Published in final edited form as:

Biol Bull. 2016 June ; 230(3): 233–242. doi:10.1086/BBLv230n3p233.

Localization of Phosphoproteins within the Barnacle Adhesive Interface

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Abstract

Barnacles permanently adhere to nearly any inert substrate using proteinaceous glue. The glue consists of at least ten major proteins, some of which have been isolated and sequenced. Questions still remain about the chemical mechanisms involved in adhesion and the potential of the glue to serve as a platform for mineralization of the calcified base plate. We tested the hypothesis that barnacle glue contains phosphoproteins, which have the potential to play a role in both adhesion and mineralization. Using a combination of phosphoprotein-specific gel staining and Western blotting with anti-phosphoserine antibody, we identified multiple phosphorylated proteins in uncured glue secretions from the barnacle *Amphibalanus amphitrite*. The protein composition of the glue and the quantity and abundance of phosphoproteins varied distinctly among individual barnacles, possibly due to cyclical changes in the glue secretion over time. We assessed the location of the phosphoproteins within the barnacle glue layer using decalcified barnacle base plates and residual glue deposited by reattached barnacles. Phosphoproteins were found throughout the organic matrix of the base plate and within the residual glue. Staining within the residual glue appeared most intensely in regions where capillary glue ducts, which are involved in cyclical release of glue, had been laid down. Lastly, mineralization studies of glue proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated that proteins identified as phosphorylated possibly induce mineralization of calcium carbonate (CaCO₃). These results contribute to our understanding of the protein composition of barnacle glue, and provide new insights into the potential roles of phosphoproteins in underwater bioadhesives.

Introduction

Barnacles are sessile crustaceans that firmly attach to a wide variety of surfaces. They adhere by secreting a proteinaceous glue that cures underwater and forms an insoluble layer (Kamino, 2006, 2008). The ability of barnacles to strongly adhere to nearly any submerged

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substrate poses significant challenges for the maritime and defense industries. Barnacles on ships cause corrosion and increase drag, decreasing performance and increasing fuel consumption (Sangeetha *et al.*, 2010; Callow and Callow, 2011). The ability of barnacle glue to cure underwater is of interest to researchers who are developing surgical adhesives. Hence, understanding the mechanisms of underwater adhesion in barnacles may inform the development of biologically inspired adhesives (Yang *et al.*, 2011; Brubaker and Messersmith, 2012), and may be used to develop coatings that reduce barnacle adhesion (*i.e.*, foul-release and antifouling coatings).

The protein fraction of barnacle glue (~90% of dry glue) is an aggregate of at least 10 major proteins, a portion of which have been isolated and sequenced (Barnes and Black stock, 1976; Kamino *et al.*, 1996, 2000; Naldrett and Kaplan, 1997; Kamino, 2001, 2006, 2008, 2010; Dickinson *et al.*, 2009; Nakano and Kamino, 2015). The major component of the glue is a cross-linked, filamentous meshwork of proteins that comprise the bulk of the adhesive (Kamino *et al.*, 2000; Dickinson *et al.*, 2009; Barlow *et al.*, 2010). Proteins in the bulk fibrils adopt beta-sheet conformation, and are amyloid-like (Barlow *et al.*, 2009, 2010). The interfacial layers of the glue facing the calcitic base plate and the substrate have a smooth appearance, which is very different from the bulk of the glue (Sangeetha *et al.*, 2010); a different suite of proteins are likely to be localized in these regions (Kamino, 2008, 2010). Proteins thought to be involved in substrate binding are dominated by the amino acids, Ser, Thr, Gly, Ala, Lys, and Val (Kamino *et al.*, 1996; Urushida *et al.*, 2007), and proteins believed to integrate the glue with the calcareous base are charged, that is, rich in His, Glu, and Asp (Kamino, 2001; Mori *et al.*, 2007).

Thickness, structural organization, chemical composition, and mechanical properties of barnacle glue vary depending on substrate chemistry and stiffness (Berglin *et al.*, 2001; Berglin and Gatenholm, 2003). When barnacles are grown on typical substrates in the marine environment, as well as on polystyrene, poly(methyl methacrylate; PMMA), poly(vinyl chloride; PVC), and glass, they produce a thin glue layer less than a micron in thickness (Wahl *et al.*, 2010). On a PMMA surface, the thin glue contains calcite particles (Berglin and Gatenholm, 2003). Yet when they are grown on (poly)dimethylsiloxane; PDMS) and certain proprietary commercial coatings, some barnacles deposit a thick, soft adhesive layer that does not contain calcite (Berglin and Gatenholm, 2003). At least on certain silicone coatings, adhesive tenacity is reduced in barnacles that express the soft glue phenotype (Holm *et al.*, 2005). The soft glue phenotype is heritable, and specific families of barnacles produce soft glue on different silicone surfaces (Holm *et al.*, 2005). Production of soft glue is attributed to the low modulus of silicones (Berglin and Gatenholm, 2003; Wiegmann and Watermann, 2003) and to disruption of glue curing by an alteration of activity of the enzymes involved in curing (Rittschof *et al.*, 2011).

While the majority of research on barnacle glue focuses on characterizing isolated and fully cured glue, a growing number of researchers have shifted their attention to analysis of glue as it cures and/or while *in situ* (Barlow *et al.*, 2009; Dickinson *et al.*, 2009; Burden *et al.*, 2012, 2014). Burden *et al.* (2012) showed that barnacle adhesion involves at least two secretions. The first, Barnacle Cement Secretion 1 (BCS1), is a non-fluorescent protein mixture that is released as the barnacle cuticle expands. Expansion is continuous and the

secretion is likely due to a release of body fluids from the barnacle beneath the cuticle (Burden *et al.*, 2014). The second secretion, BCS2, is fluorescent with ultraviolet (UV) excitation, and is released episodically from capillaries, likely related to the molting cycle. BCS1 and BCS2 each contribute about 50% of the total adhesion strength. BCS2 is secreted into existing cured BCS1, and appears to be related to the tree ring-like growth pattern of the barnacle base plate (Burden *et al.*, 2014).

Results obtained from *in situ* studies and glue protein sequencing and characterization efforts have led to two major questions. First, are multiple chemical mechanisms involved in the adhesion of barnacles to substrates? Second, within the barnacle glue interface, does the glue itself serve as a substrate for mineralization of the base plate? Phosphorylated proteins show promise with respect to both surface adhesion and mineralization (George and Veis, 2008; Flammang *et al.*, 2009). Phosphoproteins are an emerging theme in aquatic bioadhesives. They have been well documented in marine mussels, sea cucumbers, sandcastle worms, and caddisfly larvae (Stewart *et al.*, 2004; Flammang *et al.*, 2009; Stewart and Wang, 2010; Wang and Stewart, 2012), and were recently identified in the permanent adhesive of barnacle cyprid larvae (Gohad *et al.*, 2014). In these systems, phosphoproteins can contribute to both adhesive and cohesive properties of the glue (Flammang *et al.*, 2009). Phosphoproteins have a strong affinity for charged surfaces (Fujisawa and Kuboki, 1998; Huq *et al.*, 2000; Wallwork *et al.*, 2002), and play important roles in the control of nucleation during biomineralization (George and Veis, 2008; Beniash, 2011; Deshpande *et al.*, 2011).

Two lines of evidence suggest that phosphoproteins may be present in barnacle glue. First, the likelihood that serine, threonine, or tyrosine residues are phosphorylated can be predicted based on kinase recognition sequences, using known protein sequences (Blom *et al.*, 1999). NetPhos 2.0 (Blom *et al.*, 1999), a phosphorylation site prediction tool, was used to assess the barnacle glue proteins that are fully sequenced and available in the NCBI database. Each protein was found to have multiple potential phosphorylation sites (Appendix Table A1). For each cement protein tested, at least 10 percent of serine residues were found to have more than a 90 percent likelihood of phosphorylation. Second, observations of calcite mineral in barnacle glue (Berglin and Gatenholm, 2003) may indicate the presence of mineral nucleators such as phosphoproteins, which have been shown to participate in mineralization in a range of taxa, including molluscs and vertebrates (Zhang and Zhang, 2006; George and Veis, 2008).

We employed a combination of biochemical and histological techniques to test the hypothesis that the barnacle adhesive interface contains phosphoproteins. Specifically, we aimed to identify phosphoproteins in uncured barnacle glue and to determine their location within the barnacle adhesive interface, using residual glue deposited by reattaching barnacles and decalcified barnacle base plates. Finally, we assessed the potential for glue phosphoproteins to nucleate calcium carbonate (CaCO₃).

Materials and Methods

Barnacle larval culture, settlement, and maintenance

The barnacle *Amphibalanus* (= *Balanus*) *amphitrite* (Darwin, 1854) was used in this study (Pitombo, 2004). Larval culture and settlement were conducted at the Duke University Marine Laboratory in Beaufort, NC, following well-established methods (Rittschof *et al.*, 1984). Barnacle cyprids were settled on glass panels measuring 7.6 cm × 15.2 cm × 0.64 cm and coated with T2 silicone (Gelest, Morrisville, PA). After 4–6 weeks' growth, barnacles were shipped overnight to the University of Pittsburgh, where they were held in artificial seawater (Instant Ocean, Blacksburg, VA), 30–32 ppt at 25 °C. Seawater was changed twice weekly. Barnacles were fed every other day with a dense solution of *Artemia* sp.

Chemicals

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA), unless otherwise stated, and were of analytical grade or higher.

Collection of uncured glue

Droplets of uncured glue were collected using the methods described by Dickinson *et al.* (2009). Barnacles were removed intact from silicone-coated panels. All shell plates were carefully cleaned with a damp cotton swab, then swabbed with 100% ethanol, rinsed in ultrapure water, and air-dried for at least 2 hours. After this time, a dissecting needle was used to gently remove cured glue from the intersection of the base plate and parietal plate, where glue is released during growth. If breakage of the base or parietal plates occurred during this process, the barnacle was discarded. The procedure resulted in the formation of defined, usually 1–2 μ l, glue droplets. Uncured glue collected in this manner contains BSC1, BSC2, or a combination of both secretions. The high level of variation among barnacles observed here and previously (Dickinson, 2008) suggests that glue droplet contents depend on the specific stage of the molting cycle and the proximity of glue ducts to the collection site.

SDS-PAGE, phosphoprotein-specific staining, and Western blotting

Uncured barnacle glue (1–2 μ l) was delivered directly into a Laemmli Sample Buffer (#161–0737; Bio-Rad Laboratories, Hercules, CA) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), under reducing conditions on a 4%–20% gradient gel (#456–1095; Bio-Rad Laboratories). The same volume of glue was used for all samples within a gel. The Laemmli Sample Buffer contained 350 mmol l⁻¹ 1-dodecanethiol (DTT), 65.8 mmol l⁻¹ Tris-HCl (pH 6.8), 2.1% SDS, 26.3% (w/v) glycerol, and 0.01% bromophenol blue. Glue samples in reducing sample buffer were denatured by heating at 95 °C for 4 min. Gels were run at 80 volts for approximately 2 h. Molecular weight standards (#161–0374; Bio-Rad Laboratories) and phosphoprotein standards (Invitrogen #P33350; Thermo Fisher Scientific) were run along with glue samples.

Phosphoprotein-specific staining was conducted using Invitrogen Pro-Q Diamond phosphoprotein gel stain (Invitrogen #MP33300; Thermo Fisher Scientific), which semi-quantitatively detects phosphoproteins down to 1 ng. The staining procedure followed the

manufacturer's recommendations, and all staining and destaining steps were protected from light. Gels were scanned on a 300-nm UV transilluminator (Kodak, Rochester, NY). As described by the manufacturer, grayscale settings of the resulting image were optimized based on phosphoprotein controls to account for non-specific staining. Following phosphoprotein staining and imaging, gels were immediately transferred to SYPRO-Ruby gel stain (Invitrogen #S12000; Thermo Fisher Scientific), following the manufacturer's recommendations. SYPRO-Ruby-stained gels were imaged on a 300-nm UV transilluminator (Kodak).

Western blotting with anti-phosphoserine antibody (#AB9332 rabbit polyclonal; Abcam, Cambridge, MA) was conducted on a separate set of gels. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (#162-0177, 0.2- μ m-pore size; Bio-Rad Laboratories) overnight at 4 °C *via* tank blotting. Tris-glycine transfer buffer (25 mmol l⁻¹ Tris, 192 mmol l⁻¹ glycine, pH 8.3), which contained 15% (v/v) methanol, was used. The blot was blocked in TBS buffer (10 mmol l⁻¹ Tris, 150 mmol l⁻¹ NaCl, pH 7.6) with 5% bovine serum albumin (BSA). All subsequent incubation and washing steps used TBS buffer containing 0.1% gelatin and 0.5% BSA (TBS++). The anti-phosphoserine antibody was used at 0.25 μ g/ μ l and the blot was incubated overnight at 4 °C. Secondary antibody (goat antirabbit-HRP, #AB6721; Abcam) was applied at 1:2500 dilution in TBS++ for 1h at room temperature. The blot was then incubated with an enhanced chemiluminescence (ECL) reagent (Pierce SuperSignal West Chemiluminescent substrate, #34077; Thermo Fisher Scientific) and exposed to film (#34088 Thermo Scientific). Control staining for non-specific binding with secondary antibody only yielded no visible bands.

Staining of residual secondary glue

To localize phosphoproteins within the barnacle glue interface, secondary glue released by the reattaching barnacles was stained with a phosphoprotein-specific stain. Barnacles were removed intact from silicone film-coated panels and cleaned as described previously, and excess water was blotted off of the shell. Barnacles were then placed on glass microscope slides that had been cleaned with ethanol and ultrapure water, then dried. The slides were placed in petri dishes, which were subsequently filled with artificial seawater (30 ppt; Instant Ocean) filtered at 0.2 μ m using a syringe filter. Barnacles that were dislodged from the slide when filtered seawater was added were placed back on the slide with forceps. Barnacles were allowed to reattach to the slide for 72 h, during which time they were not fed. A 72-h reattachment interval was used, because this time frame was found to be sufficient for the development of concentric rings of capillary structures (Burden *et al.*, 2012). After 72 h, the barnacles were carefully removed with forceps. All barnacles had released glue that provided resistance to removal from the slide.

After removal of the barnacles, glass slides containing residual glue were washed for 3 $\times$ 10 min in ultrapure water, and divided into two sets to 1) undergo incubation with phosphoprotein-specific (ProQ Diamond, Invitrogen #MP33300; Thermo Fisher Scientific) and general protein (Coomassie blue R250, #161-0400; Bio-Rad Laboratories) stains; or 2) to remain unstained as a control for autofluorescence. Stained samples were first stained for phosphoproteins. Slides were incubated for 1 h at room temperature in phosphoprotein stain

(Invitrogen #MP33300; Thermo Fisher Scientific) while protected from light, destained for 3×30 min with a destaining solution specific to the stain (Invitrogen, #P33310; Thermo Fisher Scientific), and washed again for 2×10 min in ultrapure water. Following this staining procedure, slides were imaged on a fluorescence microscope in the tetramethylrhodamine (TRITC) channel (Nikon TE2000, Melville, NY). Excitation and emission maxima for the TRITC filter (557 and 576 nm) correspond closely to those of the ProQ Diamond stain (550 and 580 nm). The same slides were then stained for general protein. For Coomassie staining, slides were incubated for 10 min at room temperature in Coomassie brilliant blue (R-250, 0.2% in 7.5% acetic acid, 50% ethanol), destained in 10% acetic acid for 3×10 min, and washed in ultrapure water for 2×10 min. Slides stained with Coomassie were imaged under bright-field illumination. The separate set of unstained slides was washed in ultrapure water for 3×10 min, then imaged both in bright-field mode and in the TRITC channel to assess autofluorescence.

Immunohistochemical staining of decalcified barnacle base plates

Phosphoprotein localization studies were also conducted on decalcified barnacle base plates. Barnacles were removed intact from silicone-coated panels and cleaned as described previously. The operculum and barnacle body were removed from the shell plates, using a scalpel and forceps. The base plate was then incubated in a decalcification solution containing 1% paraformaldehyde and 5% ethylenediaminetetraacetic acid (EDTA) at 4 °C. The solution was changed every other day for two weeks until the whole shell was transparent. Shells were then rinsed in PBS (P4477; Sigma-Aldrich) overnight at 4 °C, followed by graded ethanol dehydration (50%, 70%, and 100% ethanol) and paraffin embedding. Paraffin blocks were sectioned perpendicularly to the glue surface in 5- μ m thick sections using a Leica RM 2255 automated rotary microtome (Leica Biosystems, Buffalo Grove, IL). The sections were floated on a water surface at 42 °C and collected on slides (3800080; Leica Biosystems); the slides were incubated on a slide heater overnight at 40 °C.

For general histological observation, a routine hematoxylin and eosin (H&E) staining procedure was used. For immunoassay, sections were de-waxed, re-hydrated, and washed before blocking with 5% goat serum (005-000-121; Jackson ImmunoResearch, West Grove, PA), 0.1% Triton X-100 (T9284; Sigma-Aldrich), and 0.15% glycine (410225; Sigma-Aldrich) in PBS for 1 h at room temperature, then rinsed twice in 0.025% Tween-20/PBS. The samples were incubated with primary rabbit anti-phosphoserine polyclonal antibodies (#AB9332; Abcam) diluted 1:1000 in 0.5% goat serum in PBS overnight at 4 °C, and rinsed 8 times in 0.025% Tween-20/PBS. The samples were incubated with secondary Alexa-Fluor 488 goat anti-rabbit antibodies (111-545-144; Jackson ImmunoResearch) diluted 1:400 in 1% goat serum/PBS for 45 min at room temperature, and rinsed 8 times in 0.025% Tween-20/PBS. Light microscopy of the samples was conducted using a Nikon Eclipse TE-2000 E (Nikon Instruments) in bright-field and epifluorescence modes.

Immunohistochemical staining of the residual glue

The glue samples were prepared as described above for residual glue staining. Samples were held in seawater after removal of the barnacles and used unfixed in the staining procedure. The immunofluorescence staining procedure was conducted in a way similar to the

procedure for staining of the sections of the demineralized base plates. However, 1.5% Sudan Black B solution (199664; Sigma-Aldrich) in 70% ethanol was used to block the autofluorescence before the slides were mounted. Control samples were treated in the same way; however, instead of the primary antibodies, non-immune rabbit serum was used.

In-gel mineralization assay

Inspired by studies that tested nucleation of mineral on monolayers (Aizenberg *et al.*, 1999; Han and Aizenberg, 2003), we developed an in-gel mineralization assay as outlined below. An SDS-PAGE gel was run as described previously using 2 μ l of uncured glue. Immediately after the gel was run, it was placed in a polypropylene container (11 $\times$ 11 $\times$ 6 cm) and fixed by incubation in 2% formalin for 1 h followed by washing in ultrapure water for 1 $\times$ 20 min (shaking), and then overnight (without shaking). The mineralization assay was conducted within the plastic container. Ultrapure water was discarded and the container was filled 100 ml with an 8 mmol l⁻¹ Ca²⁺ solution (CaCl₂·2H₂O, pH 8.0). To this a CO₂ source was added: a small polystyrene weigh boat containing approximately 500 mg ammonium bicarbonate (NH₄HCO₃). The washed gel was then stained with 1% alizarin red (Sigma-Aldrich), a specific stain for Ca²⁺, for 10 min, followed by several washes in ultrapure water. As the background began to fade, defined CaCO₃ crystals associated with gel bands were observed. The gel was then imaged on a flatbed scanner.

Results

Identification of phosphoproteins by phosphoprotein-specific gel staining and Western blotting

To test our hypothesis that barnacle glue contains phosphoproteins, we stained proteins of uncured barnacle glue, separated by SDS-PAGE, using ProQ Diamond, a phosphoprotein-specific stain (Fig. 1B, D). Complementary staining of the collected glue with a general protein stain, SYPRO-Ruby, showed a banding pattern similar to previously published stained gels of uncured barnacle glue (Dickinson *et al.*, 2009) (Fig. 1A, C). The phosphoprotein-specific stain revealed phosphoproteins at approximately 130, 100, and 80 kDa. A faint band was also observed at approximately 28 kDa (Fig. 1), which may be a proteolytic degradation product of the larger phosphoproteins. Importantly, the intensity of the phosphoprotein staining and SYPRO-Ruby staining varied among individual barnacles (Fig. 1A–D). Variability in intensity of phosphoprotein staining of the 80-kDa band was particularly distinct; this band was either very intense or very weak.

To further verify the presence of phosphoproteins in barnacle glue, Western blotting with an anti-phosphoserine antibody was conducted (Fig. 1F, H), along with general protein staining by Ponceau S (Fig. 1E, G). Phosphorylated proteins at approximately 100 and 28 kDa that were observed by ProQ Diamond phosphoprotein stain were identified by Western blot. In addition, two proteins, one at approximately 68 kDa and one at 18 kDa, that had not been identified by ProQ Diamond, were found *via* Western blot (Fig. 1F, H).

The results of the ProQ Diamond and Western blotting are summarized in Table 1. Differences in gel staining between ProQ Diamond and Western blotting could be due to the

binding properties of the antibody *versus* the gel stain. Since Western blotting is specific to phosphoserine, the approximately 130 and 80 kDa proteins stained by ProQ Diamond, not Western blotting, may contain only phosphothreonine (phospho-Thr) and/or phosphotyrosine (phospho-Tyr) residues.

Localization of phosphoproteins within the barnacle glue interface

If phosphoproteins are present in barnacle glue, a logical question to ask is: where are they located within the barnacle glue interface? To address this question, we conducted phosphoprotein localization studies using secondary glue secreted onto glass slides and histochemical analyses of the decalcified base plates. When secondary glue was tested using a phosphoprotein-specific stain, staining was confined primarily to regions where the barnacle had laid down a new ring of glue ducts, which were clearly differentiated structurally from the homogeneous layer between glue ducts (Fig. 2A–C; arrowheads mark the glue duct). As shown in Figure 2C, staining is most intense within the glue duct itself, less intense but still present in regions of glue adjacent to the new duct, but not present at all in regions farther from the glue ducts. This finding was in contrast to general protein staining (Coomassie; Fig. 2B), in which staining was found throughout the glue layer and it varied in intensity with the density of the glue layer. Of note is a region indicated with an arrow in Figure 2A–C, which stained intensely with Coomassie but showed no phosphoprotein staining, suggesting specificity of the phosphoprotein stain.

To confirm the presence of phosphorylated proteins in the secondary barnacle glue, we conducted immunofluorescence staining with antiphosphoserine antibodies of the secondary glue attached to a glass slide. A strong fluorescence signal in the samples labeled with antiphosphoserine antibody was observed, while no signal was detected in the rabbit serum controls (Fig. 2D–G).

Localization of phosphoproteins within the barnacle glue interface was also assessed on sections of decalcified barnacle base plates, using immunofluorescence staining with anti-phosphoserine antibodies. Decalcification of the base plate revealed a porous structure, perforated with a network of radial canals and capillary glue ducts, which were embedded within the calcified base plate in the native interface (Fig. 3). The glue layer was structurally distinct from the base plate, and was observed to delaminate during the decalcification process (Fig. 3A). Positive staining for antiphosphoserine was found throughout the glue layer and the organic matrix of the base plate, with staining more intense in the latter (Fig. 3G). Negative controls (no antibody, secondary antibody only) yielded very minimal or no fluorescence, confirming staining specificity (Fig. 3C, E).

In-gel mineralization assays

To test our hypothesis that proteins in barnacle glue, including phosphoproteins, can induce mineralization of glue at the interface within the base plate or stiff substrates, we conducted CaCO_3 mineralization assays on uncured glue separated by SDS-PAGE. Methods were based on studies that tested nucleation of mineral on monolayers (Aizenberg *et al.*, 1999; Han and Aizenberg, 2003). Several protein bands were stained with alizarin red, including a diffuse staining band at 28kDa and a number of bands with a granular staining pattern at

approximately 130, 100, 80, and 68 kDa (Fig. 4). This granular staining pattern was due to the stain having bound to individual calcite crystals. The fact that the crystals are specifically associated with certain protein bands suggests that these proteins have strong nucleation potential. Interestingly, three of four bands with granular staining patterns had been identified as phosphorylated proteins either by phosphoprotein gel stain or Western blot (Fig. 4). As for the diffuse band at 28 kDa, it is possible that the staining was non-specific; this band contains a large amount of protein and, since alizarin red can bind proteins to some extent, this staining may have reflected simply a large concentration of protein rather than its nucleation potential.

Discussion

Our study has demonstrated the presence of several phosphoproteins in the adult barnacle adhesive interface. The presence of phosphoproteins was detected in SDS-PAGE of uncured glue by ProQ Diamond staining and Western blot with anti-phosphoserine antibodies. Phosphoproteins have been detected in a number of underwater bioadhesives, including marine mussels, sea cucumbers, sandcastle worms, caddis fly larvae, and barnacle cyprid larvae (Stewart *et al.*, 2004; Flammang *et al.*, 2009; Stewart and Wang, 2010; Gohad *et al.*, 2014). Our identification of several phosphoproteins in barnacle glue adds to this list and supports the hypothesis that phosphoproteins are widely utilized in adhesives of many invertebrate phyla. Their wide-spread presence suggests that phosphoproteins possess certain properties that are advantageous to forming an effective underwater glue. Phosphoproteins form strong ionic bonds with mineral surfaces (Goldberg *et al.*, 2001; George and Veis, 2008), a clear benefit both in terms of binding to the barnacle's calcitic base plate and to the wide diversity of charged substrates in estuarine and marine environments. Indeed, acidic mineral-binding motifs containing phosphoserine residues have been found in the adhesive pad of *Mytilus edulis*, and are likely involved in adhesion to calcareous substrates (Waite and Qin, 2001). Phosphoproteins also play a role in the cohesive properties of aquatic adhesives *via* mechanisms of Ca^{2+} cross-bridging and electrostatic association of phosphorylated blocks (Stewart *et al.*, 2004; Flammang *et al.*, 2009; Stewart and Wang, 2010; Wang and Stewart, 2012).

We have also observed significant variations among individuals in overall protein composition and phosphoprotein composition of the glue. These variations may have been due to different ratios of BCS1 and BCS2 fractions contained in glue collected from different individuals, at molting, or other biological cycles. Such variation is consistent with previous observations of changes in glue production during the course of the molting cycle (Fyhn and Costlow, 1976). Variations in the presence and abundance of phosphoproteins may suggest significant differences in the adhesive and/or mineralization properties of glue secretions among barnacles, and at different stages of the molting and growth cycle.

As shown by immunolocalization studies and Pro-Q Diamond staining, phosphoproteins were identified throughout the organic matrix of the decalcified base plate and within secondary glue secretions. In the latter, staining intensity appeared greatest in the region where new capillary glue ducts had been laid down. Based on the phosphoprotein gel staining and western blots for phosphoserine and localization studies, we propose that glue

ducts may carry phosphoproteins from the glue glands to just behind the growing edge of the barnacle, where they are released cyclically.

At this point, we can only surmise how the phosphorylated proteins that were identified in this study relate to the previously identified barnacle glue sequences. Considering that the 100 kDa proteins described in *Megabalanus rosa* (Kamino *et al.*, 2000) and in *Amphibalanus amphitrite* (He and Zheng, NCBI direct submission) contain multiple phosphorylation sites based on our NetPhos 2.0 analysis of the published sequences (Appendix Table A1), it is plausible that these are homologs of the 100 kDa phosphorylated protein identified in this study. At the same time, such assumptions should be made cautiously, as phosphorylation and other posttranslational modifications may significantly change mass and charge of a protein.

Berglin and Gatenholm (2003) demonstrated the presence of calcite mineral in barnacle glue formed on stiff substrates (Berglin and Gatenholm, 2003). The results of our mineralization experiments suggest that the glue proteins—and specifically phosphoproteins— can play a role in the mineralization of the glue. This is the first report on the potential role of the glue proteins in biomineralization. Although granular staining was very specific to identified phosphoproteins, the results were highly variable among individual barnacles. Additional studies are needed to gain a better understanding of this process.

It is noteworthy that, in previous studies, phosphoserine was absent in the adhesive apparatus of a stalked barnacle *Lepas anatifera* (Jonker *et al.*, 2012, 2015). The adhesive interface in stalked barnacles differs from Amphibalanoid barnacles in that there is no calcified base plate; rather, the fleshy stalk (peduncle) adheres directly to the substrate with no mineral at the interface. The adhesive is comprised of a foam-like structure and mechanical properties (elastic modulus, hardness, and tensile stress) are lower than that of acorn barnacles with a calcareous base plate (Zheden *et al.*, 2015). Identification of phosphoproteins in a species with a mineralized base plate—while a species without a mineralized base plate lacks phosphoproteins—further supports the notion that phosphoproteins play a role in mineralization.

In conclusion, we have shown that the adhesive interface of the barnacle *Amphibalanus amphitrite* contains phosphoproteins. We demonstrated that protein composition of the glue, including phosphoproteins, varies among barnacles and over time, possibly due to cyclical changes in glue secretion. Mineralization studies indicate that glue proteins, specifically phosphoproteins, have the potential to induce CaCO₃ mineralization. Overall, these results provide new information about the protein composition of barnacle glue and insight into the roles of phosphoproteins in underwater bioadhesives. Ongoing and future research will probe the role of these phosphoproteins in the adhesive functionality of the glue, and clarify their role in biomineralization.

Acknowledgments

The authors gratefully acknowledge funding from the U.S. Office of Naval Research, grants N00014-11-1-0180 (DR) and N00014-14-1-0491 (GHD); the McGowan Institute for Regenerative medicine (FW); and NIH/NIDCR grant DE06703 (EB).

Appendix Table A1

Phosphorylation site prediction using NetPhos 2.0 (Blom et al., 1999)

Protein name	Percentage (%) of residues with phosphorylation potential > 0.9		
	Ser	Thr	Tyr
Cement protein-114k (<i>Amphibalanus amphitrite</i>)	15.0	3.6	3.3
Cement protein-100k (<i>Amphibalanus amphitrite</i>)	14.0	6.4	4.1
Cement protein-100k (<i>Megabalanus rosa</i>)	11.6	10.6	3.3
Cement protein-52k (<i>Amphibalanus amphitrite</i>)	19.6	4.4	4.6
Cement protein-52k (<i>Megabalanus rosa</i>)	30.9	0.0	7.3
Cement protein-20k (<i>Megabalanus rosa</i>)	23.1	11.1	0.0
Cement protein-19k (<i>Amphibalanus amphitrite</i>)	15.4	0.0	0.0
Cement protein-19k (<i>Megabalanus rosa</i>)	19.1	9.1	0.0

Note: Barnacle glue protein sequences were accessed through the database of the National Center for Biotechnology Information (NCBI).

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Figure 1. Total protein and phosphoprotein staining of SDS-PAGE of uncured barnacle glue. Results from different barnacles illustrate the variability in phosphoproteins among individuals. Lanes (A) and (C): general protein stain with SYPRO-Ruby; (E) and (G): general protein stain Ponceau S; (B) and (D): phosphoprotein-specific ProQ Diamond staining (Thermo Fisher Scientific); and (F) and (H): Western blot with antiphosphoserine antibodies. Bands containing phosphoproteins are marked with arrows.

Figure 2.

Localization of phosphoproteins in residual barnacle glue left on glass slides after barnacles were removed. (A) bright-field micrograph of the residual glue layer; (B) bright-field micrograph of the residual glue stained with the nonspecific Coomassie blue protein stain; (C) epifluorescence micrograph of the same sample stained with ProQ Diamond phosphoprotein-specific stain (Thermo Fisher Scientific). ProQ diamond selectively stains the glue ducts, and staining is strongest in the regions associated with growth of new glue ducts (arrowheads). Arrows point to a dense region that does not stain for phosphoprotein, but is heavily stained with Coomassie blue, suggesting specificity of the phosphoprotein stain. Micrographs in (A–C) were taken at the same magnification. (D) Bright-field micrograph of the residual glue layer, and (E) epifluorescence micrograph of the same sample incubated with normal, nonimmune rabbit serum instead of antiphosphoserine antibody (control for non-specific binding). (F) Bright-field micrograph of the residual glue layer; and (G) epifluorescence micrograph of the same area labeled with the antiphosphoserine antibody. Micrographs (D–G) were taken at the same magnification.

Figure 3.

Histology and immunohistochemistry of decalcified barnacle base plates. For all images, the decalcified base plate was sectioned perpendicularly to the glue surface. (A) Hematoxylin and eosin (H&E) staining of the adhesive interface, showing a structurally distinct glue layer (left) and base plate (right). (B) (D) (F) Phase-contrast images of the stained sections. (C) Control for autofluorescence (no antibodies) and (B) corresponding phase-contrast image; (E) control for non-specific binding (incubated with secondary antibody only) and (D) corresponding phase-contrast image; and (G) immunofluorescence with antiphosphoserine antibodies of a decalcified base plate section; and (F) a corresponding phase-contrast image. Scale bars for A = 50 μm ; B–G = 100 μm .

Figure 4.

In-gel mineralization assay with uncured barnacle glue. Representative examples of general protein (SYPRO-Ruby), phosphoprotein ProQ Diamond stain (Thermo Fisher Scientific), and antiphosphoserine Western blot are shown for comparison. Alizarin red staining identified the presence of CaCO_3 crystals associated with gel bands. Binding of Alizarin red to individual CaCO_3 crystals results in the distinct granules observed.

Table 1

Molecular weights of the proteins stained by ProQ Diamond phosphoprotein stain and reacting with antiSerP antibodies

Molecular weight (kDa)	ProQ Diamond	antiSerP Western blot
130	+	
100	+	+
80	+	
68		+
28	+	+
18		+

Proteins that stained using both techniques are shown in bold. (ProQ Diamond; Thermo Fisher Scientific, Waltham, MA) anti-SerP, antiphosphoserine antibodies.