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Immunological studies on the settlement-inducing protein complex (SIPC) of the barnacle *Balanus amphitrite* and its possible involvement in larva–larva interactions

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Immunological investigation has revealed that a settlement-inducing protein complex (SIPC), which induces cypris settlement of the barnacle *Balanus amphitrite*, is synthesized during larval development and accumulates in the cypris larva. We previously purified the SIPC from adult *B. amphitrite*, which was active when bound to a substratum. The SIPC is a glycoprotein of high molecular mass, consisting of three major subunits of 76, 88 and 98 kDa with lentil lectin (LCA)-binding sugar chains. In the present study, we prepared antiserum against each LCA-binding subunit of SIPC, and performed immunoblot analyses. Immunoblotting of adult extracts showed that anti-76-kDa antibody reacted only with the 76-kDa protein, whereas anti-88-kDa and anti-98-kDa antibodies reacted with both the 88-kDa and the 98-kDa proteins. Immunoblotting of larval extracts indicated that reactivity of the 76-kDa protein to anti-76-kDa antiserum increased during larval development and cyprid extracts reacted strongly. Moreover, by using immunostaining we found that the SIPC was contained in 'footprints' of cyprids, which have been shown to act as a settlement-inducing pheromone, and is secreted onto the antennular attachment discs. The results suggest that the SIPC (or SIPC-like proteins) is involved in both adult–larva and larva–larva interactions during settlement of the barnacle *B. amphitrite*.

Keywords: barnacle; settlement; glycoprotein; larva; 'footprints'; immunoblot

1. INTRODUCTION

The gregarious settlement of barnacles has long been known to have a chemical basis (Knight-Jones 1953; Crisp & Meadows 1962), although, undoubtedly, other factors are involved (Crisp 1955; Rittschof *et al*. 1984; Wethey 1984). Since gregarious settlement was first described for *Elminius modestus* by Knight-Jones & Stevenson (1950), the chemical factors that elicit this behaviour by cypris larvae have been studied primarily using *Semibalanus balanoides* (Crisp & Meadows 1963; Larman *et al*. 1982). Aqueous extracts of the adults of this species, when applied to slate surfaces, induced the settlement of cyprids, but only in an adsorbed conformation, not in a solution (Crisp & Meadows 1962, 1963). The 'settlement factor' in the adult extract was identified as a polymorphic system of closely related proteins derived from subunits of molecular mass between 5000 to *ca*. 6000 and 18 000 daltons (Larman & Gabbott 1975; Larman *et al*. 1982; Gabbott & Larman 1987; Clare 1995).

More recently, attention has focused on a more tractable species for settlement studies, *Balanus amphitrite*, which, unlike *S. balanoides*, is a multibrooder and a major fouling species (Clare 1996). The settlement inducer of *B. amphitrite* has been purified and termed the settlement-inducing protein complex (SIPC) by Matsumura *et al*. (1998*b*). Like the settlement factor of *S. balanoides*, the SIPC is active when substratum-bound. The SIPC is a glycoprotein of high molecular mass, consisting of three major subunits of 76, 88 and 98 kDa, all with lentil lectin (*Lens culinaris* agglutinin, LCA)-binding sugar chains. Each LCA-binding subunit, when isolated by preparative sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), was found to induce larval settlement as effectively as the intact SIPC. LCA was subsequently shown to inhibit adult extract-induced settlement of *B. amphitrite* cypris larvae (Matsumura *et al*. 1998*a*), suggesting that the LCA-binding sugar chain of SIPC is important in settlement induction (Matsumura *et al*. 1998*b*).

In the present study, we used anti-SIPC antisera to examine the SIPC expression during larval development. Western blot analysis showed that such an expression was maximal at the cyprid stage. Moreover, immunocytochemical staining revealed a positive relationship between

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the SIPC and cyprid temporary adhesive, a known settlement inducer. The SIPC (or SIPC-like proteins) may thus be involved in adult–larva and larva–larva interactions at settlement.

2. MATERIALS AND METHODS

(a) *Larval culture and preparation of larval and juvenile extracts*

Larvae of *B. amphitrite* were cultured through to cyprids, in the laboratory, using a well-established methodology (Yamamoto *et al*. 1995; Clare 1996). In most cases, larvae were obtained from adults that were collected at Lake Hamana, Shizuoka, Japan (Yamamoto *et al*. 1995; Matsumura *et al*. 1998*a*). For whole-mount immunocytochemistry, cyprids were reared at Plymouth, UK, from larvae released from laboratory broodstock (Clare 1996). Larval extracts were prepared each day for what would normally be five days of larval culture to the cyprid stage. After washing in artificial seawater (van't Hoff formula ASW: 460 mM NaCl, 10.1 mM KCl, 9.2 mM $CaCl_2$, 35.9 mM $MgCl_2$, 17.5 mM $MgSO_4$, 10 mM Tris-HCl, pH 8.2), day 0 to day 5 nauplii and day 0 cyprids (day 5 of larval culture) were stored at −80 °C until the preparation of extracts. To prepare juvenile extracts, cyprids were incubated in ASW containing 10 μg ml^{-1} adult extract in a glass Petri dish at 25 °C. After 24 h incubation, most larvae had settled and metamorphosed. The metamorphosed juveniles were removed gently with a needle, washed in ASW and stored at −80 °C until extraction. For the latter, the frozen larvae and juveniles were homogenized in 50 mM Tris-HCl, pH 7.5 (0.2 μl per larva), centrifuged at 20 000 *g* for 30 min and the resultant supernatants were used as extracts. All of the extraction procedures were carried out either at 4 °C, or on ice.

(b) *Preparation of adult extracts and purification of larval settlement-inducing protein complex (SIPC)*

Crude extracts of whole adult *B. amphitrite* were prepared and the SIPC was purified, as previously described (Matsumura *et al*. 1998*a*,*b*). Extracts of shells and soft tissues of adult barnacles were prepared separately. The adults were carefully removed from the substratum and the soft tissues were separated with forceps. The shells were rinsed with ASW. The extracts were prepared by homogenizing in two volumes of 50 mM Tris-HCl, pH 7.5, then centrifuged at 40 000 *g* for 30 min, and the resultant supernatants were stored at −80 °C. All of the extraction procedures were carried out at 4 °C, or on ice.

(c) *Protein determination*

The protein concentration of each sample was determined by the method of Bradford (1976) with γ-globulin as standard.

(d) *Preparation of anti-SIPC antisera*

Three major subunits of purified SIPC, of 76, 88 and 98 kDa, were prepared by preparative SDS–PAGE (Matsumura *et al*. 1998*b*). An antiserum against each SIPC subunit was raised in rabbits by Sawady Technology Co. Ltd (Tokyo, Japan).

(e) *Electrophoresis and immunoblot analysis*

SDS–PAGE was done using a 10% or 7.5% acrylamide slab gel by the method of Laemmli (1970), in the presence of β-mercaptoethanol. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (CBB) to detect the separated protein bands.

For immunoblot analysis, samples (5 μg protein per lane) were subjected to SDS–PAGE and transferred electrophoretically to a polyvinylidene-difluoride (PVDF) membrane, using an Horizeblot AE-6675 apparatus (Atto Co., Japan). The membranes were rinsed with Tris-buffered saline, TBS (0.5 M NaCl, 20 mM Tris-HCl, pH 7.5) and incubated in blocking buffer (3% gelatin in TBS) for 60 min. After blocking, they were rinsed with TBS and incubated overnight in the first antibody solution (1/1000 anti-SIPC antiserum in TBS containing 1% gelatin). After being rinsed with distilled water, the membranes were washed with TTBS (TBS containing 0.05% Tween 20) and incubated with a secondary antibody solution (1/3000 peroxidase-conjugated anti-rabbit IgG goat antibody (A-0545, Sigma, St Louis, USA) in TBS containing 1% gelatin) for 60 min. The membranes were then rinsed with TTBS and distilled water, and the reactive bands were visualized with diaminobenzidine (0.5 mg ml^{-1}).

(f) *Visualization of footprints on nitrocellulose membrane*

An untreated nitrocellulose membrane (Advantec Toyo Co. Ltd, Tokyo, Japan) was fixed, with carbon adhesive tape, to the bottom of a polypropylene container (117 mm × 84 mm × 57 mm). Two hundred cyprids, which had been stored at 4 °C for one to two days, were released into 100 ml of ASW in the container. After 24 h at 25 °C, during which time the cyprids explored the membrane using their antennular attachment discs and the temporary adhesive on them, the membrane was removed and rinsed with distilled water. To visualize the 'footprints' (the temporary adhesive), the membrane was immersed in 0.1% CBB for 5 min and destained with 25% methanol and 7% acetic acid.

Immunostaining of footprints was done using the anti-76-kDa antiserum. After washing in distilled water, the membrane was rinsed with TBS, incubated in blocking buffer (3% gelatin in TBS) for 60 min, and then incubated overnight in the first antibody solution (1/200 anti-76-kDa antiserum in TBS containing 1% gelatin). Subsequent incubation with secondary antibody solution and visualization with diaminobenzidine were carried out according to the immunoblotting methodology described in § 2e. After staining, the membranes were observed under a binocular microscope.

(g) *Whole-mount immunocytochemistry*

Cyprids were fixed in 100% ethanol containing 0.1% H_2O_2, for 48 h at 4 °C. The fixed samples were rehydrated with 75, 50, and 25% ethanol and 0.1 M phosphate-buffered saline (PBS) containing 0.01% thimerosal and 0.05% Tween 20 (PBST). After incubation in a blocking buffer (1% bovine serum albumin in PBST) for 16 h at 4 °C, the samples were incubated with anti-76-kDa antiserum (diluted 1/200) in the blocking buffer for 48 h at 4 °C. They were washed in PBST and incubated for 16 h at 4 °C in a blocking buffer containing 1/300 dilution of peroxidase-conjugated anti-rabbit IgG antibodies developed in goat (A-0545, Sigma, St Louis, USA). The samples were washed in PBST and 0.1 M Tris-HCl, pH 7.5, and incubated with 0.05% diaminobenzidine (DAB) containing 0.03% H_2O_2 as a peroxidase substrate. Controls were incubated with blocking solution instead of first antibody (anti-76-kDa antiserum).

3. RESULTS

(a) *Immunoblot analysis of adult extracts*

Immunoblotting of crude adult extracts (5 μg protein per lane) was done using antiserum against each SIPC

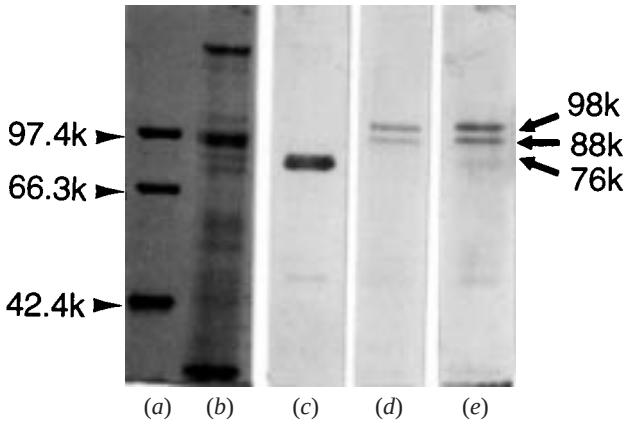

Figure 1. Immunoblotting of adult extracts using antisera against SIPC subunits of 76, 88 and 98 kDa. Molecular mass marker (*a*) and crude adult extracts (5 μg protein per lane) (*b–e*) were subjected to SDS–PAGE using 7.5% acrylamide slab gels in the presence of β-mercaptoethanol, and the separated proteins were transferred to PVDF membranes. The membranes were stained with CBB (*a*,*b*) for proteins. For immunostain, the membranes were treated with anti-76-kDa (*c*), anti-88-kDa (*d*) or anti-98-kDa (*e*) antisera. After treatment with peroxidase-conjugated secondary antibody, the reactive bands were visualized with diaminobenzidine (see § 2). Numbers with arrows (←) indicate molecular masses.

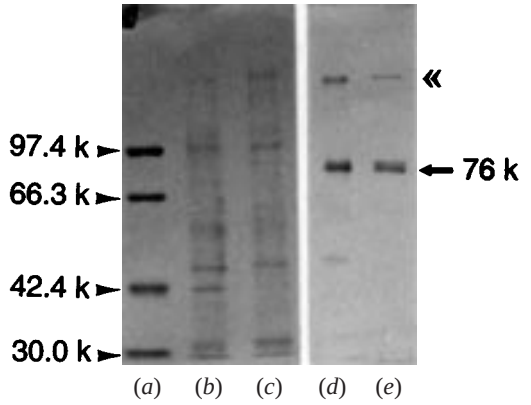

Figure 2. Immunoblotting of shells and soft tissues. Shells and soft tissues were separated from whole barnacles, and extracted. Each extract (5 μg protein per lane) was analysed by immunoblotting using anti-76-kDa antiserum as described in figure 1. (*a*) Molecular mass markers stained with CBB; (*b*) shell extracts stained with CBB; (*c*) soft tissue extracts stained with CBB; (*d*) shell extracts stained with anti-76-kDa antiserum; (*e*) soft tissue extracts stained with anti-76-kDa antiserum. Numbers with arrows (←) indicate molecular masses; «, 180-kDa band detected on occasion.

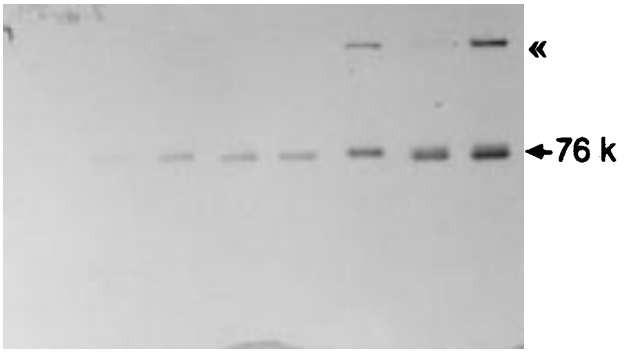

Figure 3. Immunoblotting of larval extracts. Day 0 to day 5 nauplii, day zero cyprids (day five of larval culture), young juveniles and adults were collected and extracted. Each extract (5 μg protein per lane) was analysed by immunoblotting using anti-76-kDa antiserum as described in figure 1. (*a*) day 0 nauplii; (*b*) day 1 nauplii; (*c*) day 3 nauplii; (*d*) day 4 nauplii; (*e*) day 5 nauplii; (*f*) day 0 cyprids (day 5 of larval culture); (*g*) juveniles (day 6 of larval culture); (*h*) adults. Numbers with arrows (←) indicate molecular masses; «, 180-kDa band detected on occasion.

polypeptide subunit (figure 1). The anti-76-kDa antiserum reacted strongly only with the 76-kDa polypeptide, whereas the anti-88-kDa and anti-98-kDa antisera each reacted with both the 88-kDa and 98-kDa polypeptides. The titre and specificity of the anti-76-kDa antiserum were, therefore, higher than the other antisera, and this antiserum was adopted for subsequent investigations. It was, however, noted that although CBB staining revealed a single band at 76 kDa (Matsumura *et al.* 1998*b*), immunostaining usually detected two bands close together. Moreover, a band of 180 kDa was also detected on occasion (see figures 2 and 3).

(b) *Immunoblot analysis of shells and soft tissues*

Shells (65 g) and soft tissues (9.5 g) were separated from 74.5 g (wet weight) of whole barnacles. The shell material yielded 132 ml of 0.67 mg protein ml^{-1} extract (88.4 mg of total proteins), and the soft tissues yielded 8.6 ml of 11 mg ml^{-1} extract (94.6 mg of total proteins).

Immunoblotting of shells and soft tissues (5 μg protein per lane) was done using the anti-76-kDa antiserum. As shown in figure 2, the 76-kDa polypeptide of both extracts (shells and soft tissues) reacted with the antiserum. Although the staining intensity of the shells' 76-kDa band was slightly higher than that of the soft tissues, the results indicate strongly that the 76-kDa subunit of the SIPC is present in both body compartments.

(c) *Immunoblotting of larval extracts*

To investigate the SIPC expression during development, extracts of larvae, juveniles and adults were prepared, and immunoblot analyses were performed using the anti-76-kDa antiserum. The protein concentration of the extracts from nauplii (stage I–II) just after hatching was 0.845 mg ml^{-1}, and the amount of protein extracted was calculated at 169 pg per larva. The amount of protein per larva gradually increased during larval development, such that the corresponding concentration value for cypris extract was 4.88 mg ml^{-1}, which is equivalent to 976 pg per larva.

The results of immunoblotting are shown in figure 3. Each sample lane contained the same amount of protein (5 μg per lane). Under these conditions, the reactive band at 76 kDa was hardly detectable at the early nauplius stage but increased during naupliar development and was expressed maximally at the cypris stage. The bands at 76 kDa of juvenile and adult extracts were also highly reactive.

(d) *'Footprints'*

'Footprints' of *B. amphitrite* cyprids deposited on the nitrocellulose membrane were stained with CBB (figure 4*a*). The outline of the stained 'footprints' was elliptical and measured 30–36 μm (long axis) by 20–25 μm (short axis).

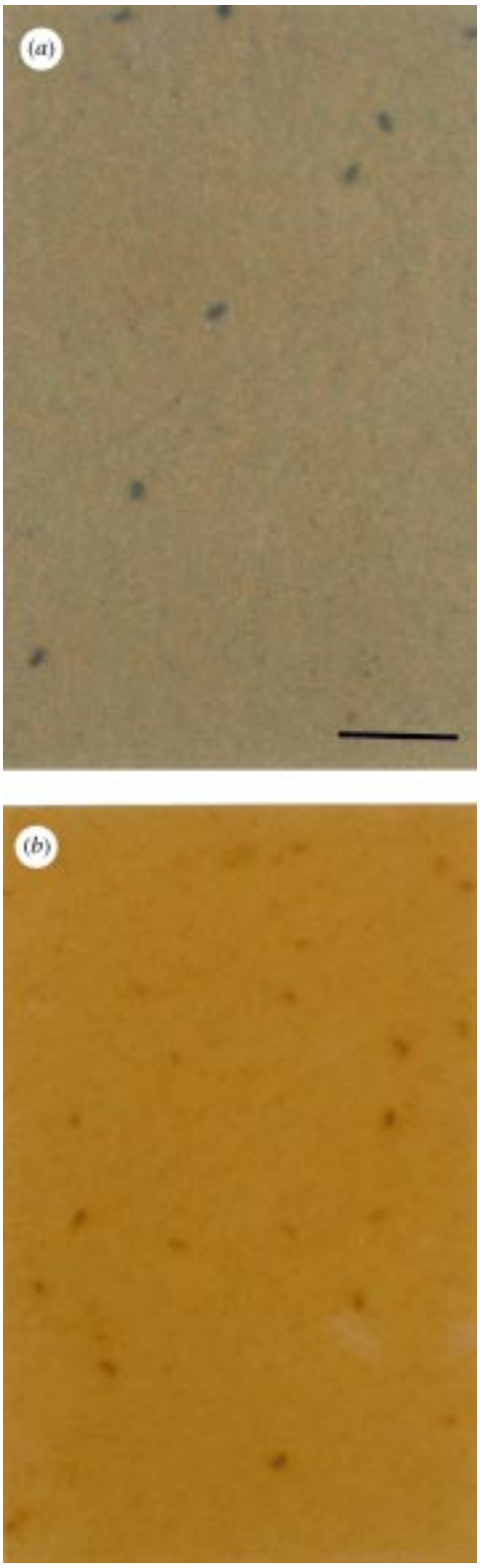

Figure 4. 'Footprints' on nitrocellulose membrane. (*a*) Cyprid 'footprints' on nitrocellulose membrane stained with CBB for proteins. (*b*) 'Footprints' stained with anti-76-kDa antiserum, peroxidase-conjugated secondary antibody and diaminobenzidine. Bar, 200 μm.

The typical pace length (distance between 'footprints') was 300–350 μm. These values agree with the results of Clare *et al*. (1994), who measured the 'footprints' of *B. amphitrite* on polystyrene dishes. 'Footprints' were also detected by immunostaining with antiserum against the 76-kDa subunit of the SIPC (figure 4*b*). The shape of the immunostained 'footprint' was similar to that stained with CBB, but the dimensions were slightly smaller: 28–34 μm (long axis) by 19–23 μm (short axis).

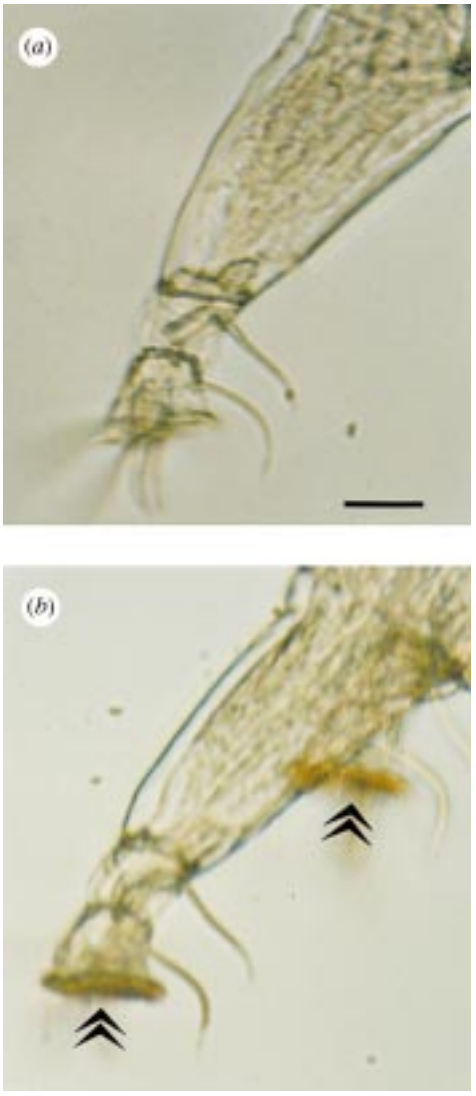

Figure 5. Immunostaining of cypris larvae antennule with anti-76-kDa antiserum. Whole-mounted cyprids were stained with anti-76-kDa antiserum, peroxidase-conjugated secondary antibody and diaminobenzidine. (*a*) Negative control incubated with blocking solution instead of first antibody. (*b*) Cypris larvae antennular attachment discs stained with anti-76-kDa antiserum. Bar, 20 μm; «, brown reaction product on attachment discs.

(e) *Immunostaining of whole-mount cyprids*

Whole-mount immunocytochemistry of cyprids was done using the anti-76-kDa antiserum, and the results are shown in figure 5. The attachment discs of the antennules stained specifically with anti-76-kDa antiserum (figure 5*b*). The stained material is presumed to be temporarily adhesive. In the control cyprids, which were treated with blocking solution instead of anti-76-kDa antiserum, the discs were unstained (figure 5*a*).

4. DISCUSSION

The present study has determined by immunological investigation that a settlement-inducing protein complex

(SIPC) of *B. amphitrite* is synthesized during larval development and is accumulated in the cypris larva. Moreover, an SIPC-like protein is contained in cyprid temporary adhesive.

We previously purified SIPC from adult *B. amphitrite* using a nitrocellulose membrane settlement assay (Matsumura *et al*. 1998*b*). SIPC is a high molecular mass (>200 kDa) glycoprotein complex. SDS–PAGE and lectin-blot analyses indicated that the SIPC consists of three major subunits of 76, 88 and 98 kDa with LCA-binding sugar chain(s). Compared with an intact SIPC, each individual LCA-binding subunit is isolated by SDS–PAGE induced larval settlement to a similar degree. In the present study, antisera were obtained against each SIPC subunit and immunological investigations were performed.

The results of immunoblotting of crude adult extracts showed that, of the three main LCA-binding subunits, the antiserum against the 76-kDa subunit reacted specifically to the 76-kDa subunit. The latter actually comprised two juxtaposed bands. The anti-88-kDa and anti-98-kDa antisera, however, reacted with both the 88-kDa and 98-kDa subunits, indicating that these subunits both contain common epitopes. The titre of the anti-76-kDa antiserum was, however, higher than the other antisera and was therefore used for further experiments.

To examine the site(s) of expression of the SIPC in adult barnacles, immunoblotting of extracts of separated shell plates and bases (including the hypodermis and some contamination by muscle) and soft body parts—the body, ovary and egg masses—was carried out. The results indicated that the shell plus the hypodermis and soft tissues contain the SIPC. This result is in accord with the results of earlier workers; for example, Knight-Jones (1953) reported that cyprids were induced to settle by extracts of the cirri and viscera of adult barnacles, and suggested that the settlement factor was probably present, not only in the epicuticle of the shell, but also throughout the body. Moreover, Larman *et al*. (1982) showed that the settlement factor in *S. balanoides* was present in the body tissues and cirri of adult barnacles. As the cuticle and associated hypodermis are the only tissues that are present in both the shell and soft body compartments, the present study supports the earlier suggestion that the 'settlement factor', or the 'SIPC', is a cuticular glycoprotein (Larman *et al*. 1982).

The synthesis of the SIPC in relation to development was examined by immunoblot analysis of larval extracts. Several stages, from nauplii just after hatching to juveniles after settlement, were examined using an antiserum against the 76-kDa subunit of the SIPC. The results showed that the 76-kDa subunit of the SIPC increased during naupliar development and was expressed strongly at the cypris stage. In the experimental design, the protein concentration, not the number of larvae, of each extract was fixed (5 μg per lane). Consequently, the increase in the SIPC per individual larva, or juvenile, is thought to be greater than the results shown in figure 3.

Walker & Yule (1984) showed that cyprids of *S. balanoides* deposit 'footprints' of proteinaceous material onto the substratum during surface exploration. Such cypris 'footprints' have also been reported for *B. amphitrite* (Clare *et al*. 1994). This material is considered to be the temporary adhesive that is produced by unicellular glands and secreted onto the antennular attachment discs (Nott & Foster 1969). In addition to serving as a temporary adhesive, the proteinaceous 'footprints' have been shown to stimulate settlement (Yule & Walker 1985; Clare *et al*. 1994). The presence of 'footprints' is thought to enhance the 'attractiveness' of a surface, resulting in gregarious settlement even in the absence of conspecific adults. However, the chemical basis of the pheromonal action has not been investigated. In the present study, we found that the 'footprints' of *B. amphitrite* cyprids were stained with the antiserum against SIPC. This result suggests strongly that the 'footprints' of *B. amphitrite* cyprids contain an SIPC-like protein and may thus explain why 'footprints' are able to stimulate settlement. The results of immunocytochemistry of whole-mount cyprids also support this conclusion. To our knowledge, this is the first report of a chemical relationship between cyprid 'footprints' and adult settlement pheromone (SIPC).

The SIPC (or pseudonyms such as arthropodin, settlement factor or settlement pheromone) has long been regarded as an adult glycoprotein that induces gregarious settlement of conspecific cypris larvae. The present study has shown that the SIPC is gradually expressed during larval development and has the highest titre at the cypris stage. The SIPC may be a structural protein, a component of the cuticle, that has acquired a secondary role in settlement. Likewise, a secondary function for the cypris temporary adhesive is to serve as a settlement pheromone. Significantly, a possible relationship between adult SIPC and cyprid temporary adhesive has been alluded to previously. Walker & Yule (1984) suggested that like arthropodin, the cyprid temporary adhesive is an integumentary protein. The present study has provided immunological evidence in support of this hypothesis.

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