic origin of Crustacea, and some hypotheses even propose that the Branchiopoda branched off the main stem of Crustacea before the insects. For a more detailed discussion of this issue, refer to Harzsch (2002) for example.

Variations of SERCA-mRNA abundance as a function of the crustacean moulting cycle as described here for the ASE of *P. scaber*, have already been demonstrated in *Procambarus* cardiac and abdominal axial muscle. These changes in SERCA mRNA could result from either regulated gene expression or variable mRNA stability. It has been shown that SERCA expression can be regulated by certain hormones and growth factors (for a review, see Hussain and Inesi, 1999). Such a connection between moulting stage and mRNA abundance therefore suggests that SERCA mRNA expression may be regulated by moulting hormones, for example 20-hydroxyecdysone (also suggested by Zhang et al., 2000a). Interestingly, the expression pattern of SERCA mRNA observed in the ASE of *P. scaber* is opposite to that observed in *Procambarus* muscle tissues. In the ASE, SERCA mRNA abundance increases from early premoult to late premoult and intramoult stages, but decreases in *Procambarus* from intermoult to pre-and postmoult stages (Zhang et al., 2000a). Thus, if the same moulting hormones regulate SERCA gene transcription, an increase of hormone concentration would induce opposite effects in the ASE of *P. scaber* and in *Procambarus* muscle. This hypothesis is supported by tissue-specific moult-related synthesis of actin protein in cheliped and walking leg muscle of crayfish (El Haj et al., 1992). A high 20-OH ecdysone titer in the haemolymph corresponds with an elevated actin expression in leg muscle and a decreased expression of actin in cheliped muscle. Possible explanations for these tissue-specific inverse effects include differential expression or activation of the ecdysteroid receptor, or, as suggested by Zhang et al. (2000a), a regulated accessibility of the receptor.

The ASE is specialized for ion transport since cuticle secretion is retarded within the anterior integument during the formation and resorption of the sternal $CaCO_3$ deposits (Messner, 1965; Ziegler, 1997). Therefore, the increase in mRNA abundance within the ASE from early premoult to late premoult and early premoult to intramoult suggests that the SERCA plays a role in epithelial Ca^{2+}-transport. This is supported by the lack of such increases in the ganglial nervous tissue, which indicates that the increase of SERCA mRNA abundance in the ASE is tissue specific, and not due to general changes of SERCA expression during the moult cycle. Our results are in accordance with previous analyses of the relative SERCA activity within permeabilised epithelial cells of the ASE employing the *in*

situ calcium oxalate technique (Hagedorn and Ziegler, 2002). SERCA activity was below the detection limit during early premoult and increased to detectable values in mid premoult (2 weeks after the anterior moult), and further increased significantly by factors of five and four from mid-premoult to late premoult and from mid-premoult to intramoult, respectively (Hagedorn and Ziegler, 2002). The concomitant increase in SERCA mRNA abundance suggests that the increase in activity of the protein is correlated with an increase in SERCA mRNA expression. This is in contrast to results obtained in vertebrate mineralising epithelial cells (Franklin et al., 2001). In the rat dental enamel epithelium, abundance of SERCA 2b isoform and the activity of the nonmitochondrial Ca^{2+}-ATPase within the microsome fraction is upregulated to high expression levels during enamel maturation, consistent with a role of SERCA in epithelial Ca^{2+}-transport. However, upregulation of SERCA was not evident at the mRNA level, suggesting post-transcriptional regulation (Franklin et al., 2001).

The nature of the SERCA's functional involvement in bulk flow of Ca^{2+} through mineralising epithelia is still unknown. Simkiss (1996) suggested that loading and discharging of membranous compartments, possibly SER cisterns, would lead to a vectorial translocation of Ca^{2+}. Such a translocation would result in only transient micromolar gradients of Ca^{2+}, which can be tolerated by the cells. However, the frequent discharge and reuptake of Ca^{2+}ions from and to the SER would require a large amount of energy. Another, more economic mechanism would be the diffusion of Ca^{2+} through the lumen of the SER, possibly facilitated by low-affinity Ca^{2+}-binding proteins. The latter mechanism is supported by the finding (Hubbard, 1996) that in rat dental enamel cells the Ca^{2+} binding proteins calreticulin and endoplasmin, both localized in the SER, are upregulated during epithelial Ca^{2+}-transport. However, this does not exclude a more passive function of the SER e.g. Ca^{2+} buffering to avoid high cytosolic Ca^{2+} concentrations during SER-independent Ca^{2+} transit mechanisms, such as vesicular transport (Nemere, 1992) or cosecretion of Ca^{2+} with matrix proteins (see Hagedorn and Ziegler, 2002, for a more comprehensive discussion).

An essential function of the SER during bulk calcium flow through the cytoplasm may be in conflict with its function in cellular Ca^{2+} signalling. Thus, in our previous study (Hagedorn and Ziegler, 2002) we suggested that different SERCA-isoforms and possibly SER subcompartments could avoid such conflicts. However, in the present study we did not find any evidence for the presence of multiple SERCA isoforms in the ASE, which raises the possibility that mechanisms connected with the SER Ca^{2+} release channels may separate putative functional conflicts.

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