Phosphorylation of serine residues is fundamental for the calcium-binding ability of Orchestin, a soluble matrix protein from crustacean calcium storage structures

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Abstract ***Orchestia cavimana*** **is a terrestrial crustacean, which cyclically stores calcium in diverticula of the midgut, in the form of calcified amorphous concretions. These concretions are associated with a proteinaceous matrix, the main constituent of the soluble matrix is Orchestin, an acidic calcium-binding protein [Testenière et al., Biochem. J. 361 (2002) 327–335]. In the present paper, we clearly demonstrate that Orchestin is phosphorylated on serine and tyrosine residues, but that calcium binding only occurs via the phosphoserine residues. To our knowledge, this is the first example of an invertebrate mineralization for which a post-translational modification is clearly related to an important function of a calcifying protein.**

Key words: Biomineralization; Calcium binding; Crustacean; Organic matrix; Phosphorylation

1. Introduction

Biomineralization is a widespread phenomenon in animals, particularly among the invertebrates [1,2]. For example, most crustaceans possess a calcified exoskeleton (or cuticle) that they have to replace periodically for growing. Thus, in these animals, growth, reproduction and calcium metabolism are tightly linked to moulting cycles. The origin of the calcium used to mineralize the cuticle after ecdysis depends on the biotope of the animal considered. If aquatic crustaceans directly take the calcium from their environment, terrestrial species have developed several calcium storage strategies [3], to compensate for the lack of direct calcium availability.

Orchestia cavimana is a terrestrial crustacean that provides a dramatic example of such a calcium storage process. During the about 15-day mean premoult period, this land crustacean stores calcium originated essentially from the old cuticle, in paired diverticula of the midgut, named posterior ceca (PC). Calcium is stored in the lumen of the PC as calcareous concretions composed of amorphous calcium carbonate [4,5], which is precipitated within an organic matrix synthesized by the cecal epithelial cells [6]. The concretions grow during the premoult period by successive additions of mineralized and unmineralized concentric layers. The organic matrix, which controls the growth of the concretions, consists essentially of proteins, some of which are soluble in EDTA buffer, the others being insoluble in this buffer [7].

In order to study this peculiar calcium storage process, we have analyzed the proteinaceous components of the EDTA-soluble organic matrix (conventionally called SM). We could characterize Orchestin, a polypeptide migrating at 23 kDa in sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). We clearly demonstrated that this acidic (p*I* 4.4) and unglycosylated protein is able to bind calcium [7]. The related cDNA and gene were obtained and sequenced [8]. The deduced amino acid sequence was enriched in acidic amino acids (ca. 30%), which suggested that the calcium-binding ability was due to carboxylic groups. However, phosphate groups are thought to play a similar role in calcium binding [9,10]. While the analysis of the sequence did not reveal canonical calcium-binding domains, numerous phosphorylation sites could be predicted. This is why we decided to further analyze these putative post-translational modifications and their relation to the function of the protein.

We report in this paper that Orchestin is phosphorylated on serine and tyrosine residues. Furthermore, we demonstrate that the calcium-binding ability of this protein only depends on phosphorylation on serine residues whereas phosphorylated tyrosine residues are not involved in this function. This is the first report of an invertebrate matrix protein, the calcium-binding ability of which is clearly related to phosphorylation, in a similar manner as phosphoproteins of the organic matrix of bones and teeth in vertebrates.

2. Materials and methods

2.1. Animals

O. cavimana were reared in the laboratory in subaquatic terraria, with a moss substrate and a daily cycle of 12 h light and 12 h dark. The moulting cycle of *O. cavimana*, a 46-day mean period for an adult specimen about 18 mm long, has been described and subdivided into 14 stages [11]. In order to avoid age- or sex-related differences, only male adult specimens were used.

2.2. Protein extraction and analysis

The calcareous concretion matrix proteins were isolated as described [7] and analyzed by SDS–PAGE [12] in a 12.5% acrylamide slab gel or by two-dimensional electrophoresis. Isoelectric focusing (IEF) was carried out using the Multiphor II system (Amersham Biosciences). Precast 13-cm linear pH 4–7 IPG strips were rehydrated overnight with 260 μl of buffer containing 8 M urea, 2% (w/v) CHAPS, 20 mM dithiothreitol (DTT), 2% (v/v) IPG buffer pH 4–7 and bromophenol blue. 60 μg/gel SM proteins, solubilized in a buffer

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containing 9.5 M urea, 2% (w/v) CHAPS, 0.5% Triton X-100, 0.1 M DTT, 2% (v/v) IPG buffer pH 4–7, were allowed to focus at 20°C with a constant voltage of 150 V for 3 h, which was then gradually increased from 300 to 3500 V over 5 h, and finally stabilized at 3500 V for 12 h. After IEF, gels were equilibrated [13] and transferred onto 12% acrylamide vertical slab gels. Electrophoresis was run at 10°C for 4 h at 100 V. Proteins were then stained with colloidal Coomassie blue [14] or transferred onto polyvinylidene difluoride (PVDF) membranes as previously described [8].

Protein concentrations were determined according to the Bradford method [15].

2.3. Western blot immunostaining

Immunostaining of Western blots was performed using antibodies raised against a recombinant Orchestin (dilution: 1/500), or using mouse monoclonal anti-phosphotyrosine antibody (dilution: 1/2500, clone no. PT-66; Sigma), as previously described [8].

2.4. Production of Orchestin in Escherichia coli

The coding sequence (without the signal peptide sequence) was obtained by polymerase chain reaction using 5′-GGATCCGATGACGATGACAAGGTGCCGTGGGACAGCGAC-3′ and 5′-TGCACTGCAGCACGCGCTAGGTGAGTGC-3′ as, respectively, forward and reverse specific primers. The 5′ primer contains a sequence encoding the bovine enterokinase-specific cleavage site. The amplified cDNA was cloned in pQE-30 expression vector (Qiagen), which contains a histidine-tagged region upstream of the cloning site. The His-tagged fusion protein was expressed in M15 *E. coli* and purified on a Ni^{2+}-nitrilotriacetate column, according to the manufacturer's instructions. Cleavage of the His tail was performed with bovine enterokinase (Novagen; 0.05 U/50 μg protein) for 16 h at 21°C in a Tris–HCl 20 mM, pH 7.4, NaCl 50 mM, $CaCl_2$ 2 mM buffer.

2.5. Calcium-binding protein detection

The calcium overlay procedure was performed according to Maruyama et al. [16], as previously described [7], with the native or dephosphorylated soluble matrix proteins or with the recombinant protein.

The Stains-all staining procedure (cationic carbocyanine dye) was carried out according to Campbell et al. [17]. After electrophoresis, gels were fixed for 16 h in 25% isopropanol and 10% acetic acid. Polypeptides were then stained by incubating the gels in the dark for 48 h in 0.025% Stains-all, 25% isopropanol, 7.5% formamide and 30 mM Tris base pH 8.8.

2.6. Phosphoprotein detection

Radiolabelled inorganic phosphate, ^{32}Pi (specific activity: 185 TBq/mmol; NEN Life Science Products), was injected (74 kBq/animal) between two segments of the body into the hemolymph of each of the five animals studied at three different stages during the premoult period. The calcareous concretions were collected just after the following ecdysis. The concretion matrix proteins were extracted and separated by SDS–PAGE. The slab gel was dried and exposed to an X-ray film (Kodak) at −80°C.

2.7. Dephosphorylation treatments

Complete dephosphorylation was performed on the whole SM proteins with the lambda protein phosphatase (Biolabs). 400 U enzyme was incubated for 1.5 h at 30°C with 15 μg proteins in 50 mM Tris–HCl pH 7.5, 0.1 mM Na_2EDTA, 5 mM DTT, 0.01% Brij 35 and 2 mM $MnCl_2$ in 20 μl final volume.

Specific Ser/Thr dephosphorylations were carried out with protein phosphatase 1 (PP1; Biolabs) in the same conditions used with the lambda protein phosphatase but with 2.5 U/reaction. Specific Tyr dephosphorylations were performed with YOP (50 U/test; Biolabs), LAR (5 U/test; Biolabs) or T-cell protein tyrosine phosphatase (10 U/test; Biolabs) in the same reaction buffer: 50 mM Tris–HCl pH 7.0, 100 mM NaCl, 2 mM Na_2EDTA, 5 mM DTT, 0.01% Brij 35 and 1 mg/ml bovine serum albumin.

Treatments with PP1 followed by one of the three Tyr phosphatases were also performed.

2.8. Phosphorylated amino acid mapping

After in vivo injection of ^{32}Pi and extraction of the whole organic matrix of the concretions as described above, acidic hydrolysis was performed on the whole soluble fraction (15 μg protein/200 μl HCl 6 N, 1.5 or 2.5 h at 110°C under nitrogen atmosphere) according to Cooper et al. [18]. Radiolabelled hydrolyzed products obtained were then separated by thin-layer chromatography. Chromatography was carried out on silica gel plates (Merck) with a solvent mixture containing chloroform/methanol/17% ammonia solution (2/2/1, v/v) in the first dimension and *n*-butanol/acetic acid/H_2O (3/1/1, v/v) in the second dimension. Radiolabelled amino acids were revealed by autoradiography. Control experiments were performed in the same conditions using a mixture of the three phosphoamino acids P-Ser, P-Tyr and P-Thr (Sigma). Standard phosphoamino acids were detected by spraying dried plates with a 0.2% ninhydrin solution in absolute ethanol (Sigma) and visualized after the plate was baked for 10 min at 60°C.

2.9. C-terminal enzymatic digestion

C-terminal enzymatic digestion was performed on the whole soluble organic matrix proteins. 30 μg protein was incubated in 16 μl solution containing 0.4 μg (0.07 U) carboxypeptidase B (Sigma). Proteolytic activity was tested from time 0 to 20 min by stopping the reaction every 2.5 min by addition of sample buffer supplemented with 0.5 mg/ml Na_2EDTA, 40 μg/ml bestatin. After heating for 10 min at 95°C, each mixture (25 μl final) was separated by SDS–PAGE and stained

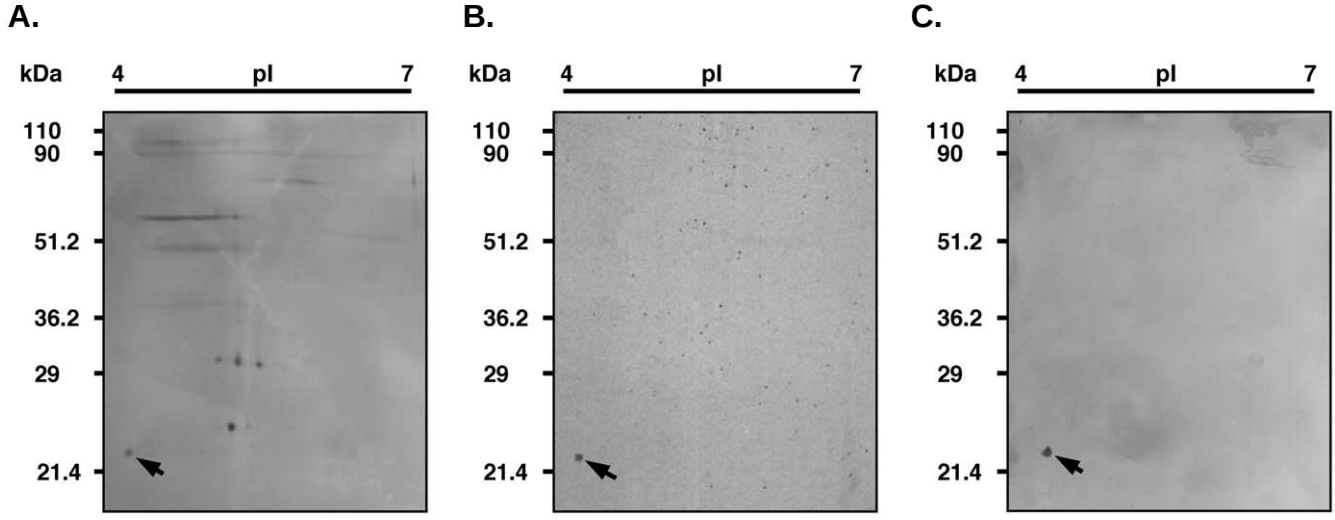

Fig. 1. Two-dimensional electrophoretic analysis of the soluble matrix proteins of the concretions (IEF/SDS–PAGE). The arrow indicates the Orchestin protein. A: Coomassie blue staining. B: Autoradiography after transfer on PVDF membrane and ^{45}Ca incubation. C: Western blotting and immunostaining with anti-recombinant Orchestin polyclonal antibodies.

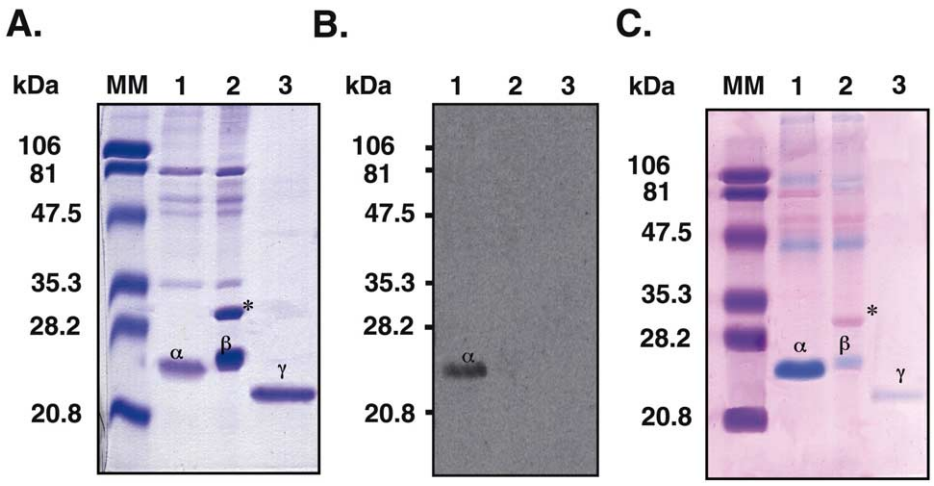

Fig. 2. Phosphorylations and calcium-binding property of Orchestin. Lane MM: molecular mass standards; lane 1: whole native soluble matrix proteins (α: Orchestin); lane 2: whole soluble matrix proteins after digestion with the lambda protein phosphatase (*; β: dephosphorylated Orchestin); lane 3: the recombinant protein (γ). A: Coomassie blue staining. B: Autoradiography after transfer on PVDF membrane and ^{45}Ca incubation. C: Stains-all staining.

with Coomassie blue or transferred onto PVDF membranes and analyzed for the calcium-binding ability by the Maruyama et al. procedure, as described in Section 2.5.

3. Results and discussion

We have previously characterized a non-glycosylated calcium-binding protein, called Orchestin, from the SM components of calcium storage structures elaborated by a terrestrial crustacean [7,8]. After extraction of this 23-kDa polypeptide, we obtained the N-terminal sequence and internal sequences, which were used to clone the cDNA then the gene encoding this protein [8]. The amino acid sequence deduced from the cDNA corresponds to a polypeptide of 12.4 kDa (p*I* 4.4). The discrepancy between the apparent and the deduced molecular masses was explained in two ways: the acidic sequence of Orchestin and the putative presence of post-translational modifications. Polyclonal antibodies raised against the recombinant protein obtained after expressing the above cDNA in *E. coli* recognized the native protein migrating at 23 kDa [8]. To ensure definitively that the polypeptide deduced from the cDNA and the calcium-binding polypeptide correspond to a single protein, we performed a two-dimensional electrophoresis of the SM fraction followed by either the calcium-binding test [16] or immunodetection using the antibodies produced against the recombinant protein. The results obtained (Fig. 1) show clearly that only one polypeptide migrating at 23 kDa for a p*I* of about 4.3 (Fig. 1A) is able to bind calcium (Fig. 1B) and is recognized by the anti-recombinant Orchestin antibodies (Fig. 1C).

In our previous study [8], we had shown that Orchestin exhibits an unusual electrophoretic migration. This might be due essentially to its richness in acidic amino acids which are known to moderately bind SDS [19]. However, analysis of the

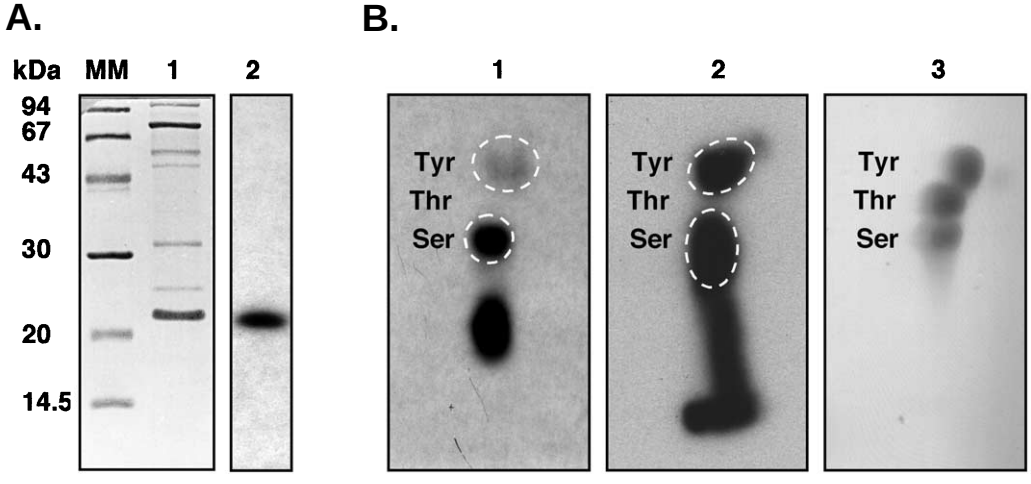

Fig. 3. Analysis of phosphorylated proteins and determination of the phosphorylated residues. A: Separation of the whole soluble matrix proteins by SDS–PAGE. Lane 1: Coomassie blue staining; lane 2: autoradiography after in vivo ^{32}Pi labelling; lane MM: molecular mass standards. B: Analysis of the phosphorylated residues by thin-layer chromatography. 1: After in vivo ^{32}Pi labelling and 2.5 h acidic hydrolysis of the soluble matrix proteins; 2: after in vivo ^{32}Pi labelling and 1.5 h acidic hydrolysis of the soluble matrix proteins; 3: control phosphorylated residues stained with ninhydrin.

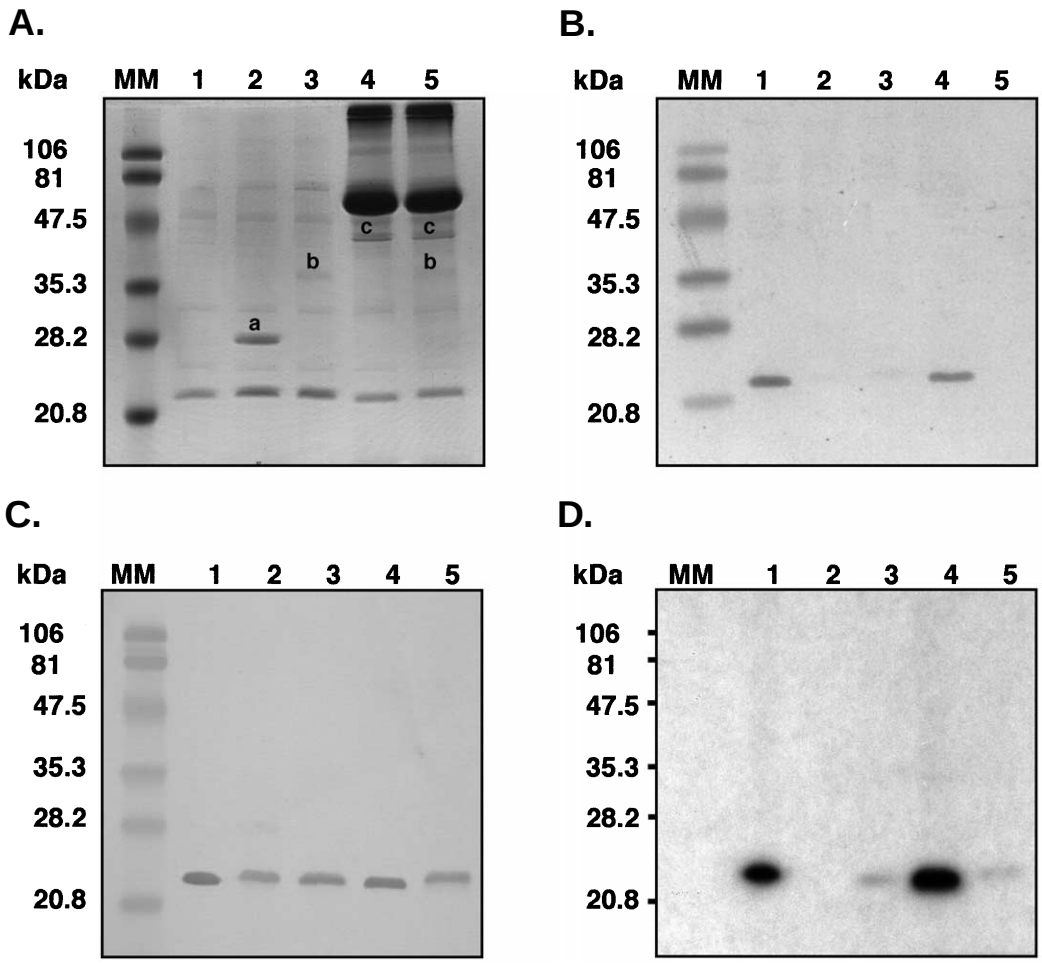

Fig. 4. Relation between phosphorylation on serines and tyrosines and the calcium-binding property of Orchestin. SDS–PAGE analysis of the whole soluble matrix proteins. Lane 1: without treatment (native proteins); lane 2: after digestion with the lambda protein phosphatase (a); lane 3: after digestion with the Ser/Thr phosphatase, PP1 (b); lane 4: after digestion with the Tyr phosphatase, YOP-PTP (c); lane 5: after digestion with PP1 (b) followed by YOP-PTP (c). Lane MM: molecular mass standards. A: Coomassie blue staining. B: Autoradiography after transfer on PVDF membrane and ^{45}Ca incubation. C: Western blotting and immunostaining with anti-recombinant Orchestin polyclonal antibodies. D: Autoradiography after ^{32}Pi in vivo injection.

comparative electrophoretic behavior of the native protein versus the recombinant protein (Fig. 2A) led us to consider that Orchestin exhibits post-translational modifications. To investigate the presence of phosphorylations, as predicted from the sequence [8], we carried out dephosphorylation treatments on the whole SM fraction. After treatment with the lambda protein phosphatase, we observed that dephosphorylated Orchestin exhibits a decrease in electrophoretic mobility of about 1 kDa as compared to the native protein (Fig. 2A, lanes 1 and 2). This difference can be explained by a loss of negative charges carried by the phosphate groups. In addition, the recombinant protein devoid of its His tag migrates 2 kDa lower than the native dephosphorylated protein (Fig. 2A, lanes 2 and 3). This could be explained by the fact that phosphorylations are not the only post-translational modifications of Orchestin. If other modifications are really present, their nature remains unknown at this time.

Then, the dephosphorylated SM was tested for its ability to bind radioactive calcium and to stain with the cationic carbocyanine dye Stains-all. This extract was compared with the native SM and with the recombinant Orchestin. Results demonstrate that the calcium-binding ability of Orchestin is dependent on its phosphorylation (Fig. 2B): when dephosphorylated, Orchestin does not exhibit any calcium-binding activity. The cationic carbocyanine staining corroborates this result because only the native protein exhibits a metachromatic blue color (Fig. 2C), which is characteristic of calcium-binding proteins [17].

Twenty phosphorylation sites (18 on serine and two on threonine residues) [8] were predicted from the amino acid primary sequence of Orchestin according to the NetPhos 2.0 program [20]. In order to assess the existence of phosphorylations, ^{32}Pi was injected to animals during the premoult period. Autoradiographic analysis of the SM proteins extracted at the following ecdysis shows that Orchestin is phosphorylated and furthermore is the only component of the SM fraction to possess this property (Fig. 3A). To further analyze this chemical characteristic, we determined precisely the nature of the phosphorylated residues after acidic hydrolysis of the ^{32}P-labelled protein followed by thin-layer chromatography. The first result obtained after 2.5 h acidic hydrolysis (Fig. 3B, 1) showed a spot at the level of P-Ser and a weaker spot at the level of P-Tyr. To verify that P-Tyr is really present, we performed the same experiment but with a shorter acidic hydro-

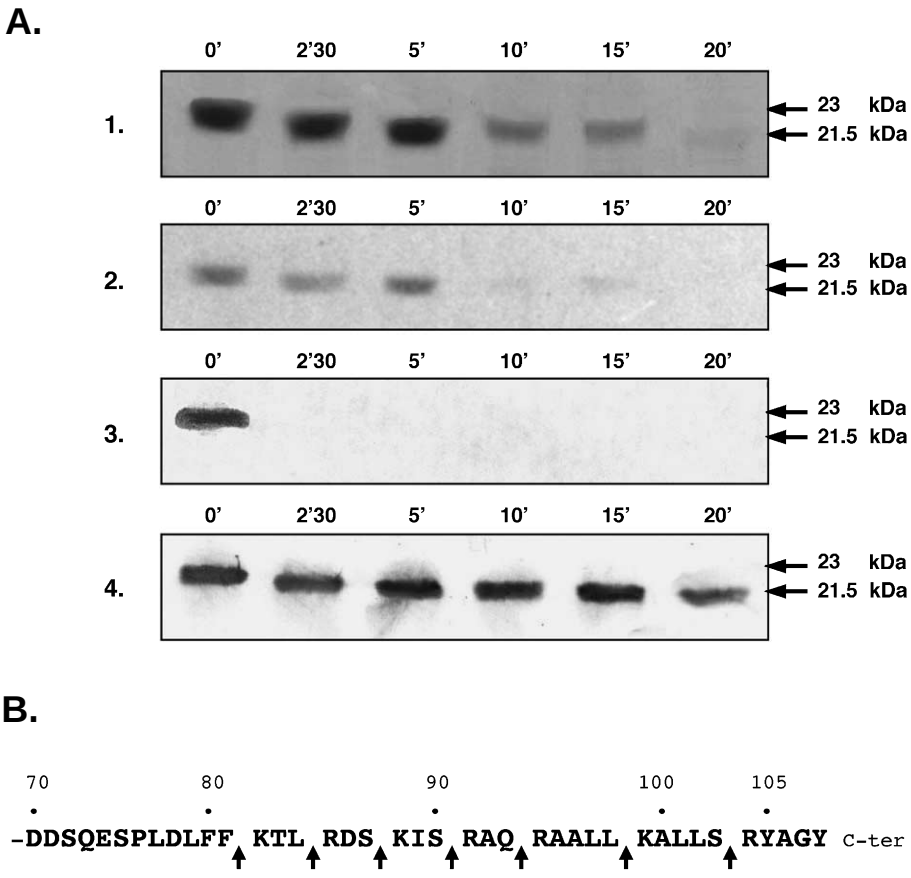

Fig. 5. Phosphorylated tyrosines and calcium binding. A: Degradation kinetics of the C-terminus of Orchestin by carboxypeptidase B. 1: Coomassie blue staining; 2: autoradiography after transfer on PVDF membrane and ^{45}Ca incubation; 3: Western blotting and immunostaining with anti-phospho-Tyr antibody; 4: Western blotting and immunostaining with anti-recombinant Orchestin polyclonal antibodies. B: Location of the carboxypeptidase B cleavage sites in the C-terminal sequence of Orchestin.

lysis (1.5 h; Fig. 3B, 2), known to be in favor of P-Tyr recovery [21]. Finally, results shown in Fig. 3B revealed that Orchestin is phosphorylated on serine residues as predicted, and not on threonine but on tyrosine residues, unlike the prediction.

As the calcium-binding capacity of Orchestin depends on phosphorylations, testing the relative involvement of each phosphorylated residue (Ser or Tyr) was required. We performed specific dephosphorylations and demonstrated a decrease of the electrophoretic mobility (Fig. 4A, lanes 2, 3, 5) and the loss of the calcium-binding ability of Orchestin after Ser dephosphorylation (Fig. 4B, lanes 2, 3, 5). The immunoblot (Fig. 4C) represents a control of the correlation between the dephosphorylated proteins, the calcium-binding proteins and the proteins recognized by the anti-recombinant Orchestin. The role of the tyrosine residues was not really clear because of a residual labelling observed after treatment with the Ser/Thr phosphatase followed by a Tyr phosphatase (Fig. 4D, lane 5), regardless of the Tyr phosphatase used (YOP, LAR or T-cell protein Tyr phosphatase). To clarify this point, we used the bovine carboxypeptidase B, a Lys/Arg carboxypeptidase, to suppress the tyrosine amino acids located at the C-terminus (Fig. 5B). The first cleavage site suppresses the two terminal tyrosine residues (Fig. 5B). We observed a shift after 2.5 min and 5 min digestion (Fig. 5A, 1,2), whereas the cleaved protein still exhibited a calcium-binding activity (Fig. 5A, 2). To ensure that the shift corresponds to the loss of a peptide really containing the two tyrosine residues, we tested anti-phospho-Tyr antibody on Orchestin after carboxypeptidase B treatment. After 2.5 min degradation, the protein no longer exhibits the P-Tyr residues (Fig. 5A, 3). From these results, we can conclude that the phosphorylated tyrosine residues are not involved in the calcium-binding ability of Orchestin. The immunoblot (Fig. 5A, 4) represents a control of the presence of similar amounts of Orchestin in each lane. In addition, it shows that there is a single band per lane. The loss of the calcium-binding ability after 10 min digestion (Fig. 5A, 3) could be explained by an important conformational change of Orchestin induced by its increasing shortening.

While the search for homology or identity with other proteins sequenced so far was unfruitful, the search for consensus motifs known in other calcium-binding proteins did not reveal conserved domains either. Nevertheless, several vertebrate matrix proteins present physico-chemical characteristics shared by Orchestin. Among the organic matrix components of dentin are the dentin phosphoproteins or phosphophoryns (DPP or Dmp2 [22,23]), which are EDTA-soluble acidic calcium-binding phosphoproteins. Phosphophoryns are rich in aspartic acid and phosphorylated serine residues. Furthermore, they exhibit DSS and SD repeats, also found in Orchestin [8]. Dmp1 (dentin matrix protein 1) represents another vertebrate dentin matrix protein [24,25], also found in bone [26], with similar characteristics: they are acidic calcium-binding phosphoproteins rich in Asp, Glu and phosphorylated Ser,

percentages of which are close to those of Orchestin (i.e. 16.7, 13.0 and 18.5%, respectively). DPP and Dmp1, secreted by odontoblasts and/or osteoblasts, are thought to be involved in tooth and bone mineral nucleation and growth [22,24]. Among invertebrates, few acidic soluble proteins associated with biominerals exhibit similarities with Orchestin. Interestingly, RP-1, a molluscan shell protein, is, to our knowledge, the sole example, which emphasizes the role of phosphorylation in calcification: in native conditions, RP-1 was found to be a potent inhibitor of calcium carbonate crystallization [27]. However, dephosphorylation of RP-1 induced a drastic loss of this property. A similar drastic effect on calcium binding is observed with Orchestin.

These features lead us to propose that the main N-terminal domain of Orchestin could be involved in calcium binding and calcium carbonate nucleation. These functions would be performed through the cooperation of the carboxylic groups of the acidic amino acids and the phosphate groups of the phosphorylated serine residues, as suggested before [9]. This would contribute to the formation of the concretion calcified layers. The hydrophobic C-terminus containing the phosphorylated tyrosine residues could be attached to components of the same fraction in the same layers, or to components of the insoluble fraction, in the non-calcified layers. Cyclical repetitions of this spatial organization of Orchestin would contribute to the concentric growth of the concretions.

In conclusion, the same function in the elaboration of calcified structures shared by Orchestin and some vertebrate matrix proteins rests on common physico-chemical features rather than on sequence homologies. Evolutionary convergence of molecular conformations adapted to the same function could represent one way to produce, since the Precambrian period over the animal kingdom, from primitive invertebrates to the mammalians, the so-called biologically controlled mineralizations [28], similar in their basic structures but very diversified in their morphology.

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