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Separation of two cell signalling molecules from a symbiotic sponge that modify algal carbon metabolism

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Abstract

Two distinct cell signals have been isolated from the sponge host of the tropical sponge/macroalga symbiotic association *Haliclona cymiformis*/*Ceratodictyon spongiosum*. These water soluble cell signals (M_r between 500 and 1000) modify separate steps in the carbon metabolism in both *C. spongiosum* and the microalga, *Symbiodinium* from the coral *Plesiastrea versipora*. The first signal, host release factor (HRF), stimulates the release of compounds derived from algal photosynthesis; the second signal, photosynthesis inhibiting factor (PIF), partially inhibits photosynthesis. Both HRF from the sponge *H. cymiformis* and HRF from the coral *P. versipora* stimulated the release of glycerol from *Symbiodinium* suggesting that they act at a similar step in the metabolism of this alga. This is the first time that such cell signals have been isolated from a sponge. We suggest that they belong to a family of similar cell signals from symbiotic invertebrates that modify algal carbon metabolism.

Keywords: Cell signals; Calmodulin; Carbon metabolism; *Ceratodictyon spongiosum*; *Haliclona cymiformis*; Glycerol; Macroalga; *Plesiastrea versipora*; Sponge; *Symbiodinium*

Cell signals regulate many biochemical steps involved in carbon metabolism in all animals and plants. However, very little is known about the animal host cell signals that control algal symbionts. One cell signal, termed host release factor (HRF), stimulates the transfer of organic carbon from alga to host thus enabling symbiotic marine invertebrates to benefit from algal photosynthesis. HRF has been found in the homogenized host tissue of several species of Cnidaria including corals [1–3] and anemones [4–8]. Glycerol is the main product of photosynthesis (photosynthate) that is released from symbiotic dinoflagellate algae, when *Symbiodinium* spp. isolated from several cnidarians are incubated in coral homogenate [1,3,4,9]. We have also found HRF activity in the host tissue of the symbiotic clam *Tridacna maxima* that stimulated photosynthate release from its own *Symbiodinium* algae (S. Frankland, unpublished results), showing that HRF is not confined to cnidarians.

HRF from one cnidarian host species can stimulate the release of photosynthate from algae isolated from other cnidarians. For example, HRF from the scleractinian coral *Plesiastrea versipora* stimulated photosynthate release from freshly isolated *Symbiodinium* of the anemone *Aiptasia pulchella* [8] and the zoanthid *Zoanthus robustus* [10] and also from *Symbiodinium* cultured from the coral *Montipora verrucosa* and the jellyfish *Cassipoeia xamachana* [10]. Similarly, HRF from the coral *Montastraea annularis* stimulated release of algal photosynthate from freshly isolated *Symbiodinium* of the anemone *Aiptasia pallida* [7]. Such cross-reactivity suggests that HRF is common to diverse cnidarians.

HRF from *P. versipora* is the cell signal that has been studied in most detail. It has been partially purified by centrifugation and cation exchange chromatography. It is a

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water soluble compound of $M_r < 1000$ that stimulates the synthesis and release of glycerol (plus some alanine) from *Symbiodinium* isolated from *P. versipora* [9].

Another cell-signalling molecule, photosynthesis inhibiting factor (PIF), has recently been isolated from *P. versipora* [8]. PIF partially inhibits photosynthetic carbon fixation in freshly isolated *Symbiodinium* from *P. versipora* and *Z. robustus* as well as in cultured algae from the coral *M. verrucosa* [10]. Thus, like HRF, *P. versipora* PIF shows cross-reactivity by modifying carbon metabolism in *Symbiodinium* from several cnidarians. HRF and PIF from *P. versipora* can be separated from one another by cation exchange chromatography [8].

Many sponges also contain photosynthesizing symbionts, including *Haliclona cymiformis* which is associated with the multicellular rhodophyte, *Ceratodictyon spongiosum* [11]. The existence of a sponge HRF cell-signalling system has been inferred from the presence of ^{14}C-labelled carbon products in sponge tissue following incubation of intact symbiotic sponge/algal associations with $NaH^{14}CO_3$ in the light [12,13]. Grant et al. [14] also found ^{14}C-labelled compounds in *H. cymiformis* tissue after 2 h incubation of intact pieces of the *H. cymiformis*/*C. spongiosum* symbiosis in the light with $NaH^{14}CO_3$.

We have now confirmed the fact that the sponge *H. cymiformis* produces the cell signal HRF as well as demonstrating that it also produces PIF. These cell signals modify the carbon metabolism of both its own symbiotic macroalga *C. spongiosum* and freshly isolated single-celled dinoflagellate algae (*Symbiodinium*) from the coral *P. versipora*.

As clotrimazole, a synthetic calmodulin (CaM) antagonist, acts as an analogue of HRF and PIF in *P. versipora* algae [15], we discuss the possibility that these cell signals may involve CaM regulated mechanisms.

Materials and methods

Collection of the H. cymiformis/C. spongiosum symbiotic association. Pieces of the symbiotic association between the sponge, *H. cymiformis* Esper (1794), and the red macroalga *C. spongiosum* Zanardini (1878) were collected from the lagoon of One Tree Reef (Latitude 23°30′S, Longitude 152°06′E), southern Great Barrier Reef, Queensland, Australia.

For scanning electron microscopy pieces (2–10 mm long) of the intact symbiosis were frozen in liquid nitrogen then viewed in a Philips SEM 505 scanning electron microscope.

Isolation of C. spongiosum from H. cymiformis and culture of C. spongiosum. In contrast to most symbiotic sponges and corals which contain intracellular symbionts, the sponge *H. cymiformis* grows over the surface of, and in between, the algal branchlets (~1 mm in diameter), so that only the tips of the branchlets are free of sponge tissue [11]. Neither symbiont physically penetrates nor inhabits the cells of the other. For HRF and PIF bioassay experiments, algal branchlets were carefully teased apart with forceps. Sponge tissue was then gently removed from between the algal branchlets, which were then washed in filtered (Whatman GF/C, 1.2 μm) seawater.

Cultures of the macroalga *C. spongiosum* were established from small branchlets (~10 mm long) isolated from pieces of the *Haliclona*/*Ceratodictyon* association, and maintained at 22 ± 1 °C under a 12:12 h light:dark cycle in Grund algal culture medium [11].

Collection and fractionation of sponge cell homogenate. Sponge cells were released into a small volume (~2 ml) of seawater by gently pressing on the soft tips of the branches with the flat end (2 cm diameter) of the plastic plunger of a 20 ml disposable syringe (Terumo) and centrifuged (1000*g* for 5 min). The sponge cell pellet was then resuspended and washed in 15 ml of filtered seawater, centrifuged again, and the pellet frozen at −70 °C for 30 min to break open the cells. After thawing, the sponge homogenate from the broken cells was filtered through Whatman GF/C and GF/F (0.7 μm) filter papers and either used immediately or frozen at −70 °C for transport to our Sydney laboratory. Sponge cell preparations from this step were used as the source of cell signals in sponge homogenate experiments with freshly isolated or cultured *C. spongiosum* and with freshly isolated *Symbiodinium* from *P. versipora*.

Centrifugal concentrators (Microseps, Pall Life Sciences) were used to prepare a low $M_r < 1000$ fraction of sponge homogenate exactly as described previously for *P. versipora* homogenate [9]. HRF and PIF were then separated using cation exchange chromatography [8]. To reduce the level of NaCl present in seawater used during sponge cell isolation, 11 ml of MilliQ water (Millipore ultrapure) was added to 11 ml of the sponge low M_r fraction and concentrated to a volume of 5 ml over a YC 05 membrane (Amicon, M_r cut-off 500) in an Amicon concentrator under pressure (40 psi). An equal volume of MQ water was added and the sample further concentrated down to 1.6 ml then diluted 1:1 with MQ water and transferred to a microfuge tube. The membrane was rinsed with a further 0.8 ml of MQ water to lift any adhered molecules from the membrane and added to the concentrated sample. This concentrate, containing molecules of M_r between 500 and 1000 (NaCl concentration now 42 mM), was acidified to pH 2.0 (20 °C) in 1% formate and added to 15 ml of cation exchange resin (Biorad AG 50W-X8 resin, hydrogen form, 200–400 mesh, prepared in 7% formate) in a BioRad Econo column which had been washed with MQ water until pH 3.3. After addition of the sponge sample, the resin was washed with 20 ml MQ water at a flow rate of 30 ml/h and fractions of 1.5 ml were collected. Bound compounds were eluted with a seawater gradient of 0–100% followed by ammonium/acetic acid buffer, pH 9.4, at 20 °C [8]. Fractions showing a high absorbance at 215 nm were freeze-dried then tested in the bioassay [3,9] with *Symbiodinium* algae (4×10^5 in 0.5 ml total volume) freshly isolated from *P. versipora*.

Collection of P. versipora and preparation of algae and homogenized coral tissue. Pieces of *P. versipora* (Lamarck) were collected from Port Jackson, NSW (Latitude 33°48′S, Longitude 151°16′E), at a depth of 3–7 m. They were maintained in aquaria at the University of Sydney, with recirculating seawater, under fluorescent lamps with a light intensity of around 60 μmol photons m^{-2} s^{-1} (12 h light:12 h dark). *Symbiodinium* [16] and coral tissue were removed from pieces of *P. versipora*, by gently brushing the coral surface with a soft toothbrush. Algae were washed four times in filtered seawater as described previously [3]. Coral homogenate, used as the source of the *P. versipora* cell signals HRF and PIF, was prepared as described in [3].

Protein of sponge and coral homogenates was measured using the modified Lowry method [17] with bovine serum albumin Fraction V as the standard. Protein values are used only as an estimate of the protein concentration in animal cell homogenate; the amount of HRF and PIF may vary between different batches of homogenate that contain equivalent amounts of protein (See Fig. 3A, C, E in [18]).

Bioassay to determine the activity of HRF and PIF in sponge and coral tissue. In this study, evidence of HRF activity is defined as the stimulation of the release of at least four times as much photosynthate as that released from algae incubated in seawater. PIF activity is defined as the capacity to inhibit photosynthetic carbon fixation by >10% relative to algae incubated in seawater only [18]. The activity of HRF and PIF in homogenates from *H. cymiformis* and *P. versipora* was determined using the bioassay, previously described for *P. versipora*, that measures the fixation of $NaH^{14}CO_3$ and release of ^{14}C-labelled photosynthetically derived compounds by algae after 2 h incubation in the light [3,9] with modifications as detailed below.

Determining the effect of sponge HRF and PIF on freshly isolated C. spongiosum. Forty-five freshly isolated algal branchlets of similar size (8–10 mm long) were added to each of six glass vials. One milliliter of

seawater was added to three vials (controls), and 1 ml of sponge homogenate in seawater was added to the remaining three vials. Fixed and released carbon were determined in the bioassay [3,9] with the following modifications. At the end of the 2 h incubation, 50 μl aliquots of the incubation medium were removed from each tube to determine released fixed ^{14}C. The algal branchlets were then washed twice in filtered seawater and transferred to 25 ml capped glass vials each containing 2 ml of methanol to stop further metabolism. They were then dried at 55 °C and weighed. Soluble compounds (water, lipid, and KOH soluble) and KOH insoluble compounds were extracted as described in [19]. The ^{14}C-labelled photosynthetically derived compounds in these fractions were counted in subsamples of each fraction and the results expressed as nmol C per mg dry weight.

Autoradiography. ^{14}C-Labelled photosynthetic products released from freshly isolated branchlets of *C. spongiosum* incubated in sponge homogenate were identified using two–dimensional paper chromatography and autoradiography, and compared with non-radioactive standards that were mixed with an equal volume of seawater to the released samples, then processed in parallel with the labelled samples, as described in detail in [9].

Determining the effect of sponge and coral HRF and PIF on cultured C. spongiosum. The effects of HRF and PIF in both sponge and coral homogenate were tested on *C. spongiosum* which had been in culture for 3 months using the bioassay [3,9]. One clump of algal branchlets, 30 ± 1 mg (wet weight), was placed into each of nine vials. Replicates ($n = 3$ for each) were incubated in 1 ml of either seawater, or sponge or coral homogenate. Chlorophyll *a* was determined as detailed in [15].

Determining the effect of sponge and coral HRF and PIF on freshly isolated Symbiodinium from P. versipora. The activities of HRF and PIF in homogenates from *H. cymiformis* and *P. versipora* were determined in freshly isolated *Symbiodinium* from *P. versipora* using the bioassay [3,9]. Each experiment used a single preparation of algae. Replicate samples of *Symbiodinium* ($n = 3$) were incubated in either: (a) seawater only (controls); (b) coral homogenate; (c) sponge homogenate; (d) the low M_r fraction of sponge homogenate; (e) sponge HRF or PIF further purified by cation exchange chromatography from the low M_r fraction.

Measurement of released glycerol. Glycerol released from *Symbiodinium* freshly isolated from the coral *P. versipora* after 2 h incubation in the light was measured in triplicate tubes of algae that were incubated (omitting $NaH^{14}CO_3$) in parallel with the triplicate samples of algae that were used to measure total carbon fixation and release. The sensitive enzymatic method that is specific for glycerol was used exactly as described in [3]. Replicate tubes containing homogenate incubated in seawater only were included in each experiment.

Results

Physical relationship between H. cymiformis and C. spongiosum

Freezing samples in liquid nitrogen prior to scanning electron microscopy avoided the artificial separation of tissues that may occur when glutaraldehyde is used as a fixative [20]. Fig. 1 shows the sponge symbiosis as it exists *in vivo* indicating the close association between the sponge *H. cymiformis* and its red macroalgal symbiont *C. spongiosum*. Calculated on the basis of dry weight, the alga forms the larger part of the symbiosis (70:30% alga:sponge [21]).

Response of freshly isolated C. spongiosum to H. cymiformis cell signals

The amounts of fixed and released carbon are expressed as nmol of carbon per mg of algal dry wt (mean $n = 3 \pm$ SEM). When freshly isolated branchlets of *C. spongiosum* were incubated in sponge homogenate, release of photosynthate into the incubation medium (0.273 ± 0.030) was eight times higher than from seawater controls (0.033 ± 0.004), indicating stimulation of release by sponge HRF. The main compounds released were provisionally identified as glycolate, glutamate and succinate (Fig. 2A). Notably, there was no release of glycerol or alanine, which are the main compounds released from the unicellular alga *Symbiodinium* isolated from the coral *P. versipora* in response to *P. versipora* HRF [9].

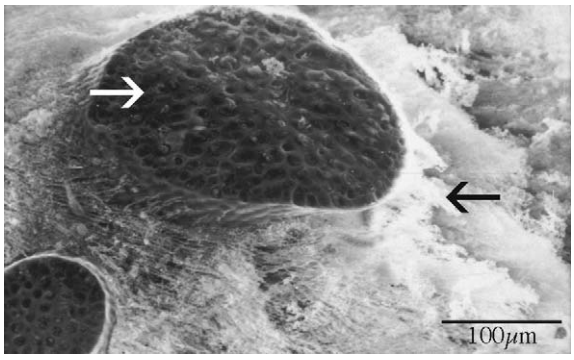

Fig. 1. A scanning electron micrograph showing *H. cymiformis* tissue growing closely between and around the branchlets of its symbiotic alga *C. spongiosum*. The white arrow on the left-hand side shows the fractured surface of an algal branchlet. The black arrow on the right-hand side shows the sponge tissue surrounding the alga. Scale bar = 100 μm.

The total amount of fixed carbon, that is the amount released into the incubation medium (Fig. 2A) plus the amount remaining inside the alga (Fig. 2B), was 16% lower in *C. spongiosum* incubated in sponge homogenate (31.82 nmol carbon/mg of algal dry wt), than in seawater controls (37.70 nmol carbon/mg of algal dry wt) indicating inhibition of carbon fixation by sponge PIF. This decrease was predominantly due to a smaller amount of fixed carbon in the KOH soluble fraction in *C. spongiosum* incubated in sponge homogenate, which was only 60% of the amount in seawater controls (Fig. 2B). There were minimal differences between *C. spongiosum* incubated in sponge homogenate and seawater controls in the amount of carbon fixed in soluble aqueous and lipid fractions, and in the KOH insoluble fraction (Fig. 2B).

Response of cultured C. spongiosum to H. cymiformis and P. versipora cell signals

The amounts of fixed and released carbon are expressed as nmol of carbon per mg chlorophyll *a* (mean $n = 3 \pm$ SEM). Release of photosynthate from branchlets of cultured *C. spongiosum* incubated in seawater (1.85 ± 0.23) or sponge (1.68 ± 0.12) or coral homogenate (1.98 ± 0.35) was similar, indicating a lack of response to HRF in both sponge and coral homogenates. This was not due to a lack of HRF, as both the *H. cymiformis* and the *P. versipora* homogenates used in this experiment stimulated photosynthate release from 1×10^6 algae isolated from the coral *P. versipora* (9 and 11 times higher than

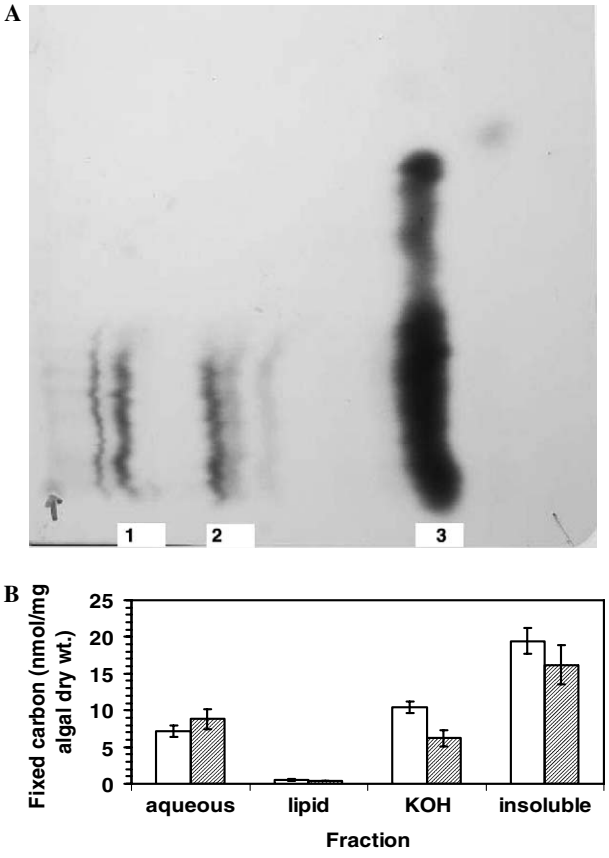

Fig. 2. Released (A) and fixed (B) carbon products from freshly isolated branchlets of *C. spongiosum* after incubation in the light in *H. cymiformis* homogenate. Freshly isolated pieces of *C. spongiosum* were prepared and incubated in seawater or in *H. cymiformis* sponge cell homogenate (1.74 mg protein per vial) for 2 h in the light as described in Materials and methods. (A) Is an autoradiogram showing the carbon products released into the medium from alga incubated in sponge cell homogenate after separation by paper chromatography: 1, glycolate; 2, glutamate; 3, succinate. The minor compounds were not identified. The arrow in the bottom left-hand corner represents the origin. (B) The various forms of intracellular fixed carbon. Extracts included aqueous, lipid, and KOH soluble material (aqueous, lipid, and KOH). The insoluble fraction is what remained after the KOH extraction step. Data are expressed as nmol carbon per mg algal dry wt. (means ± SEM, $n = 3$). Seawater (open columns); sponge cell homogenate (hatched columns).

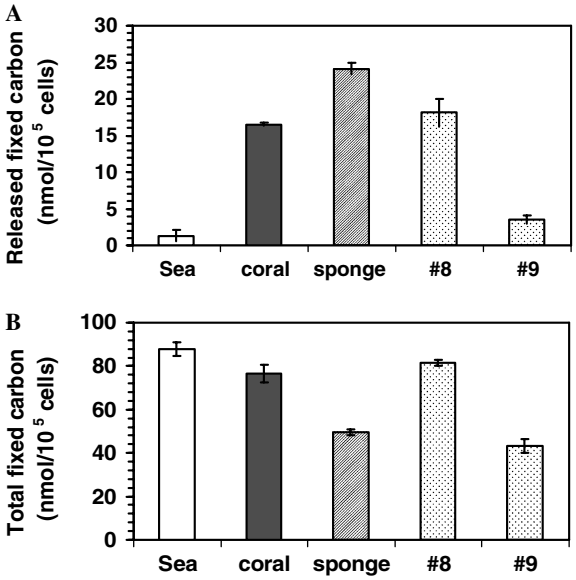

Fig. 3. Released (A) and total fixed (B) carbon in *Symbiodinium* from the coral *P. versipora* after incubation in seawater or in *P. versipora* homogenate or partially purified *H. cymiformis* homogenate. *P. versipora* algae were incubated for 2 h in the light in seawater (open columns) or homogenate (120 μg protein) from *P. versipora* (filled columns) or the low M_r fraction (66 μg protein) from *H. cymiformis* (hatched columns) or in # 8 (18 μg protein) or # 9 (18 μg protein) separated from the low M_r fraction of *H. cymiformis* by the ion exchange chromatography step (dotted columns) as described in Materials and methods. Data are expressed as nmol carbon per 10^5 cells (means ± SEM, $n = 3$).

seawater controls, respectively). However, carbon fixation of cultured *C. spongiosum* was inhibited by PIF in both sponge (25.58 ± 1.4) and coral homogenate (35.09 ± 11.78), by 66% and 53%, respectively, compared with seawater controls (75.31 ± 10.20).

Response of freshly isolated Symbiodinium from P. versipora to H. cymiformis and P. versipora cell signals

Symbiodinium algae isolated from *P. versipora* were used in seven separate experiments that tested the effects of sponge cell HRF and PIF in either (a) sponge cell homogenate, or (b) the low $M_r < 1000$ fraction prepared from sponge homogenate or (c) cation exchange fractions prepared from the sponge low M_r fraction.

Fig. 3 is a representative experiment that shows the separation of sponge HRF and PIF following cation exchange chromatography of the low M_r fraction. Fractions 8 and 9 (M_r between 500 and 1000) showed a strong absorbance at 215 nm and eluted near the end of the MQ water wash before the start of the seawater gradient.

Photosynthate release from *Symbiodinium* incubated in the low M_r fraction from the sponge *H. cymiformis* was 19 times higher than from seawater controls. Fraction 8 isolated from this low M_r fraction by cation exchange chromatography stimulated release 14-fold (Fig. 3A) indicating strong HRF activity. Fraction 8 had no PIF activity (showing <10% inhibition of carbon fixation, Fig. 3B). Carbon fixation was inhibited by sponge PIF in the low M_r fraction by 43% and by PIF in fraction 9 from the chromatography step, by 51% (Fig. 3B); fraction 9 had minimal HRF activity (<3 times higher release than seawater controls) compared with fraction 8 (Fig. 3A).

Coral HRF in *P. versipora* homogenate stimulated the release of 13 times more photosynthate than seawater controls (Fig. 3A) but PIF activity was minimal (Fig. 3B).

Stimulation of glycerol release from freshly isolated Symbiodinium from P. versipora

Fig. 4 shows a typical example of three separate experiments each of which showed that both *H. cymiformis* and

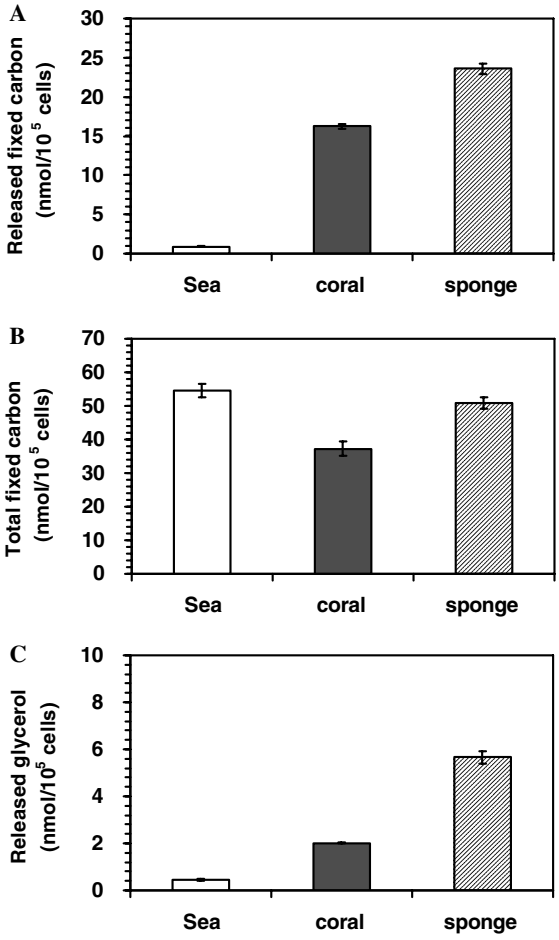

Fig. 4. Released (A) and total fixed (B) carbon and released glycerol (C) in *Symbiodinium* from the coral *P. versipora* after incubation in seawater or in *P. versipora* or *H. cymiformis* homogenate. *P. versipora* algae were incubated for 2 h in the light in seawater (open columns) or homogenate (157 μg protein) from *P. versipora* (filled columns) or (158 μg protein) *H. cymiformis* (hatched columns) as described in Materials and methods. Data are expressed as nmol carbon or glycerol per 10^5 cells (means ± SEM, $n = 3$).

P.versipora HRF stimulated glycerol release from *P. versipora* algae.

HRF was active in both *P. versipora* and *H. cymiformis* preparations; release of fixed carbon was, respectively, 18 and 27 times greater than in seawater controls (Fig. 4A). In this experiment, there was no inhibition of carbon fixation by incubation in *H. cymiformis* homogenate (inhibition <10%) indicating that this sponge preparation lacked PIF (Fig. 4B) whereas PIF activity was present in the *P. versipora* homogenate (33% inhibition of carbon fixation).

Fig. 4C shows that HRF in both *H. cymiformis* and *P. versipora* stimulated glycerol release (13 and 4 times, respectively, higher than seawater controls).

In this experiment there was no detectable glycerol in *P. versipora* homogenate; the amount of glycerol in *H. cymiformis* homogenate was less than 0.017 nmol glycerol and was deducted from the amount released from algae incubated in *H. cymiformis* homogenate for 2 h.

Discussion

Stimulation of the release of algal photosynthate by the cell signal HRF from cnidarians has been known for more than 40 years. More recently, a second cell signal, PIF, has been isolated from the coral *P. versipora* [8]. These coral cell signals regulate different steps in the carbon metabolism of freshly isolated *Symbiodinium* sp. from *P. versipora* and several other cnidarians [8,10].

While the idea of sponges containing HRF was considered more than twenty years ago [12], this is the first study that has confirmed the activity of the cell signal HRF in a sponge with an algal symbiont It is also the first study to show the existence of a sponge PIF. While both HRF and PIF activities were clearly demonstrated when freshly isolated *C. spongiosum* branchlets were exposed to *H. cymiformis* homogenate, routine use of this method to monitor purification and to study their mechanism of action is impractical; the separation of sufficient fresh branchlets from the sponge host tissue requires at least 20 h for a single experiment using three seawater controls plus three test (homogenate) samples. As a more practical alternative, we next tested the effects of sponge homogenate on cultured *C. spongiosum*. However, while isolated branchlets of *C. spongiosum* could be cultured in the laboratory, we noticed that they became thinner and fragile, indicating that in the absence of the animal host, the physiology of the alga had changed. Indeed, we observed that although cultured *C. spongiosum* responded to both sponge and coral PIF there was no response to sponge or coral HRF.

As we had found that the cell signals of HRF and PIF from *P. versipora* showed cross-reactivity through their actions on S*ymbiodinium* from several cnidarian/algal associations [8,10], we tested the effects of cell signals in *H. cymiformis* homogenate on freshly isolated *Symbiodinium* from *P. versipora*. We found that *P. versipora* algae responded to both HRF and PIF cell signals from *H. cymiformis* (Figs. 3 and 4). Thus it seems likely that these two sponge cell signals belong to a family of molecules similar to those of *P. versipora*.

Haliclona cymiformis HRF also stimulated the release of glycerol from *Symbiodinium* (Fig. 4), suggesting that HRF from both coral and sponge modify a similar step in the carbon metabolism of this alga. However, there was no stimulation of glycerol release by *H. cymiformis* HRF from its own alga *C. spongiosum* (Fig. 2A). Rather, the main compound released from *C. spongiosum* was succinate suggesting that the pathway of photosynthetic carbon metabolism in this rhodophyte alga differs from that of the dinoflagellate *Symbiodinium*.

It has been observed that HRF regulates algal metabolism in a manner that supplies carbon for the host's own needs, and it has been assumed that this is its only role. However, in a recent study of aposymbiotic *P. versipora*, we found that both the cell signals, HRF and PIF, were still produced in corals that had lacked photosynthesizing algal symbionts for almost two years [18]. That study raised the

question of whether these cell signals are produced by the animal host for its own metabolism but can also be used by the host to control the metabolism of photosynthesizing algae when they are present. We know that HRF modifies the carbon metabolism of *Symbiodinium* from *P. versipora* only when the algae are exposed to light [3], i.e., only when they are photosynthesizing. Therefore, in these algae, HRF must regulate a light-activated enzyme; for example, an enzyme such as chloroplast NADP linked glyceraldehyde 3-phosphate dehydrogenase (E.C.1.2.1.13, [22]).

The class of cell signals that HRF and PIF belong to is still unknown. However, we have some evidence which suggests that *P. versipora* HRF and PIF may act as calmodulin (CaM) antagonists. We have found that the synthetic CaM antagonist, clotrimazole (1-α-2 chlorotrityl imidazole, 100 μM), stimulated the release of the same compounds (glycerol and alanine) from *P. versipora* algae as HRF and also inhibited photosynthesis [15]. Thus, clotrimazole acted in a similar fashion to both HRF and PIF from *P. versipora*. There is a large family of Ca^{2+}-calmodulin mediated signalling mechanisms in both animal and plant systems [23] which include diversity in both target recognition and activation mechanisms of many Ca^{2+} regulated functions [24,25]. When CaM binds Ca^{2+}, hydrophobic domains on its surface are exposed allowing it to also bind to specific sites on target proteins which include a variety of enzymes. Both hydrophobic and electrostatic interactions between CaM and target proteins may be involved [26] and CaM binding can lead to activation or inactivation of target enzymes [24].

Although the primary structure of CaM has been highly conserved among mammals, immunoassays have shown that there may be small variations between different species in the COOH terminal region that contains one of the Ca^{2+} binding domains [27]. However, multiple isoforms of CaM may exist in a single plant species [28]. In symbioses between animals and plants, it is therefore likely that cell signals such as naturally occurring CaM antagonists play a role in modifying both animal and plant biochemistry.

We have further evidence which confirms a role for CaM antagonists in regulating carbon metabolism in *Symbiodinium*. The synthetic CaM antagonists W7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide HCl, 159 μM) and chlorpromazine (2-chloro-10-[3(-dimethylamino) propyl] phenothiazine monohydrochloride, 16 μM) also stimulated photosynthate release and inhibited carbon fixation in *Symbiodinium* freshly isolated from the zoanthid *Z. robustus* (our unpublished results). However, whereas these CaM antagonists triggered both HRF and PIF response pathways, we have separated HRF and PIF in both *P. versipora* [8] and *H. cymiformis* (this study). We have shown that they are distinct signalling molecules that regulate two separate pathways in *Symbiodinium* carbon metabolism, indicating that these invertebrate cell signals are more specific than the synthetic CaM antagonists.

This study has made a new contribution to the field of invertebrate cell signals that control carbon metabolism. It is the first study to demonstrate the functioning of animal cell signals in a sponge-algal symbiosis, and in a rhodophyte symbiosis. Chemical identification of these cell signals will greatly facilitate the study of their mode of action. As in the case of *P. versipora*, we were able to separate these two cell signals of *H. cymiformis* by cation exchange chromatography. However, amassing sufficient amounts of pure HRF and PIF (from both animal hosts) for full chemical analysis has been fraught with difficulty due to temperature lability and loss of activity when all salt is removed for analysis. Nevertheless, continuing improvements in the sensitivity of analytical biochemical techniques hold promise for identification of these cell signals in the near future.

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References

[1] L. Muscatine, R.R. Pool, E. Cernichiari, Some factors influencing selective release of soluble organic material by zooxanthellae from reef corals, Mar. Biol. 13 (1972) 298–308.
[2] D.C. Sutton, O. Hoegh-Guldberg, Host-zooxanthellae interactions in four temperate marine invertebrate symbioses: assessment of effect of host extracts on symbionts, Biol. Bull. 178 (1990) 175–186.
[3] A.J. Grant, M. Rémond, J. People, R. Hinde, Effects of host tissue homogenate of the scleractinian coral *Plesiastrea versipora* on glycerol metabolism in isolated symbiotic dinoflagellates, Mar. Biol. 128 (1997) 665–670.
[4] R.K. Trench, The physiology and biochemistry of zooxanthellae symbiotic with marine coelenterates. III. The effects of homogenates of host tissues on the excretion of photosynthetic products *in vitro* by zooxanthellae from marine coelenterates, Proc. Ry. Soc. Lond. Ser. B 177 (1971) 251–264.
[5] D. Schlichter, A. Svoboda, B.P. Kremer, Functional autotrophy of *Heteroxenia fuscescens* (Anthozoa: Alcyonaria): carbon assimilation and translocation of photosynthates from symbiont to host, Mar. Biol. 78 (1983) 29–38.
[6] J.-T. Wang, A. E Douglas, Nutrients, signals and photosynthate release by symbiotic algae: the impact of taurine on the dinoflagellate alga *Symbiodinium* from the sea anemone *Aiptasia pulchella*, J. Plant Physiol. 114 (1997) 631–636.
[7] C.B. Cook, S. Davy, Are free amino acids responsible for the 'host factor' effects on symbiotic zooxanthellae in extracts of host tissue? Hydrobiologia 461 (2001) 71–78.
[8] A.J. Grant, M. Rémond, K.J.T. Withers, R. Hinde, Inhibition of algal photosynthesis by a symbiotic coral, Hydrobiologia 461 (2001) 63–69.
[9] A.J. Grant, M. Rémond, R. Hinde, Low molecular-weight factor from *Plesiastrea versipora* (Scleractinia) that modifies release and glycerol metabolism of isolated symbiotic algae, Mar. Biol. 130 (1998) 553–557.
[10] A.J. Grant, D. Trautman, S. Frankland, R. Hinde, A symbiosome membrane is not required for the actions of two host signalling compounds regulating photosynthesis in symbiotic algae isolated from cnidarians, Comp. Biochem. Physiol. (Part A) 135 (2003) 337–345.

[11] I.R. Price, R.L. Fricker, C.R. Wilkinson, *Ceratodictyon spongiosum* (Rhodophyta), the macroalgal partner in an alga-sponge symbiosis, grown in unialgal culture, J. Phycol. 20 (1984) 156–158.
[12] C.R. Wilkinson, Nutrient translocation from symbiotic cyanobacteria to coral reef sponges. In: C. Levi, N. Boury-Esnault (Eds.) Biologie des spongiaires. Colloques Internationionaux du Centre National de la Recherche Scientifique no. 291, Paris, 1979, pp. 373-380.
[13] C.R. Wilkinson, Nutrient translocation from green algal symbionts to the freshwater sponge *Ephydatia fluviatilis*, Hydrobiologia vol.75 (1980) 241–250.
[14] A.J. Grant, R. Hinde, M. Borowitzka, Evidence of transfer of photosynthate from a red algal macrophyte to its symbiotic sponge, Memoirs of the Queensland Museum, Proceedings of the 5th International sponge symposium, 44, Origin and Outlook, Brisbane, 1998, p. 204.
[15] R.J. Ritchie, A.J. Grant, K. Eltringham, R. Hinde, Clotrimazole, a model compound for the host release factor of the coral *Plesiastrea versipora* (Lamarck), Aust. J. Plant Physiol. 24 (1997) 283–290.
[16] J.W. Udy, R. Hinde, M. Vesk, Chromosomes and DNA in *Symbiodinium* from Australian hosts, J. Phycol. 29 (1993) 314–320.
[17] F.K. Shakir, D. Audilet, A.J. Drake, K.M.M. Shakir, A rapid protein determination by modification of the Lowry procedure, Anal. Bioch. 216 (1994) 232–233.
[18] A.J. Grant, T. Starke-Peterkovic, K.J.T. Withers, R. Hinde, Apo-symbiotic *Plesiastrea versipora* continues to produce cell signalling molecules that regulate the carbon metabolism of symbiotic algae, Comp. Biochem. Physiol. (Part A) 138 (2004) 253–259.
[19] C.J. Crossland, D.J. Barnes, M.A. Borowitzka, Diurnal lipid and mucus production in the staghorn coral *Acropora acuminata*, Mar. Biol. 60 (1980) 81–90.
[20] D.A. Trautman, R. Hinde, M.A. Borowitzka, The role of habitat in determining the distribution of a sponge-red alga symbiosis on a coral reef, J. Expt. Mar. Biol. Ecol. 283 (2003) 1–20.
[21] S.K. Davy, D.A. Trautman, M.A. Borowitzka, R. Hinde, Ammonium excretion by a symbiotic sponge supplies the nitrogen requirements of its rhodophyte partner, J. Expt. Biol. 205 (2002) 3505–3511.
[22] S. Scagliarini, P. Trost, P. Pupillo, V. Valenti, Light activation and molecular-mass changes of NAD(P)-glyceraldehyde-3-phosphate dehydrogenase of spinach and maize leaves, Planta 190 (1993) 313–319.
[23] T. Yang, B. Poovaiah, Calcium/calmodulin-mediated signal network in plants, Trends Plant Sci. 8 (2003) 505–512.
[24] M. Brini, E. Carafoli, Calcium signalling: a historical account, recent developments and future perspectives, Cell. Mol. Life Sciences 57 (2000) 354–370.
[25] K.P. Hoeflich, M. Ikura, Calmodulin in action: diversity in target recognition and activation mechanisms, Cell 108 (2002) 739–742.
[26] K.T. O'Neil, W.F. DeGrado, How calmodulin binds its targets: sequence independent recognition of amphiphilic α-helices, TIBS 15 (1990) 59–64.
[27] K. Zuklys, J. Kramer, M. Magocsi, J. Ovadi, Structurally distinct mammalian calmodulins, Imm. Lett. 24 (1990) 87–92.
[28] R.E. Zielinski, Calmodulin and calmodulin-binding proteins in plants, Ann. Rev. Plant Physiol. Plant Mol. Biol. 49 (1998) 697–725.