Characterization and spatiotemporal expression of *orchestin*, a gene encoding an ecdysone-inducible protein from a crustacean organic matrix

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We report the characterization of a new gene encoding an acidic protein named Orchestin. This protein is a component of the organic matrix of calcium storage structures (calcareous concretions) elaborated during the moulting cycles of the terrestrial crustacean *Orchestia cavimana*. The deduced molecular mass of Orchestin is estimated to be 12.4 kDa and the pI to be 4.4, whereas the native protein extracted from the calcium deposits migrates as a 23 kDa band on SDS/PAGE. This discrepancy is probably due to the richness of this protein in acidic amino acids (approx. 30 %). The protein obtained by expressing the Orchestin cDNA in *Escherichia coli* presents an electrophoretic mobility of 25 kDa. Antibodies raised against the recombinant protein recognize the 23 kDa native protein exclusively among the organic-matrix components. Spatiotemporal analysis of the expression of the *orchestin* gene shows that it is expressed only in the storage organ cells when the concretions are elaborated during the premoult period and also, to a smaller extent, during the postmoult period. The translation products are expressed in accordance with the transcript expression during both the premoult and postmoult periods. Study of the hormonal stimulation of *orchestin* reveals that 20-hydroxyecdysone induces this gene as a secondary-response or late-response gene.

Key words: biomineralization, calcium metabolism, ecdysteroid, hormonal regulation, *Orchestia cavimana*.

INTRODUCTION

Arthropods, like many other invertebrates, possess an outer unstretchable skeleton, the cuticle, which they have to replace periodically to permit growth. In most crustaceans the cuticle, also called the carapace, is usually calcified. Thus the whole physiology of these animals, for example their growth, reproduction and calcium metabolism, is overlaid with moulting cycles, each characterized by the complete renewal of the exoskeleton.

The origin of the calcium used to mineralize the cuticle after moulting (ecdysis) depends on the way of life of the particular animal. Most aquatic crustaceans take calcium from the water, whereas terrestrial species have developed several storage strategies, of which food represents a minor contribution to calcification. The stored calcium originates essentially from the old cuticle, from which the mineral is partly reabsorbed during the premoult period. The storage is realized in different organs, in forms as diverse as granules in the haemolymph or the hepatopancreas, sternal plates at the level of some sternites, gastroliths in the stomach wall or concretions in diverticula of the midgut. All the calcified deposits correspond to biomineralized structures, and more precisely to biologically controlled mineralizations [1]. Most biomineralizations are composed of a mineral precipitated within an organic matrix synthesized by the tissues forming them [2]. The stored calcium is then reabsorbed to calcify the new cuticle rapidly. Thus crustaceans are a group of invertebrates in which calcium metabolism is particularly active because of the balance occurring during each moulting cycle between the exoskeleton and an endogenous and/or exogenous source of calcium [3–5].

Orchestia cavimana is a terrestrial amphipod that provides a dramatic example of such a calcium storage process. During the approx. 16-day-long premoult period, this land crustacean stores calcium in paired diverticula of the midgut, named the posterior caeca (PC) [6]. Calcium is precipitated as calcium carbonate within an organic matrix synthesized by the caecal epithelial cells in the lumen of the PC.

After ecdysis, the calcified transepithelial spherules, transiently elaborated to resorb the intraluminal concretions, are composed of calcium carbonate and calcium phosphate precipitated within an organic matrix [7].

Thus *Orchestia* is of great interest not only for studying a particular calcium-transporting epithelium, the centre of an important calcium turnover, but also for understanding the method of elaboration of biologically controlled calcifications.

One way of performing such a study was to analyse the organic-matrix components of these biomineralizations with the object of finding proteinaceous markers of the storage and reabsorption processes. An analysis of the organic matrix of the calcareous concretions, after decalcification in a buffer containing EDTA, revealed that this matrix is, as described previously [8], composed of two fractions with regard to their solubility in this buffer [9]. All the proteins of the soluble organic-matrix fraction (the SM fraction) are acidic, as are other SM proteins found in comparable invertebrate mineralized structures [10,11]. Furthermore, a comparative electrophoretic analysis of the two fractions led us to characterize an acidic polypeptide specific for

Abbreviations used: 20E, 20-hydroxyecdysone; GAMP, gastrolith matrix protein; PC, posterior caeca; RACE, rapid amplification of cDNA ends; RT–PCR, reverse-transcriptase-mediated PCR; SM, soluble matrix.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under the accession number AF124526.

the soluble fraction, which is not glycosylated but is able to bind calcium (as determined by the test of Maruyama et al. [12]) and whose molecular mass was estimated to be 23 kDa with regard to its electrophoretic mobility [9]. These interesting physicochemical properties led us to undertake the characterization of the gene encoding this protein.

Crustaceans forming calcium deposits during each moulting cycle in addition to the cyclic calcification of their cuticle also offer the opportunity to investigate the hormonal regulation of the elaboration and resorption of these biomineralizations. In particular, calcareous concretions synthesized by *Orchestia* in the lumen of specific storage organs composed of a unistratified epithelium constitute a very good and convenient model for such a prospect. The first family of hormones thought to be involved in the calcium metabolism events, essentially during the premoult period, is ecdysteroids that control moulting cycles in arthropods. Notably, 20-hydroxyecdysone (20E) is considered to be the active hormone in most of the insects and crustaceans. In *Orchestia* previous experiments have shown that 20E is involved not only in cuticle renewal but probably also in calcium metabolism [13,14]. Furthermore, ecdysteroid titre fluctuations during a moulting cycle have been determined by RIA and show an increasing rate during the premoult period, concomitant with the calcium storage [15].

In the present study we obtained the cDNA and the genomic sequences encoding the water-soluble organic-matrix protein characterized previously [9]. We decided to name this protein Orchestin. The study of the developmental expression of Orchestin was realized by Western blotting with antibodies raised against a recombinant protein obtained in a prokaryotic expression system. We also analysed the spatial and developmental expression of the gene encoding Orchestin and undertook the study of its hormonal regulation.

MATERIALS AND METHODS

Animals

O. cavimana specimens were reared in the laboratory in subaquatic terraria and staged as described by Graf [16]. To avoid age-related or sex-related differences, only male adult specimens were employed.

Protein extraction procedures

The whole PC proteins were extracted as described [9] with the same lysis buffer but devoid of EDTA. The calcareous concretion matrix proteins were isolated as described [9]. These two sets of proteins were then analysed by SDS/PAGE as described by Laemmli [17] in a 12.5% (w/v) polyacrylamide slab gel.

The 23 kDa polypeptide, which appeared as a unique spot after two-dimensional electrophoresis [9], was eluted from the polyacrylamide gel with NH_4HCO_3 [18]. To concentrate the protein and remove any SDS, the eluate was diafiltered seven times with ultrapure water with Nanosep™ 10K microconcentrators (Pall Filtron Corporation).

Protein concentrations were determined by the Bradford method [19].

Protein microsequencing

The matrix proteins separated by SDS/PAGE were electroblotted on to a PVDF membrane as described by Towbin et al. [20]. The polypeptide band containing Orchestin was detected by staining with Coomassie Blue and excised from the membrane. The polypeptide (10 μg) was subjected to enzymic digestion *in situ* by trypsin or Lys-C endoprotease (Boehringer) as described [21]. The resulting peptides were separated by reverse-phase HPLC on a Vydac C_{18} column. Sequencing of the N-terminal polypeptide portion or of internal digest fragments was performed with an Applied Biosystems Model 470 Gas-Phase Sequencer in accordance with the manufacturer's recommendations. The sequences of the N-terminal portion and of two internal peptides were determined.

RNA extraction from PC epithelium and poly(A)$^+$ RNA purification

Approximately 20 PC pairs were removed from animals during the calcium storage period and homogenized in 2 ml of lysis buffer containing 2% (w/v) SDS, 0.1 M Tris/HCl, pH 9.5, 0.2 M EDTA, 5% (v/v) ethanol, 0.2 mg/ml proteinase K (Sigma) and 0.4 mg/ml heparin (Sigma). After several extractions in phenol and phenol/chloroform (1:1, v/v), RNA species were precipitated with ethanol and the RNA pellet was dissolved in ultrapure water. The poly(A)$^+$ RNA was purified by affinity with oligo(dT)-cellulose (Sigma) [22]. The amount and purity of RNA from each sample were estimated by spectrophotometric measurements at 260 and 280 nm.

Reverse-transcriptase-mediated PCR (RT–PCR) and cDNA cloning

Poly(A)$^+$ RNA (1 μg) was reverse-transcribed with oligo(dT) as primer and 200 units of SuperScript™ II reverse transcriptase (Life Technologies) in accordance with the manufacturer's recommendations. The cDNA was amplified by PCR with the degenerate forward primer 5′-GAYGAYGAYGARGARUCN-GAYGAYGA-3′, corresponding to the presumed N-terminal peptide sequence of the protein [9], and oligo(dT) as a reverse primer. Each cycle comprised 1 min at 94 °C, 1 min at 48 °C and 2 min at 72 °C; the last cycle was a 15 min extension step at 72 °C. Reaction was performed in a thermal cycler (PTC-200; MJ Research) in 50 μl of PCR buffer [20 mM Tris/HCl (pH 8.4)/50 mM KCl] containing each primer at 2 μM, 0.2 mM deoxynucleotides, 1.5 mM $MgCl_2$ and 1 unit of *Taq* polymerase (Life Technologies). The PCR products were separated on an agarose gel and analysed by Southern blotting on a nylon membrane (Amersham) under alkaline conditions. The cDNA of interest was detected by hybridization with pools of degenerate oligonucleotides (^{32}P-labelled by T4 polynucleotide kinase with [γ-^{32}P]ATP deduced from the first internal peptide sequence of Orchestin. The hybridization was performed under the same conditions used for Southern blot analysis (as described below) with minor modifications. The hybridization temperature was 45 °C and the membrane was washed in 2 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% SDS. The 800 bp fragment detected was subcloned into T/A cloning pMOSblue (Amersham). cDNA species from several clones were sequenced on both strands.

5′ Rapid amplification of cDNA ends (RACE) and Orchestin cDNA cloning

To obtain the 5′ end of the cDNA, 5′ RACE was performed. Poly(A)$^+$ RNA (1 μg) was reverse-transcribed with a specific primer (5′-TGGGCGCGACTGATCTTG-3′) and the first-strand cDNA was poly(C)-tailed with terminal transferase (Life Technologies). The 5′ cDNA end was amplified by PCR as described above with a nested second specific primer (5′-GGGC-TCTCCTGCGAGTCATC-3′) and an oligo(dG/dI). The 400 bp fragment obtained was subcloned into T/A cloning pMOSblue (Amersham) and several clones were sequenced on both strands.

The full-length cDNA sequence was then verified with the use of oligonucleotides corresponding to the 5′-end cDNA from clone B (+1 to +22; see Figure 1) and oligo(dT) as a reverse primer, and after subcloning (clone C) into T/A cloning pMOS blue (Amersham). Several clones were sequenced on both strands.

Isolation of genomic DNA

Adult animals were ground in homogenization buffer [10 mM Tris/HCl (pH 7.5)/60 mM NaCl/0.15 mM spermine/0.15 mM spermidine/5 % (w/v) sucrose/10 mM EDTA/1 mg/ml proteinase K/0.1 mg/ml RNase A] and incubated for 1 h at 65 °C after the addition of an equal volume of lysis buffer [100 mM Tris/HCl (pH 9.5)/0.5 M EDTA/1 % (w/v) SDS]. After several extractions in phenol and phenol/chloroform, genomic DNA was precipitated with ethanol and the DNA pellet was dissolved in ultrapure water.

Genomic library construction and screening

A genomic library was made by using the λ EMBL3/*Bam*HI kit from Stratagene. Genomic DNA was partly digested by *Sau*3A and size-fractionated by centrifugation on a sucrose density gradient (10–40 %). The 15–25 kb fraction was ligated to EMBL3 arms and packaged with Gigapack II plus Packaging Extracts (Stratagene). After amplification, three genome equivalents were plated, transferred on to nylon membranes and hybridized with the ^{32}P-labelled cDNA under the same conditions as those used for Southern blot analysis. One positive phage clone was isolated and amplified; the phage DNA was restriction-mapped. A 2500 bp *Eco*RI–*Sal*I restriction fragment containing *orchestin* was then subcloned into pBluescript SK$^-$ (Stratagene).

Southern blot analysis

After digestion by restriction endonucleases, the DNA fragments were separated by electrophoresis and transferred on to a nylon membrane under alkaline conditions. DNA was then hybridized with a ^{32}P-labelled *Hae*II–*Eco*RI restriction fragment (see Figure 3) obtained after amplification and enzymic digestion of the 2500 bp *Eco*RI–*Sal*I genomic fragment described above. Hybridization was performed at 65 °C in a hybridization buffer containing 6 × SSC, 0.5 % SDS and 5 × Denhardt's solution. The membrane was washed at 20 °C in 1 × SSC/0.1 % SDS and at the hybridization temperature in 0.1 × SSC/0.1 % SDS,then dried and exposed to X-ray film.

Northern blot analysis

Total RNA species (10 μg) were size-fractionated on a formaldehyde/agarose (1 % w/v) gel and then transferred by capillary action on to a nylon membrane. The presence of *orchestin* transcripts was detected by hybridization of total RNA species with the ^{32}P-labelled cDNA. Hybridization was performed as described for Southern blot analysis.

Expression of Orchestin cDNA in *Escherichia coli*

The coding sequence without the sequence encoding the signal peptide was obtained by PCR from clone C and inserted into the pQE-30 expression vector (Qiagen), in frame with a 5′ polyhistidine-coding region. The expression of the His-tagged protein was induced with 1 mM isopropyl β-D-thiogalactoside in M15 [pREP-4] *Escherichia coli* transformed by the recombinant plasmid. The recombinant protein was then extracted and purified on a Ni^{2+}-nitrilotriacetate column in accordance with the manufacturer's instructions.

Antibody production

Polyclonal antibodies were raised in two rabbits by injecting 125 μg of His-tagged fusion protein in 0.5 ml of phosphate buffer first emulsified with 0.5 ml of Freund's complete adjuvant. Booster injections were given 14, 28 and 56 days later with the same amount of protein but containing Freund's incomplete adjuvant. The final bleeding was made 80 days after the first injection. Preimmune (from a day 0 bleeding) and final sera were drawn off, divided into aliquots and stored at −70 °C.

Western blot analysis

After electrophoresis, the whole PC proteins, the recombinant protein or the organic-matrix proteins were transferred on to a PDVF membrane [19]. Transfer membranes were incubated overnight at 4 °C in TBS-T blocking buffer [10 mM Tris/HCl (pH 7.5)/0.9 % NaCl/0.05 % (v/v) Tween 20 containing 5 % (v/v) skimmed milk]. After being washed, they were incubated for 45 min at 20 °C in primary antibody solution (rabbit anti-Orchestin, 1:500 dilution). After being washed four times in TBS-T, membranes were incubated for 1 h at 20 °C with secondary antibody solution (alkaline-phosphatase-conjugated goat anti-rabbit IgG, 1:30000 dilution; Sigma). After being washed five times in TBS-T and once in TBS, immunoreactive bands were revealed with 100 mM Tris/HCl (pH 9.5)/100 mM NaCl/5 mM $MgCl_2$ containing 0.3 mg/ml Nitro Blue Tetrazolium and 0.225 mg/ml 5-bromo-4-chloroindol-3-yl phosphate. Controls were made with the preimmune serum as a primary antibody solution or with the secondary antibody solution alone.

In situ hybridization

An oligonucleotide (5′-GGGCTCTCCTGCGAGTCATC-3′), ^{32}P-labelled by T4 polynucleotide kinase, complementary to the +369 to +388 cDNA region (see Figure 1) was used as a probe for hybridization *in situ* with mRNA species in transverse abdominal cryosections of animals deposited on poly(L-lysine)-coated slides. After three washes in PBS [130 mM NaCl/7 mM Na_2PO_4/3 mM NaH_2PO_4 (pH 7)], sections were incubated in 0.2 M HCl for 10 min and acetylated with acetic anhydride, then dehydrated in increasing concentrations of ethanol. Desiccated sections were incubated for 1 h at 26 °C in prehybridization buffer (4 × SSC/1 × Denhardt's solution). The probe, dissolved in hybridization buffer [50 % (v/v) formamide/10 % (v/v) dextran sulphate/4 × SSC/0.5 × Denhardt's solution/0.5 mg/ml salmon-sperm DNA], was deposited on each section for 16 h at 26 °C. Sections were washed six times (25 min each) in 4 × SSC, twice (45 min each) in 2 × SSC and twice (15 min each) in 1 × SSC. Slides were then desiccated, coated with Ilford K5 emulsion and exposed for 24 h.

Hormonal treatment

A saline solution of 20E (Sigma) [5 mg/ml hormone in 10 % (v/v) ethanol/150 mM NaCl] was used. The injections (10 μg per animal) were performed with a microsyringe between two abdominal tergites of animals at different stages of a moulting cycle. Control injections were made with saline solution alone. For the study of gene expression, total RNA species were extracted from the PC 3 h after injection and *orchestin* expression was analysed by Northern blotting as described above.

For the analysis of protein expression, the whole proteins were extracted 24 h after injection with 20E and Western blotting was performed as described above.

Cycloheximide experiments

Animals at the $D_{1^{-}a}$ stage were treated with an injection of 3 μg of the protein synthesis inhibitor cycloheximide (Sigma) dissolved in 0.15 M NaCl immediately followed by 10 μg of 20E. The *orchestin* expression rate was then estimated after extraction of total RNA species 3 h later, as described above. The efficiency of cycloheximide was estimated by measuring the percentage of protein synthesis inhibition by 370 kBq of [^{35}S]methionine (NEN Life Science Products; specific activity: 43.5 TBq/mmol) injected into animals treated with cycloheximide in comparison with untreated animals. Protein concentrations were determined by the method of Bradford [19] and the radioactivity of trichloroacetic-acid-precipitable ^{35}S-labelled proteins was measured with a scintillation counter (Beckman) and evaluated per mass of total protein. The putative toxicity of cycloheximide was tested by comparing the incorporation of [^{35}S]methionine into untreated and cycloheximide-treated animals 24 and 48 h after the cycloheximide injection.

RESULTS

Isolation of Orchestin cDNA by RT–PCR and RACE PCR and deduced amino acid sequence

In a previous paper [9] we described the N-terminal sequencing of a polypeptide of 23 kDa, as estimated by SDS/PAGE, isolated from the SM fraction of calcareous concretions elaborated by the terrestrial crustacean *O. cavimana*. Two internal peptidic sequences were also obtained after enzymic digestion *in situ*. Degenerate oligonucleotides designed from the putative N-terminal sequence and a poly(dT) nucleotide allowed us to obtain, by RT–PCR, a 785 bp cDNA that hybridized with a probe corresponding to one of the internal sequences. After cloning (clone A) and sequencing, the cDNA of interest was found to contain the other small microsequenced fragment that encoded exactly the C-terminal end of the protein (YAGY; single-letter amino acid codes). However, the molecular mass of the protein deduced from this cDNA was smaller than expected. One possible explanation is that the cDNA obtained was incomplete. We therefore performed 5′ RACE PCR. After sequencing from several clones, the approx. 400 bp fragment obtained (clone B) revealed that the first cDNA (clone A) was not full-length and that the N-terminal sequence previously obtained by microsequencing (i.e. WDDDEESDERRSD [9]) was not exactly correct (VPWDSDESSDERLSD). In fact the 12 last bases of the 3′ end of oligonucleotides designed from the N-terminal fragment previously sequenced matched exactly a sequence located downstream of the real N-terminal sequence (+248 to +259, dotted underline in Figure 1). This probe therefore hybridized easily with this region and not with the first putative N-terminal end whose nucleotide sequence was revealed to be different from the exact N-terminal one.

Finally, the full-length cDNA was composed of 1042 bp from the putative transcriptional initiation site to the base pairs located just before the poly(A) tail (Figure 1). It comprised an open reading frame of 384 bp (the first 60 bp fragment corresponding to the signal peptide) preceded by a 102 bp 5′-untranslated region. Downstream, the stop codon was followed by a 533 bp 3′ untranslated region. The first amino acid residues obtained after a new N-terminal microsequencing were in agreement with the first deduced residues of the secreted protein.

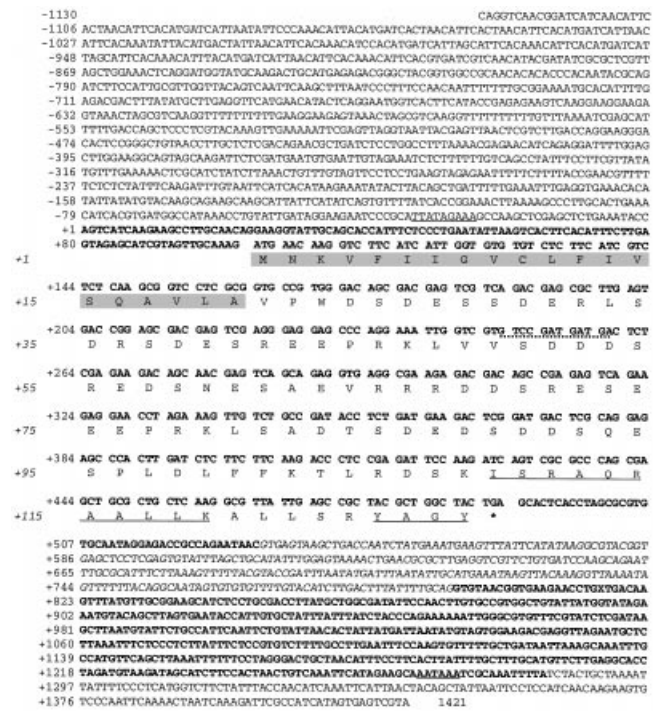

Figure 1 Sequences of the *orchestin* gene and cDNA and deduced amino acid sequence

Nucleotides are numbered from the putative transcription initiation site (+1). The cDNA sequence is in bold letters. X^{+816} is G or C. The sequence in italics corresponds to the intron. The TATA box and the putative polyadenylation signal element are underlined. The deduced amino acid sequence is indicated with the single-letter amino acid code and the signal peptide is boxed in grey. The underlined amino acid sequences correspond to the internal fragments obtained by enzymic digestion of Orchestin *in situ*. The dotted underlined sequence (+248 to +259) corresponds to the bases matching with the oligonucleotides designed from the isolated N-terminal sequence. The TGA triplet marked with an asterisk is the stop codon.

The deduced polypeptide sequence (Figure 1) was composed of a 20-residue signal peptide followed by a 108-residue sequence corresponding to the secreted protein. The molecular mass estimated from this primary sequence was 12.4 kDa and the isoelectric point was 4.4. The amino acid composition of the secreted protein was rich in acidic residues, such as aspartic acid (16.7 %) and glutamic acid (13 %). A comparison of this sequence with sequence databases revealed no identity with any other proteins sequenced so far. Prosite analysis of the putative post-translational modification sites showed only one glycosylation site (an acid periodic Schiff test on the native protein was negative [9]), whereas numerous phosphorylation sites were predicted from the sequence with the use of the NetPhos 2.0 website [23]. Analysis of the putative tandem or periodic repeats with the SAPS program [24] revealed several aligned matching blocks: SDES (amino acid positions 5–8 and 17–20), DDSRE (positions 32–36 and 48–52), ESR(/E)EEPRKL (positions 19–27 and 52–60). Other repeats were observed: SDE (four), SRE (three) and also SD and DS (seven of each).

Production of a recombinant protein and of antibody

Orchestin was then expressed in *E. coli* after insertion in the pQE-30 vector of the cDNA corresponding to the sequence of the secreted protein obtained as described above. The recombinant protein (histidine-tagged and lacking any post-trans-

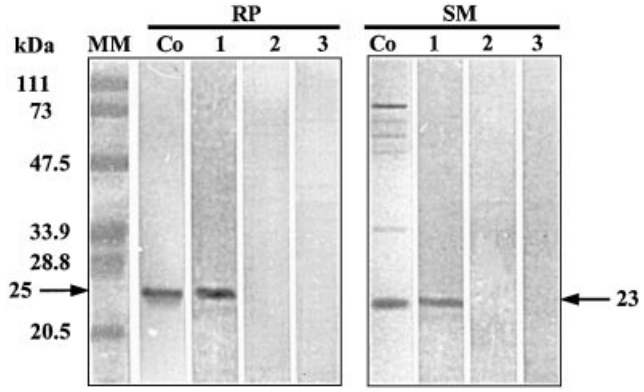

Figure 2 Analysis of the anti-recombinant Orchestin antibody specificities by Western blotting

Abbreviations: RP, recombinant protein; Co, proteins stained with Coomassie Brilliant Blue. Lanes 1, immunodetection with polyclonal anti-RP antibodies; lanes 2, control (second antibodies alone); lanes 3, control (preimmune serum as the first antibody solution); lane MM, molecular-mass standards.

lational modifications) showed almost the same electrophoretic behaviour as the native protein and migrated with an apparent molecular mass of 25 kDa (Figure 2, RP). The antibodies produced against the fusion protein specifically recognized the 23 kDa protein from the SM fraction (Figure 2, SM, lane 1).

Genomic sequence and Southern blot analysis

The screening of an *Orchestia* genomic library constructed in λ EMBL3 led us to subclone and sequence a 2.5 kb fragment containing the complete transcription unit of the *orchestin* gene. The nucleotide sequence (Figure 1) contained two exons and a single 260 bp intron located in the 3′ non-coding region, 47 bp downstream of the stop codon, and a polyadenylation signal. The 5′ untranslated region contained a TATA box but no CG or CAAT elements. The transcribed sequence matched the cDNA sequence completely except for one nucleotide in the 3′ untranslated region (G or C in position +816 from the putative transcription initiation site).

To determine the number of copies of the *orchestin* gene, genomic DNA was extracted independently from two animals and digested with *Eco*RI, *Hin*dIII or *Hae*II separately or in combination. Southern blot analysis of the restriction fragments obtained (results not shown), with the *Hae*II–*Eco*RI genomic

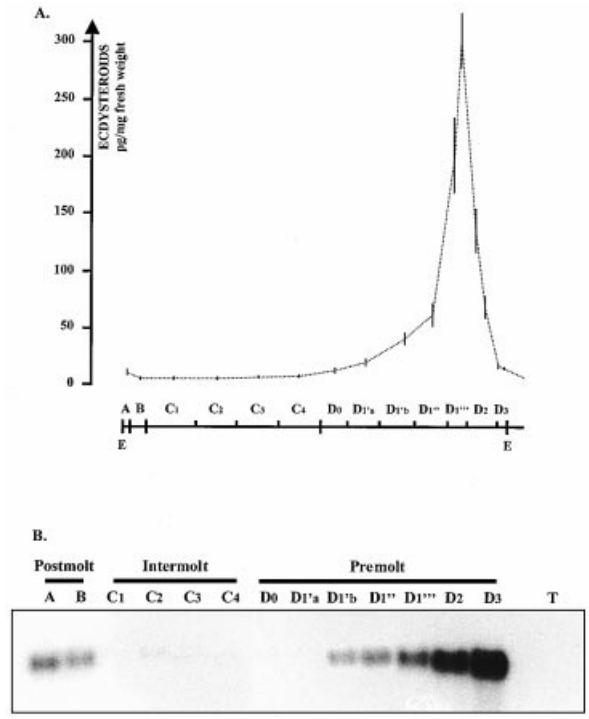

Figure 4 Ecdysteroid titre fluctuations and *orchestin* expression during a moulting cycle of *O. cavimana*

(**A**) Ecdysteroid titre fluctuations as determined by RIA [14]. (**B**) Total RNA species extracted from the PC of animals at different stages (A to D_3) of a moulting cycle were hybridized with the full-length ^{32}P-labelled cDNA. The quantity of RNA used for each stage (10 μg) was controlled by ethidium bromide staining of the ribosomal RNA species. RNA species from digestive caeca were used as a negative control (lane T).

fragment as a probe, led us to propose a unique restriction map of the *orchestin* locus (Figure 3) showing that the genome of *Orchestia* contains only one copy of the *orchestin* gene.

Spatiotemporal expression

A study of the temporal expression of *orchestin* was realized by Northern blotting, which revealed the presence of a single band corresponding to an approx. 1000 bp fragment, in agreement with the size expected for the *orchestin* transcript.

The autoradiograph shown in Figure 4(B) reveals that the transcript, undetectable in intermoult, became detectable at

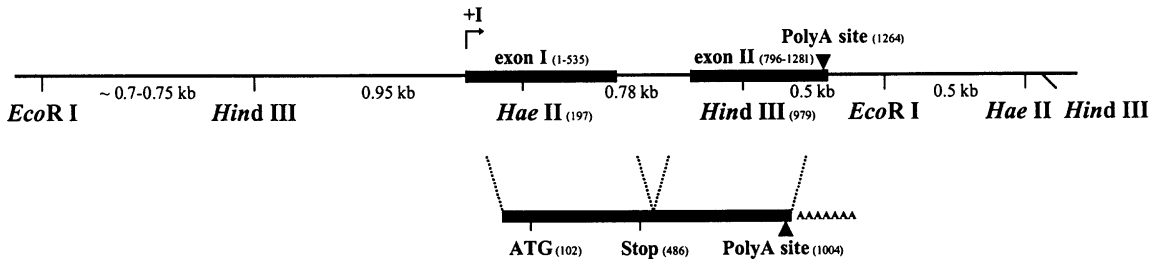

Figure 3 Genomic restriction map of *orchestin*

Hybridization of the genomic restriction fragments obtained after single or double digestions led to this deduced restriction map of the genomic region containing *orchestin*. The structure of the mature *orchestin* transcript is also shown. The *Hae*II–*Eco*RI genomic fragment was used as a probe.

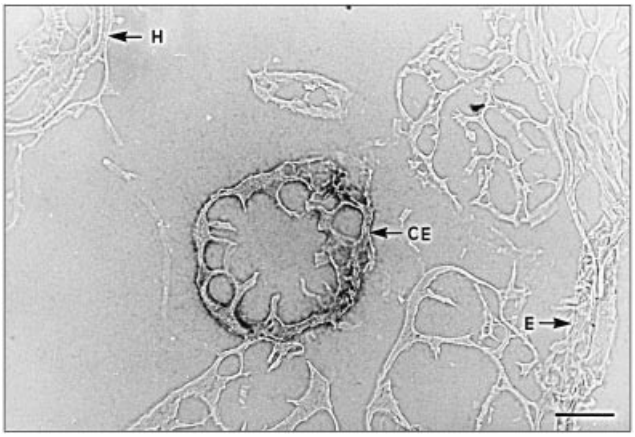

Figure 5 Localization of *orchestin* transcripts during the premoult period by *in situ* hybridization

Transverse abdominal cryosections of animals were hybridized with ^{32}P-labelled oligonucleotides complementary to the +369 to +388 cDNA region. Abbreviations: CE, caecal epithelium; E, epidermis; H, hindgut. Scale bar, 30 μm.

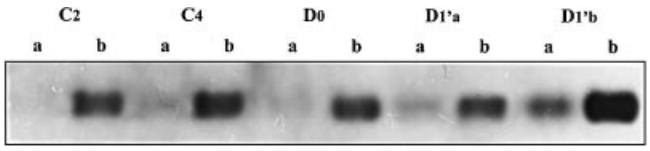

Figure 6 Expression of *orchestin* after stimulation *in vivo* for 3 h by 20E

Animals at different stages of a moulting cycle received injections of 10 μg of 20E; PC RNA species were extracted 3 h later. Expression of *orchestin* was analysed by Northern blotting as described in the legend to Figure 4. Lanes a, control [animals injected with in 10% (v/v) ethanol/0.15 M NaCl alone]; lanes b, animals treated with 20E.

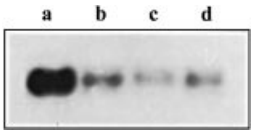

Figure 7 Effect of cycloheximide on *orchestin* expression

The experiment was performed with animals at stage $D_{1'a}$. Lane a, animals treated with 20E alone; lane b, animals treated with cycloheximide and then 20E; lane c, animals treated with cycloheximide alone; lane d, control.

the beginning of the premoult period, concomitantly with the increasing 20E titre (Figure 4A). It accumulated during the premoult period ($D_{1'b}$ to D_3 stages), corresponding to the calcium storage period. After ecdysis, the transcripts were still detectable in the postmoult period, although in much smaller amounts, and became undetectable in intermoult. The expression of *orchestin* during the postmoult period is somewhat surprising, because the concretions were resorbed during this period. Nevertheless, the temporal analysis of Orchestin translation (see Figure 8A) corroborated the result of the Northern blot: the translation products began to be detectable at the $D_{1'b}$ stage and remained detectable during the premoult period and also after ecdysis. The rate decreased during the postmoult period and became undetectable at the beginning of intermoult (see Figure 8A, lane C).

As the whole physiology of *O. cavimana* is involved in an important and cyclic turnover of calcium, we looked for the expression of *orchestin* in other organs and notably those concerned with the calcium balance occurring between the storage organs and the cuticle. Transverse cryosections of animals at three different stages (one from each of the three main periods) of a moulting cycle were subjected to hybridization *in situ*. The autoradiograph obtained corroborated the temporal expression results: the gene was expressed only during the premoult period (Figure 5) and the postmoult period (results not shown). Furthermore, *orchestin* was organ-specific: the transcripts were detectable only in the PC epithelium regardless of the period.

Hormonal regulation

Ecdysteroid titre fluctuations during a moulting cycle (Figure 4A) [15], determined by RIA, showed increasing rates during the premoult calcium storage period. The titre reached a maximum before the end of this period ($D_{1''}$ stage) and decreased just before ecdysis until the postmoult and intermoult level. Northern blot analysis also showed an increasing expression of *orchestin* during the premoult period. We therefore thought that it would be of interest to test the effect of 20E on *orchestin* expression.

Animals were injected with 20E at various times (C_4 to $D_{1'b}$ stages) during the critical period when the PC switched to calcium storage activity (intermoult–premoult period; see Figure 4). At the $D_{1'a}$ stage, the PC began to store calcium; the *orchestin* transcripts were first detectable at the $D_{1'b}$ stage (Figure 4A). This period also corresponded to the beginning of the increase in 20E titre. We also tested, as a control, the C_2 stage in the middle of intermoult. When injected, animals naturally eject a few microlitres of haemolymph. Therefore, to ensure that each animal received an amount of hormones sufficient to exert an effect, a supra-physiological dose was chosen (10 μg per animal; the peak value estimated by RIA was approx. 30 ng per animal) [15]. The results are shown in Figure 6. For each of the stages studied a significant enhancement of expression was obtained, indicating that *orchestin* expression was sensitive to this hormone. Although 3 h was a short stimulation time, the possibility remains that this effect was not direct. To investigate this point further, animals received an injection of the protein synthesis inhibitor cycloheximide before the hormone. The dose injected was considered to be non-toxic (the incorporation of [^{35}S]methionine was similar in treated animals and in control animals 48 h after the cycloheximide injection) and efficient (approx. 95% inhibition). The results show an inhibition of the 20E stimulation when compared with animals treated with 20E alone or with cycloheximide alone (Figure 7). Thus, if *orchestin* can be considered to be an 20E-inducible gene, the stimulation pathway was probably indirect.

We also studied the effect of injection of 20E on *orchestin* translation. When injected during the premoult period, animals showed markedly accelerated calcium storage and exuviated more quickly than untreated animals. When injected at the critical storage period $D_{1'a}$, small calcareous concretions were visible 24 h later in the distal part of the PC, whereas no concretions were present in control animals. Similarly, animals injected at $D_{1'b}$ showed small concretions all along the storage organs, whereas control animals exhibited these structures only in the distal part of the PC. Moreover, as described previously [14], a significant modification of the electrophoretic pattern of the whole PC proteins was still observable 24 h after injection during the same period. The Western blot analysis corroborated these observations. We analysed first the relative physiological levels of Orchestin during a moulting cycle (Figure 8A), then

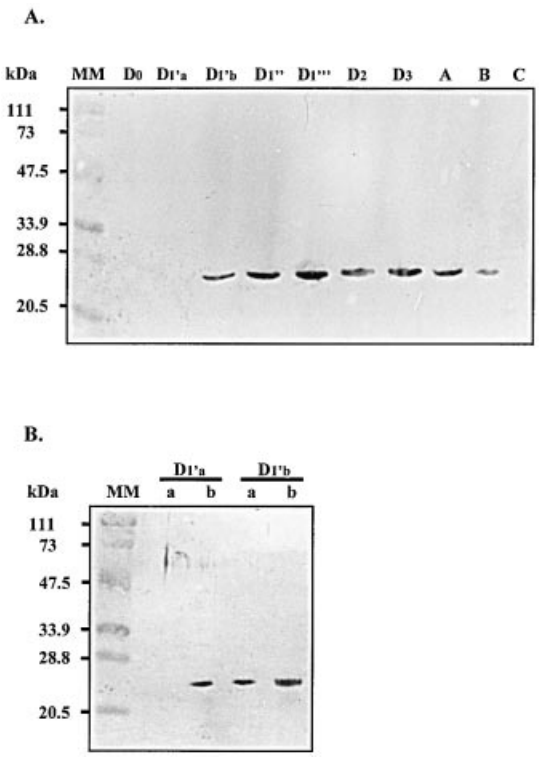

Figure 8 Temporal expression of Orchestin during a moulting cycle and the effect of 20E on this expression

(**A**) Total proteins were extracted from the PC at different stages of a moulting cycle and separated by SDS/PAGE (30 μg of proteins per lane). Orchestin was revealed with the anti-Orchestin antibody raised against the recombinant protein. Lanes D_0 to D_3, premoult period; lanes A and B, postmoult period; lane C, intermoult. (**B**) Animals were injected with 20E during the $D_{1'a}$–$D_{1'b}$ period, when animals begin to elaborate concretions. Proteins were extracted 24 hours below and the level of expression was analysed by Western blotting. lanes a, control animals [injection of 2 μl of 10% (v/v) EtOH/0.15 M NaCl]; lanes b, stimulated animals [injection of 10 μg of 20E in 2 μl of 10% (v/v) EtOH/0.15 M NaCl per animal]. Lanes MM, molecular-mass standards.

the effect of an injection of 20E at the critical period when the translation products became detectable. At 24 h after hormonal injection in $D_{1'a}$ or $D_{1'b}$ animals, the rate of translation of Orchestin was modified. Whereas Orchestin remained undetectable in the $D_{1'a}$ control animals (Figure 8B, $D_{1'a}$, lane a), it became detectable among the PC proteins of the animals treated at $D_{1'a}$ (Figure 8B, $D_{1'a}$, lane b). Similarly, the level of expression of Orchestin seemed enhanced in the animals treated at $D_{1'b}$ (Figure 8 B, $D_{1'b}$, lane b) in comparison with the untreated ones at $D_{1'b}$ (Figure 8B, $D_{1'b}$, lane a).

DISCUSSION

Analysis of the genomic sequence reveals that *orchestin* possesses two exons and a single intron in the 3′ untranslated region (Figure 3) and is probably present as a single copy in the genome of *Orchestia*. This gene, as well as the deduced protein, does not share significant similarity with any known genes and proteins or calcium-binding motifs sequenced so far. Another 23 kDa protein named CCBP-23 was previously isolated from the abdominal muscle of a crustacean [25] but Orchestin shares no identity with this protein. Moreover, CCBP-23 is a member of the EF-hand calcium-binding protein family and reveals no discrepancy between the molecular masses deduced from the amino acid sequence and those obtained by MS.

No similarities were found to other sequenced proteins that are involved in biomineralized structures in invertebrates. The organic-matrix proteins characterized and sequenced so far originate essentially from echinoderms, molluscs and crustaceans. Among these, candidate calcium-binding proteins are the nacrein extracted from oyster pearls [26] and two proteins extracted from the nacreous layer of mollusc shells (MSI60 and MSI31) [27]. Another calcium-binding protein from the extrapallial fluid of a mollusc was recently characterized and its N-terminal sequence was determined [28]. The involvement of these two proteins in the calcification process is suggested but is not clearly demonstrated.

In crustaceans an insoluble matrix protein, named gastrolith matrix protein (GAMP), extracted from the gastroliths (another calcium storage form) of a crayfish, was sequenced and well characterized by Nagasawa and co-workers. GAMP is suspected to have a role in the calcification process of these storage structures [29–32]. Inoue et al. [33] also characterized, from the exoskeleton of a crayfish, an interesting polypeptide with anti-calcification and chitin-binding properties. Finally, four cDNA species were sequenced that encode crustacean proteinaceous components of the exoskeleton (DD4, DD9A, DD9B and DD5) [34–36]. Among these, only DD4 [34] clearly binds calcium, as demonstrated by the test of Maruyama et al. [12].

A characteristic of known invertebrate organic-matrix proteins is the presence of repeated domains in their sequence. If Orchestin does not possess real domains, repeated blocks are present whose significance remains unknown. Nevertheless, all of these repeats comprise essentially acidic amino acids, as previously observed, for example, in molluscan soluble matrix proteins.

The difference observed between the deduced molecular mass and the estimate by SDS/PAGE might have two explanations. First, the native protein could be a dimer of two 12.4 kDa subunits. In this case, the two monomers are not linked by disulphide bridges, because these are reduced by SDS/PAGE; neither does the sequence contain cysteine. Furthermore, treatment by 8 M urea followed by heating did not affect the electrophoretic mobility (results not shown). We also never obtained bands in SDS/PAGE that could correspond to the monomer form, as is generally observed in this case. The second hypothesis is related to atypical binding of SDS. Strongly biased amino acid compositions, such as very acidic domains, induce a decrease in the electrophoretic mobility and consequently give an overestimate of molecular mass [37–39]. Similarly, post-translational modifications, such as glycosylations and/or phosphorylations can modify electrophoretic behaviour [40]. Cuticular proteins, for example, have been noted for their aberrance in this [41,42]. Nacrein, mentioned above [26], also exhibits this abnormal migration on SDS/PAGE. Another example is provided by GAMP, the insoluble matrix protein characterized from crustacean gastroliths: it is rich in acidic residues and its molecular mass has been estimated to be 94 kDa by SDS/PAGE and 50.5 kDa by MS [30].

Orchestin is rich in acidic residues (approx. 30% of the sequence), which could be sufficient to explain the discrepancy between the estimated and calculated molecular masses. On the one hand, this hypothesis is supported by the fact that Orchestin expressed in *E. coli* migrates with an apparent molecular mass of 25 kDa, which is greater than expected (12.4 kDa). On the other hand, the antibodies produced against the fusion protein recognize, in Western blotting, the sole 23 kDa protein from the SM fraction. The discrepancy observed in SDS/PAGE between the molecular masses of the native (23 kDa) and fusion (25 kDa)

proteins is due to the presence of the polyhistidine tail and probably also to the presence of putative post-translational modifications (such as phosphorylations). Taken together, these results demonstrate that the polypeptide (Orchestin) migrating at 23 kDa on SDS/PAGE is clearly encoded by the gene related to the 12.4 kDa polypeptide. This gene can therefore be confidently named *orchestin*.

In a previous paper [9] we reported the ability of a polypeptide migrating at 23 kDa to bind calcium, as determined by the procedure of Maruyama et al. [12]. We are not yet confident that this protein corresponds to Orchestin, as characterized in the present paper, because we failed to demonstrate any calcium-binding activity of the recombinant protein. We cannot exclude the possibility that Orchestin was co-purified with the actual calcium-binding protein. However, the putative Ca^{2+}-binding ability of Orchestin might require specific post-translational modifications that are not properly made in *E. coli*. Additional experiments are therefore required to establish that Orchestin participates in calcium binding in the organic matrix of the concretions.

The formation of these mineralized structures is quite obviously tightly controlled; in particular, neuropeptides and hormones are likely to be involved in their regulation. In gastropods, insulin-like substances [43] and members of the secosteroid hormone family [44] are thought to regulate shell growth and calcification. In crustaceans, both cuticle calcification and the formation and resorption of gastroliths (calcium storage forms in some decapods) are believed to be dependent on ecdysteroid [31,45].

The *orchestin* gene is clearly stimulated by the injection of 20E *in vivo*. Moreover, after stimulation with 20E, the translation of Orchestin is clearly induced or enhanced. This is in good agreement with an earlier result [14] showing a modification of the electrophoretic pattern of the total PC proteins in response to the injection of 20E. However, the stimulation of *orchestin* by 20E is indirect, as demonstrated by its dependence on protein synthesis (inhibition of stimulation with cycloheximide). Thus, according to the reclassification of steroid-responsive genes by Dean and Sanders [46], *orchestin* belongs to the secondary-response gene category. In insects, studies on the effect of ecdysone on the puffing pattern of polytene chromosomes have provided insights into sequential gene activation regulating metamorphosis [47]. With regard to the model proposed by Ashburner et al. [48] for the regulatory hierarchy of the puffing response in *Drosophila*, *orchestin* can be considered a late-response gene. Moreover, analysis of the 5′ untranslated region does not reveal any consensus sequence for the binding of the ecdysone-receptor complex that is necessarily formed in the primary response to this hormone.

Two additional conclusions can be drawn. First, a stimulation effect is observed when the animals are in intermoult (C_2 or C_4), which suggests that the PC cells are competent for this hormone even at times when they are not involved in calcium storage. Secondly, the temporal expression analyses performed with Northern and Western blotting reveal that *orchestin* is not only transcribed but is also translated after ecdysis during the period when the PCs are no longer involved in calcium storage and the amount of 20E is very low (Figure 4A). One explanation is that Orchestin might also be a component of the organic matrix of the calcified transepithelial spherules elaborated to resorb the stored calcium [7]. In this case we speculate that this second phase is controlled not by 20E but possibly by other factors.

We have thus characterized a new gene encoding an EDTA-soluble organic-matrix protein from an invertebrate. This gene is activated cyclically during each premoult period by the moulting hormone of this terrestrial crustacean. In comparison with the other organic-matrix components described so far, Orchestin seems to be a new organic-matrix protein that does not possess canonical repeated domains and is not glycosylated. Experiments are currently being performed to determine whether Orchestin has calcium-binding activity, which might be important for understanding the involvement of this matrix protein in the formation of the calcium storage structures elaborated by *O. cavimana*.

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