Polyphosphoprotein-Containing Marine Adhesives

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Protein phosphorylation is an important regulator of both cellular and extracellular events. Recently, protein phosphorylation has also emerged as an important process in biological adhesives. During the last decade, Herbert Waite and his group have indeed characterized several polyphosphoproteins from the adhesive secretions of two different marine organisms, mussels and tube-building worms. This suggests the possibility that polyphosphoproteins could be important components of several bioadhesives and may, therefore, be widely distributed throughout the animal kingdom. Many amino acids can be targets for phosphorylation but only phosphoserine (pSer) has been detected to date in marine adhesive proteins. We investigated whether monoclonal antibodies directed against pSer could be used to specifically label polyphosphoproteins in marine adhesives. Antibodies were applied on histological sections through the foot of the mussel Mytilus edulis *and through the building organ of the tube-worm* Sabellaria alveolata*. In both cases, anti-pSer binding was detected in the adhesive glands (phenol gland and cement gland, respectively). However, the intensity of the immunolabeling was different between the two species, being weak in the former and strong in the latter. With the use of these antibodies, a new pSer-rich bioadhesive has been detected in Cuvierian tubules, the sticky defense organs of sea cucumbers. Immunoblots and amino acid analyses confirmed the presence of polyphosphoproteins in the adhesive secretion of the Cuvierian tubules from three species of sea cucumber. These findings bring to three the number of animal groups in which adhesive processes involve polyphosphoproteins and raise interesting questions about the convergent evolution of these adhesives.*

"Adhesion is a way of life in the sea"
Herbert Waite (1983)

1. INTRODUCTION

Protein phosphorylation is an important regulator of cellular and extracellular events. Within the cell, the phosphorylation or dephosphorylation of a protein can alter its behaviour in almost every conceivable way. These include modulation of its intrinsic biological activity, subcellular location, half-life, and docking with other proteins [1]. Regulation of proteins by phosphorylation is, therefore, one of the most common modes of regulation of protein function and of intracellular signal transduction. Within the extracellular matrix, many of the acidic non-collagenous proteins of mineralized tissues are frequently phosphorylated. These phosphorylated proteins have been implicated in various aspects of the mineralization process, though the exact functions of the phosphorylation are not well understood [2]. Recently, protein phosphorylation has also emerged as an important process in biological adhesives [3]. During the last decade, Herbert Waite and his group have indeed characterized several polyphosphoproteins from the adhesive secretions of two marine organisms, mussels and tube-building worms [4–7].

Many amino acids, including Ser, Thr, Tyr, His, and Asp, can be targets for phosphorylation but only phosphoserine (pSer) has been detected to date in marine adhesive proteins [3]. pSer is essentially a monophosphoester with two ionizable hydroxyls at pK_1 2.1 and pK_2 6.5. At the pH of seawater (8.2), both are largely charged, rendering this residue and the sequence surrounding it highly polar (Fig. 1A [3]). In the adhesive secretions of marine invertebrates, phosphorylation is thought to impart a potential for both cohesive (by Ca^{++} bridging) and adhesive contributions to the glue [7,8]. In the latter case, it has been proposed that phosphorylation could be an adaptation for adhesion to calcareous substrata [4]. Moreover, adhesive protein condensation in secretory granules may depend on a process called complex coacervation, in which oppositely charged proteins and divalent cations coalesce and condense into an electrically neutralized dense phase [5,6]. Secretion is accompanied by a jump in pH from ∼5 in the secretory granule to 8.2 in seawater that could trigger a change in bonding between Ca^{++} and phosphate from electrostatic to ionic, the effect of which would be to harden spontaneously and solidify the adhesive (Fig. 1A, [3]). Finally, pSer may
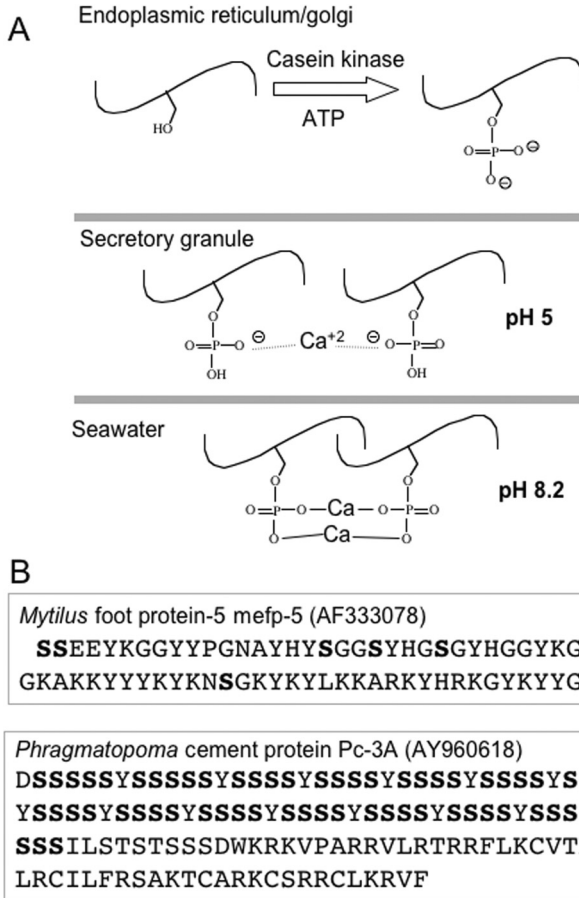
FIGURE 1 (A) Formation and (B) occurrence of phosphoserine in secreted polyphosphorylated adhesive proteins from marine organisms (reprinted from [3] with permission). (A) Phosphorylation requires ATP and casein kinases and occurs in the endoplasmic reticulum and perhaps in the Golgi. In the mature secretory granules, H^+ pumping ATPases drop the pH to about 5 and Ca^{++} is accumulated approaching mmol/L concentrations. Upon secretion into seawater the pH jumps to 8.2. The Ca^{++} and phosphate interactions thus go from mostly electrostatic at pH 5 to ionic bonds at pH 8. (B) Sequences of two polyphosphoproteins, Mefp-5 and Pc-3A (with GenBank accession numbers). Targeted serines are highlighted in bold.

also be involved in protein-protein cross-linking: it is thought to condense with His to form histidinoalanine crosslinks with the loss of phosphate [9].

To secure themselves to the substratum, mussels produce an extra-organismic holdfast, the so-called byssus [10]. The byssus consists of a bunch of proteinaceous filaments connecting the animal to the substratum. Each filament is made up of a proximal thread, functioning as a mooring line, and a distal attachment plaque, securing the filament on the substratum. A complex blend of proteins (the foot proteins, or fps) constitutes the attachment plaques and, to date, six specialized adhesive proteins have been identified in byssal plaques: fp-1, -2, -3, -4, -5, and -6 [11,12]. Among fps, fp-5 is a relatively small protein with a molecular mass of about 9 kDa, presenting a basic pI, and containing pSer and another modified amino acid, DOPA [11]. It was first identified from *Mytilus edulis* (Mefp-5, Fig. 1B [4]) and, subsequently, was also reported to be present in *M. galloprovincialis* (Mgfp-5 [13]) and *M. californianus* (Mcfp-5 [7]). The protein appears to be distributed near the plaque-substratum interface [11] and is usually variably phosphorylated in that the isolated protein is a mixture of variants having between 0 and 8 pSer [4,7]. Another fp, Mcfp-6 is the most recent adhesive-related byssal plaque protein identified from *M. californianus* [7]. This small protein (11.6 kDa) is also polyphosphorylated (6 pSer residues). Its suggested role in adhesion may be to provide a link between the different fps present in byssal attachment plaques [7].

Sabellariids are tube-dwelling marine polychaetes which live in the intertidal zone. They are commonly called honeycomb worms or sandcastle worms because they are gregarious and the tubes of all individuals are closely imbricated to form large reef-like mounds [5,14]. To build their tube, they collect sand grains or mollusk shell fragments in their surroundings, dab them with spots of cement, and assemble them into a rigid composite tube. The cement of sabellariids such as the California sandcastle worm *Phragmatopoma californica* is comprised of at least three proteins, referred to as Pc1-3, and significant amounts of Mg^{2+} and Ca^{2+} [6,15]. There may be additional as-yet unidentified components. Among these proteins, Pc3 is an unusual hyperphosphorylated protein which exists in at least two major variants, Pc-3A (13.9 kDa) and Pc-3B (30.5 kDa) (Fig. 1B). Both variants are rich in serine (72.9 mol %) and careful calculations indicated that up to 90% of these serine residues are phosphorylated [6]. Pc3 is, therefore, a remarkably acidic protein (pI 0.5–1.5).

The occurrence of polyphosphoproteins in two unrelated adhesive systems suggests the possibility that such proteins could be important components also in other bioadhesives, and may, therefore, present a wider distribution within the animal kingdom. In this article, we

report that monoclonal antibodies directed against pSer can be used to specifically label polyphosphoproteins in marine adhesives. With the use of these antibodies, a new polyphosphoprotein-containing bio-adhesive is pinpointed in the Cuvierian tubules, the peculiar sticky defense organs of sea cucumbers. The presence of polyphosphoproteins in the adhesive secretion of the Cuvierian tubules from three species of sea cucumbers is further confirmed by immunoblots and amino acid analyses.

2. MATERIALS AND METHODS

Mussels (*Mytilus edulis* Linnaeus, 1758) were collected intertidally at Audresselles (Pas-de-Calais, France) and honeycomb worms (*Sabellaria alveolata* Linnaeus, 1767) were sampled from the Champeaux reef located in the eastern part of Mont-Saint-Michel Bay (Manche, France). Sea cucumbers were collected at depths ranging from 10 to 30 m by scuba diving: individuals of *Holothuria forskali* (Delle Chiaje, 1823) at Banyuls-sur-Mer (Pyrénées-Orientales, France), and specimens of *Bohadschia subrubra* (Quoy & Gaimard, 1833) and *Pearsonothuria graeffei* (Semper, 1868) at the south point of the Great Reef of Toliara (Madagascar).

2.1. Immunohistochemistry

Mussel feet, tubeworm anterior parts, and sea cucumber Cuvierian tubules were fixed in Bouin's fluid, dehydrated in graded ethanol, embedded using a routine method in paraffin wax, and sectioned at 7 μm. A few sections were stained with Heidenhain's azan trichrome stain [16]; the others were subjected to an indirect immunohistochemical staining method according to the following protocol. Non-specific background staining was blocked by section pre-incubation in phosphate-buffered saline containing 0.5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) (PBS-BSA). Monoclonal anti-phosphoserine antibodies (clone PSR-45, mouse ascites fluid; Sigma-Aldrich, St. Louis, ref. P 3430), diluted 1:5000 in PBS-BSA, were then applied for 1 h at room temperature. After several washes in PBS, the sections were incubated for 30 min in biotinylated goat anti-mouse immunoglobulins (Dako, Glostrup, Denmark) diluted 1:50 in PBS-BSA. Following a final wash in PBS, immunoreactivity was visualized by an immunoperoxidase technique using the avidin–biotin peroxidase complex (Dako, Glostrup, Denmark) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO, USA) as a chromogen substrate. The sections were lightly

counterstained with hemalum and luxol blue. Alternatively, FITC-conjugated goat anti-mouse immunoglobulins (Dako, Glostrup, Denmark) were used as secondary antibodies and the sections were observed in epifluorescence. Control reactions were performed by substituting the primary antibody with PBS-BSA and/or by using the primary antibody saturated with its specific antigen (antibodies diluted in PBS containing 0.5% casein [Sigma-Aldrich, St. Louis], a milk polyphosphoprotein). Sections were observed with a Zeiss Axio-scope A1 microscope (Carl Zeiss MicroImaging, Göttingen, Germany).

2.2. Collection of Cuvierian Tubule Adhesive and Amino Acid Analysis

The discharge of the Cuvierian tubules was induced mechanically by pinching the dorsal integument of sea-cucumbers with forceps. The expelled tubules (usually between 10 and 20) were collected in clean glass Petri dishes (diameter 15 cm) filled with filtered seawater (0.22 μm). After the tubules adhered firmly on the bottom of the Petri dishes, their collagenous cores were detached manually using fine forceps [17]. The Petri dishes were then thoroughly rinsed in distilled water and freeze-dried. The lyophilized tubule print material (TPM) was scraped off using a glass knife and stored at $-20°C$.

Samples of TPM were suspended in 6 M HCl with 5% phenol and hydrolyzed under vacuum in sealed tubes for either 2 or 20 h at 110°C. Amino acid concentrations were measured on a Beckman 6300 amino acid analyzer (Beckman Instruments, Fullerton, CA, USA). The analyser was calibrated using a standard amino acid mixture and an O-phosphoserine standard (Sigma-Aldrich; St. Louis).

2.3. Electrophoresis and Immunoblotting

Proteins were extracted from dissected Cuvierian tubules and TPM according to the procedure developed by Kamino *et al.* [18] to characterize the barnacle cement, with minor modifications. Briefly, weighted quantities of tubules and TPM were suspended in a 10 mM sodium phosphate buffer (pH 6.0) containing 6 M GdnHCl. After centrifugation, the supernatant ("Fraction 1", F1) was collected and the pellet was re-suspended in a 1.5 M Tris-Cl buffer (pH 8.5) containing 7 M GndHCl, 0.5 M DTT, and 20 mM EDTA at 60°C for 1 h. After centrifugation, the resulting supernatant was recovered as "Fraction 2" (F2). Both fractions were dialysed against 1% acetic acid at 4°C before being evaporated to a minimal volume. They were then suspended in a sample buffer containing 6 M urea and 2% βMSH, heated for 2 min at

90°C, and loaded onto two 8% SDS–PAGE gels. As a positive control, 0.5 μg of casein were also loaded onto the gels. After electrophoresis, one of the gels was stained with Coomassie R-250 stain.

Proteins separated on the other gel were blotted onto a PVDF membrane using 90 mM Tris-borate, 2.5 mM EDTA, 0.1% SDS, and 25% methanol as transfer buffer. The membrane was washed with Tris-buffered saline containing 0.05% Tween (TBS-Tween) and then blocked overnight at 4°C in TBS-Tween with 3% BSA (TBS-Tween-BSA). The membrane was incubated for 1h30 with monoclonal anti-pSer antibodies diluted 1/5000 in TBS-Tween-BSA. After several washes in TBS-Tween, peroxidase-conjugated anti-mouse immuno-globulins (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) diluted 1:10000 in TBS-Tween-BSA were applied for 1h. Finally, the membrane was washed again in TBS-Tween and immunoreactive bands were visualized by an ECL immunoblot detection system (GE Health-care Bio-Sciences, Piscataway).

3. RESULTS

3.1. Histology and Imunohistochemistry of the Adhesive Organs

To investigate whether the monoclonal antibodies directed against pSer (clone PSR-45, mouse ascites fluid; Sigma-Aldrich, P 3430) could be used to specifically label polyphosphoproteins in marine adhesives, they were applied on paraffin sections through the adhesive organs of the mussel *Mytilus edulis* and of the sabellariid polychaete *Sabellaria alveolata*. The antibodies were then used on sections of the Cuvierian tubules of three species of sea cucumbers. In all species, a specific anti-pSer binding was detected in the adhesive organs. Although no quantitative measurements were made, the immunolabeling appeared to be much stronger in tube-worms and sea cucumbers than in mussels. Except for mussel feet (see below), no labeling was observed on sections in which the primary antibody was omitted and all labeling was suppressed by pre-incubation of the antibodies with the poly-phosphoprotein casein.

3.1.1. Mussels

Mussel byssal filaments are produced by the foot of the animal, their different constituting proteins being secreted and assembled into a groove running along the ventral side of the foot [19]. Four glands, distributed along the foot, contribute to the formation of the filaments: the mucous gland, the phenol gland, the accessory gland, and the
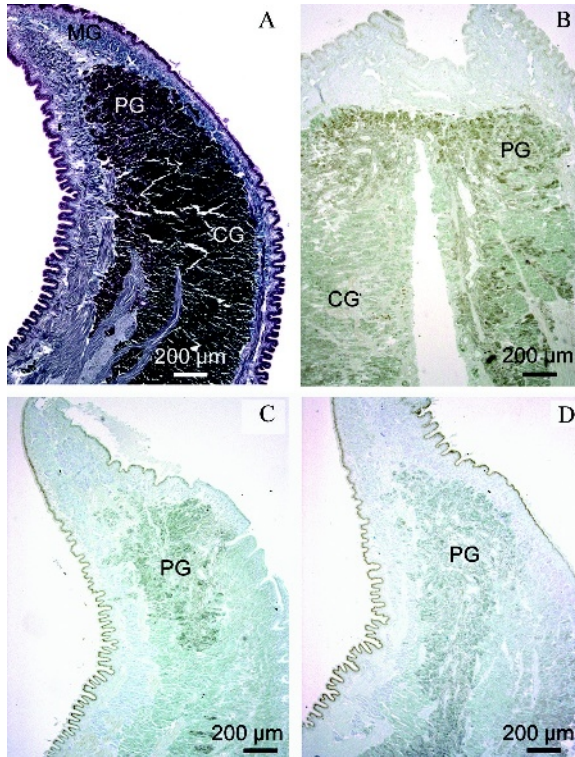
FIGURE 2 (A, C, D) Sagittal and (B) longitudinal sections through the foot of the mussel *Mytilus edulis*. (A) Tip of the foot showing the different glands involved in byssus formation. (B) Immunoperoxidase labeling of the foot with anti-pSer antibodies (immunoreactive cells are labeled in brown). (C, D) Feet immunolabeled with anti-pSer antibodies and with anti-pSer antibodies preincubated with casein, respectively (immunoperoxidase method). *Abbreviations:* CG, collagen gland; MG, mucous gland; PG, phenol gland.

collagen gland ([10]; Fig. 2). Since a previous study localized the polyphosphoproteins in the attachment plaque, fp-5 and fp-6 are probably synthesized and released by the phenol gland [7]. This gland is indeed located at the tip of the foot and caps the distal depression of the groove in which the attachment plaque is formed [10,19]. In *M. edulis*, the phenol gland is the only gland whose content is lightly immunolabeled with anti-pSer antibodies (Fig. 2B, C), suggesting that it is presumably the site where Mefp-5 is produced and stockpiled (no fp-6 has been described to date in *M. edulis*). This immunostaining is difficult to detect because the phenol gland already appears light

brown in the control sections (Fig. 2D), probably due to the oxidation of DOPA-containing proteins. Moreover, the phenol gland is strongly autofluorescent, precluding the use of immunofluorescence (results not shown). With the azan trichrome, the secretory cells of the phenol gland are stained in red (Fig. 2A; see also [20]).

3.1.2. Tube-Worms

In sabellariid tube-worms, the cement that holds the composite tube together is secreted at the level of a pulpy, crescent-shaped bilobed organ near the mouth, the so-called building organ [21]. The two lobes of the building organ are only the external part of a complex secretory organ made up of bouquets of cement cells located deep within the thorax of the worms ([14]; Fig. 3A, B). According to the literature, these
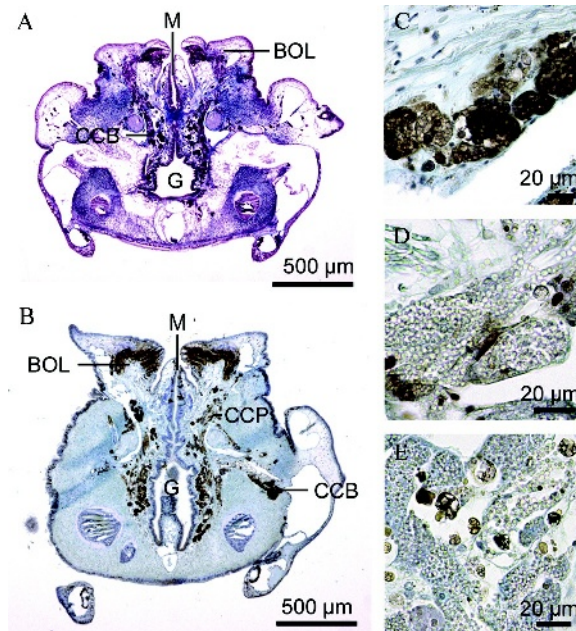
FIGURE 3 Adhesive glands of the tube-worm *Sabellaria alveolata*. (A, B) Transverse sections through the thorax of the worm stained with azan trichrome and immunolabeled with anti-pSer antibodies (immunoperoxidase method), respectively. (C-E) Immunoperoxidase labeling of clusters of cement cell bodies with the anti-pSer antibodies, with the anti-pSer antibodies preincubated with casein, and with PBS (antibodies omitted), respectively. *Abbreviations:* BOL, building organ lobe; CCB, cement cell bodies; CCP, cement cell processes; G, gut; M, mouth.

cells produce morphologically distinct secretory granules, described as homogeneous and heterogeneous [14]. The secretory granules travel up through duct-like extensions of the cement cells to the tip of the building organ lobes where their contents are applied as small dabs to the mineral particles held in the building organ. In *S. alveolata*, the cement cells are strongly and specifically labeled with the anti-pSer antibodies (Fig. 3B–E). This suggests that, in this species, a polyphosphoprotein homologous to Pc-3 would be present in the cement gland secretory granules. The extensive anti-pSer staining of the granules allows one to follow their path within the cement cells, from the basal cell bodies through their processes up to the lobes of the building organ (Fig. 3B). Cement cell granules stain bright red with the azan trichrome (Fig. 3A).

3.1.3. Sea Cucumbers

Cuvierian tubules are present in several species of sea cucumbers, in which they occur in great numbers in the posterior part of the body cavity [22]. Cuvierian tubules consist of, from the inside to the outside, an epithelium surrounding the narrow lumen, a thick connective tissue layer and a mesothelium lining the surface of the tubule that is exposed to the coelomic cavity ([22]; Fig. 4A). The mesothelium is responsible for adhesion. In quiescent tubules, it is a pseudostratified epithelium made up of three superposed cell layers; an outer layer of peritoneocytes, a middle layer of granular cells which is highly folded along the long axis of the tubule, and, in some species, an inner layer of vacuolar cells ([22,23]; Fig. 4D). It was previously demonstrated that granular cells are filled with densely packed secretory granules enclosing a proteinaceous material [17]. They stain red with the azan trichrome (Fig. 4B, D). In the three species considered, Cuvierian tubules are specifically immunolabeled with the anti-pSer antibodies (Fig. 4). The anti-pSer staining is entirely restricted to the mesothelium. Granular cells present the strongest labeling but other mesothelial cell types, the peritoneocytes and the vacuolar cells, also show some anti-pSer binding (Fig. 4E, G).

3.2. Composition of the Cuvierian Tubule Adhesive

3.2.1. Amino Acid Composition

The amino acid compositions of the protein fraction in *H. forskali* and *B. subrubra* indicate that their adhesives are closely related (Table 1). Both are rich in small side-chain amino acids, especially glycine, and in charged and polar amino acids (see also [22]). For the two species, a peak eluted with a retention time identical to that of pure pSer.
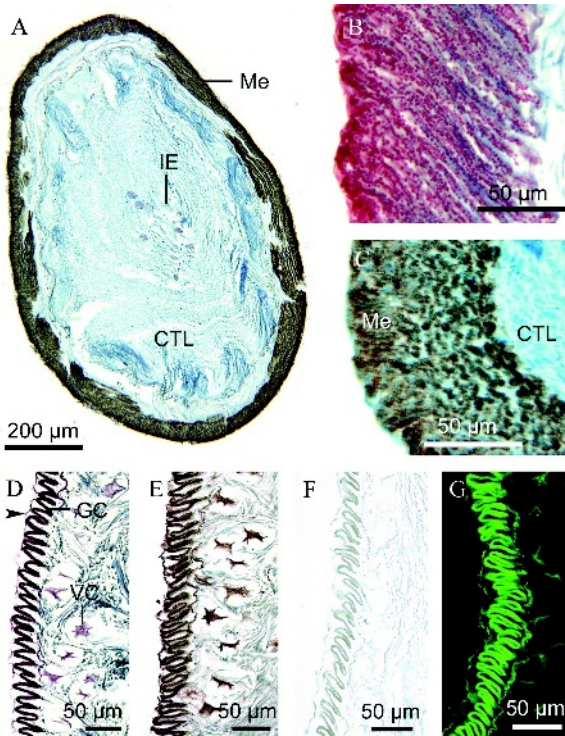
FIGURE 4 Cuvierian tubules of the sea cucumbers (A) *Holothuria forskali*, (B, C) *Pearsonothuria graeffei*, and (D–G) *Bohadschia subrubra*. (A) Immunoperoxidase labeling of a transverse section through a tubule. (B, C) Longitudinal and transverse sections through the mesothelium stained with azan trichrome and immunolabeled with anti-pSer antibodies (immunoperoxidase method), respectively. (D–G) Longitudinal sections through the mesothelium, respectively stained with azan trichrome, immunolabeled with anti-pSer antibodies (immunoperoxidase method), immunolabeled with anti-pSer antibodies preincubated with casein (immunoperoxidase method), and immunolabeled with anti-pSer antibodies (immunofluorescence method). The arrowhead indicates mesothelial peritoneocytes. *Abbreviations*: CTL, connective tissue layer; IE, inner epithelium; GC, granular cells, Me, mesothelium; VC, vacuolar cells.

When converted to mol %, this peak amounted to about 1.8 pSer residues per 100 residues whatever the species considered (Table 1). Unexpectedly, however, this peak did not increase with decreasing hydrolysis time. On the contrary, after 2 h hydrolysis the amount of pSer was reduced to 0.7 mol % in *H. forskali* and to 1.4 mol % in *B. subrubra*.

TABLE 1 Amino Acid Compositions in mol % (Residues Per 100 Residues) Determined by Amino Acid Analysis of Adhesive Prints from the Cuvierian Tubules of Two Sea Cucumber Species (20 h Hydrolysis)

Amino acid	*Holothuria forskali*	*Bohadschia subrubra*
pSer	1.86	1.83
Asx	7.43	6.87
Thr	6.02	8.26
Ser	6.26	7.50
Glx	10.07	9.05
Pro	6.67	4.65
Gly	28.20	24.00
Ala	10.06	7.86
Cys/2	0.00	0.00
Val	3.30	4.21
Met	0.00	0.00
Ile	2.41	4.50
Leu	3.57	4.40
Tyr	1.14	1.50
Phe	1.68	2.58
His	1.86	2.38
hLys	0.41	0.22
Lys	3.78	3.81
Arg	4.53	4.02

3.2.2. Protein Composition

Considering the two successive extracts (F1 and F2) together, the SDS-PAGE analysis revealed that whole tubule extracts were composed of about 25 major protein bands in *H. forskali* and 15 in *B. subrubra* (Fig. 5A). As for TPM extracts, they separated into about 15 major protein bands in *H. forskali* and seven in *B. subrubra*. In the two species, and whatever the fraction considered, all the protein bands from TPM extracts corresponded to protein bands present in tubule extracts. Differences were found between the two fractions. Indeed, the SDS-PAGE analysis of F2 revealed bands already found in F1 but also other additional bands. For instance, in *H. forskali*, the 98 kDa, 110 kDa, and 480 kDa bands were observed in the two fractions while the others were specific to each fraction. In *B. subrubra*, only the 32 kDa band was present in the two fractions. Based on the band pattern, it was difficult to show protein similarities between the two species. However, the 45, 68, 98, and 110 kDa bands present in F2 appear to be common to the two species as well as to the two samples, whole tubules and TPM.

After their separation by SDS-PAGE, tubules and TPM extracts were blotted onto PVDF membranes and probed for phosphoproteins
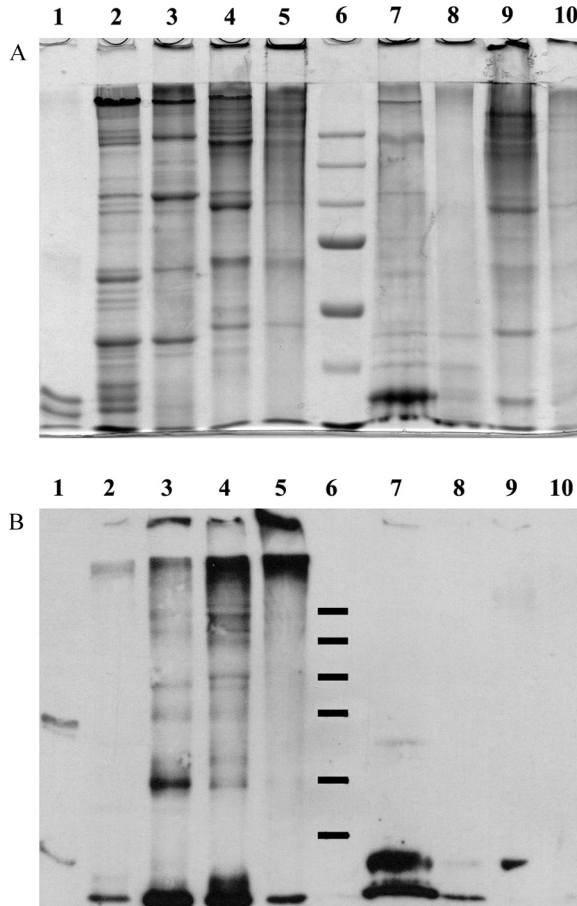
FIGURE 5 Proteins and phosphoproteins from the Cuvierian tubules and TPM of *Holothuria forskali* and *Bohadschia subrubra* identified by (A) SDS-PAGE and (B) Western blot, respectively. (A) is stained for protein using Coomassie Blue R-250 and (B) is immunostained with anti-phosphoserine antibodies. Lane 1: 0.5 µg casein. Lanes 2 and 3: F1 from the tubules and TPM of *H. forskali*, respectively. Lanes 4 and 5: F2 from the tubules and TPM of *H. forskali*, respectively. Lane 6: molecular weights (from top to bottom: 250, 150, 100, 75, 50, and 37 kDa). Lanes 7 and 8: F1 from the tubules and TPM of *B. subrubra*, respectively. Lanes 9 and 10: F2 from the tubules and TPM of *B. subrubra*, respectively.

using the monoclonal anti-pSer antibodies (Fig. 5B). Several labeled bands were present in the extracts from the two species, though many more were found for *H. forskali*. Among them, the most strongly

labeled are a protein of 49 kDa and a protein located just above the migration front. Most positive bands were highly immunostained, presenting a labeling equal to or more intense than that of casein bands. In *H. forskali*, a similar pattern of immunoreactive bands was found in TPM F1 and in whole tubules F2. Moreover, an intense labeling was also observed at the top and the bottom of the lanes, corresponding, respectively, to the bottom of the loading wells and the migration front, as well as at the interface between the stacking and running gels. Most of these labeled bands were undetectable on gels stained with Coomassie blue (compare Fig. 5A and B). In *B. subrubra*, the antibodies labeled the 32 kDa protein in the tubule extract and in the TPM extract. A faint labeling was also found for the 62 kDa protein from whole tubules F1.

4. DISCUSSION

To date, polyphosphoproteins have been described in the adhesives of two different marine organisms: mussels and sabellariid tube-worms [3]. Although these proteins have in common the presence of many phosphorylated serine residues in their sequence, they appear to be unrelated phylogenetically. Yet, this shared post-translational modification allows the use of a single anti-pSer monoclonal antibody to specifically label these otherwise non-homologous proteins. In the mussel *M. edulis*, the anti-pSer staining is weak and restricted to the phenol gland at the tip of the foot, indicating that this gland is the location where Mefp-5 is presumably produced and stockpiled. In *M. californianus*, it was already suggested that the plaque proteins Mcfp-5 and Mcfp-6 would originate from the phenol gland [7]. In the tube-worm *S. alveolata*, an extensive immunolabeling is observed in the thoracic cement glands as well as in their processes located in the building organ. No information is available about the composition of the cement in this species but, in the light of cement composition in the closely-related species *P. californica* [6], it is very likely that the cement cells of *S. alveolata* would secrete a polyphosphoprotein homologous to Pc-3. Anti-pSer antibodies, therefore, appear as a powerful tool to specifically label adhesive polyphosphoproteins. Moreover, the intensity of the labeling is seemingly proportional to the pSer content of the adhesive. Indeed, sabellariid cement, which contains hyperphosphorylated proteins and in which pSer accounts for more than a quarter of the amino acids in the whole cement [6], is the most strongly immunolabeled adhesive. On the other hand, labeling of the mussel phenol gland is weak, presumably in relation with the low pSer content in the byssal adhesive plaque as a whole (J.H. Waite, personal communication).

The use of the same antibodies on sea cucumber Cuvierian tubules reveals that their adhesive is also rich in pSer residues, a strong immunolabeling being detected in the adhesive cells from the tubules of three different species (*B. subrubra, H. forskali,* and *P. graeffei*). These three species possess sticky Cuvierian tubules that function in defense against predators [24]. Amino acid analyses further confirmed the presence of pSer in the tubule print material (secreted adhesive) of two of these species, *B. subrubra* and *H. forskali*, which both contain about 2% pSer (value measured after 20 h hydrolysis). However, contrary to what occurs in both mussel and tube-worm adhesives [4,5], shorter hydrolysis times do not increase the pSer yield. The most plausible explanation for this unexpected behavior is that pSer residues could be shielded from hydrolysis in the Cuvierian tubule adhesive.

Cuvierian tubule secreted adhesive consists mostly of proteins [17]. In this study, a two-step extraction procedure was used to solubilize the Cuvierian tubule adhesive of *B. subrubra* and *H. forskali*, which separated into about seven protein bands in the former and 15 in the latter. Among those proteins, four appear to be common to both species. The protein band pattern obtained in *H. forskali* is similar but not identical to that reported by De Moor *et al.* [17] for the same species. Differences in the apparent molecular weight of the proteins could, however, be explained by the different extraction and separation protocols used. When the proteins were transferred to PVDF membranes and probed for pSer residues with the monoclonal antibodies, several immunoreactive bands were detected, indicating the presence of phosphorylated proteins in the adhesive. Among those, two proteins with apparent molecular masses of 32 and 49 kDa were particularly conspicuous in the adhesive extracts from *B. subrubra* and *H. forskali*, respectively. The intensity of the immunolabeled bands compared with that of casein suggests that several Cuvierian tubule adhesive proteins would be polyphosphoproteins. Moreover, comparison between the number of immunoreactive bands and the total number of proteins in the different extracts shows that the 2% pSer residues measured in the whole adhesive would actually originate from a low number of polyphosphorylated proteins.

The occurrence of polyphosphoproteins in the adhesive of sea cucumber Cuvierian tubules indicates that it could share some molecular mechanisms with the adhesives of mussels and sabellariid tube-worms, even though they represent different types of adhesion. Indeed, mussel byssal plaques and sabellariid cement are both permanent adhesives which are initially secreted as fluids and then gradually solidify to form glues possessing high adhesive and cohesive

strength [22]. Curing involves another modified amino acid that mussel and polychaete adhesives have in common in their adhesive proteins, DOPA [6]. Cuvierian tubules, on the other hand, are a typical example of instantaneous adhesion, a type of adhesion relying on single-use organs or cells which are used in functions requiring a very fast formation of adhesive bonds, such as prey capture and defense reactions [22]. Instantaneous adhesives are not designed to last and do not appear to be cured [24,25]. Yet, many of the functions proposed for polyphosphoproteins in the adhesives of mussels and tube-worms may apply to their equivalents in Cuvierian tubules as well. For instance, the three adhesives present short setting times: less than 1 min in mussels [10], less than 30 s in sabellariids [26], and less than 10 s in sea cucumber tubules [27]. In *P. californica*, it has been proposed that the setting mechanism likely exploits the pH differential between secretory granules (pH 5) and seawater (pH 8.2) [5,26], the potential pH trigger mechanism being a change in the nature of the bonding between Ca^{++} with the phosphate sidechains of Pc-3 from electrostatic interactions to stronger more specific ionic interactions at the higher pH of seawater [3]. Although the involvement of calcium in Cuvierian tubule adhesion has never been investigated, it has already been demonstrated that tubule adhesiveness is strongly dependent on salinity and pH, being always maximum at the values of seawater [24,25].

From a morphological point of view, the secretory granules of the adhesive cells are strikingly similar in the mussel phenol gland, the tube-worm building organ, and the sea cucumber Cuvierian tubules. In light microscopy, they stain with acidic dyes such as eosin or azo-carmin [14,20, present work], while in transmission electron micro-scopy they reveal a homogeneous electron-dense content [14,23,28]. Adhesive granules with an acidophilic and osmiophilic content are relatively common in the animal kingdom [29], suggesting that polyphosphoprotein-containing marine adhesives might be even more widely distributed. A comparative immunohistochemical survey using anti-pSer antibodies is currently in progress to assess the distribution of polyphosphorylated proteins in the adhesive organs of marine invertebrates.

5. CONCLUSION

In the marine environment, attachment mechanisms developed by animals usually rely on highly viscous or solid adhesive secretions, which all contain specialized proteins. Functional convergences are noted among marine animals, particularly in terms of the type of

adhesion used: permanent, temporary, or instantaneous. Although marine adhesive proteins from non-related organisms do not present any sequence homologies, molecular convergences have been recognized, and some adhesive motifs have been found to be shared by phylogenetically different animals. DOPA has long been known as one such motif. Now, another modified amino acid, phosphoserine (pSer), is emerging as an important motif in biological adhesives. Indeed, our findings bring the number of polyphosphoprotein-containing marine adhesives to three. The occurrence of high levels of pSer in adhesive systems from totally unrelated animals, which moreover use different types of adhesion, raise questions about the convergent evolution of these adhesives.

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