ELSEVIER

Comparative Biochemistry and Physiology Part A 126 (2000) 33–44

CBP

www.elsevier.com/locate/cbpa

Symbiosis-enhanced gene expression in cnidarian-algal associations: cloning and characterization of a cDNA, sym32, encoding a possible cell adhesion protein

W.S. Reynolds, J.A. Schwarz, V.M. Weis *

Department of Zoology, Oregon State University, Corvallis, OR 97331, USA

Received 4 October 1999; received in revised form 10 February 2000; accepted 25 February 2000

Abstract

Mutualistic endosymbioses between two partners are complex associations that are regulated by the genetic interactions of the partners. One important marine symbiosis is that between various cnidarians, such as corals and anemones, and their photosynthetic algal symbionts. We have been interested in characterizing cnidarian host genes that are expressed as a function of the symbiotic state, using the temperate sea anemone *Anthopleura elegantissima* as a model. In this study, we report on symbiosis-enhanced expression and synthesis of sym32 in anemones. We characterized the full-length *sym32* cDNA, obtained by RT-PCR, and demonstrated, by semi-quantitative RT-PCR, that *sym32* transcript was much more abundant in symbiotic than in non-symbiotic host anemone RNA. Further, using immunoblots, we determined that an antibody made to a sym32 fusion protein labeled a 32 kD band much more strongly in symbiotic compared to non-symbiotic anemone protein homogenates. Databank searches revealed that the sym32 deduced amino acid sequence shares significant homology with the fasciclin I (Fas I) family of homophilic cell adhesion proteins, present in a variety of organisms ranging from bacteria to humans. This strong homology with the Fas I family suggests that sym32 is involved in regulation of the symbiosis by mediating cell-cell interactions.

Keywords: Anemone; Anthopleura; Cnidarian; Fasciclin I; Symbiosis; Zooxanthellae

1. Introduction

Symbiotic associations between two or more unrelated organisms are found throughout every ecosystem. These relationships encompass a spectrum of lifestyles, from mutualistic to parasitic, and from extracellular to intracellular (Smith and Douglas, 1987; Douglas, 1994). Among the most significant marine mutualisms are those between corals and anemones and their dinoflagellate endosymbionts *Symbiodinium* spp. (referred to as zooxanthellae), that together form the trophic and structural foundation of the entire coral reef ecosystem (see Dubinsky, 1990). The relationship is considered mutualistic; zooxanthellae provide hosts with reduced organic carbon (Falkowski et al., 1984; Muscatine, 1990) , while hosts provide zooxanthellae with inorganic carbon (Weis, 1993; Goiran et al., 1996; Furla et al., 1998), inorganic nitrogen (reviewed in Wang and Douglas, 1998), a high light environment and refuge from herbivory. Such tight nutrient cycling between sym-

* Corresponding author. Tel.: +1-541-7374359; fax: +1-541-7370501.

E-mail address: weisv@bcc.orst.edu (V.M. Weis)

1095-6433/00/$ - see front matter
PII: S0742-8413(00)00099-2

biont and host promotes high productivity in otherwise nutrient-poor marine environments. As a result, gross productivity of algae contained in their hosts makes *Symbiodinium* a major contributor to global carbon fixation (Hatcher, 1988; Muscatine and Weis, 1992; Hatcher, 1996). A great deal is known about corals on a macroscale level, such as their global distribution, population dynamics, ecology and energetic physiology. In contrast, microscale genetic processes of interpartner communication and regulation that occur during the initiation, establishment and maintenance of these endosymbioses remain largely unexplored.

These molecular conversations, where the partners reciprocally regulate numerous genes that are essential in establishing and maintaining a stable association, have been examined in other symbiotic associations. The most extensive genetic studies have been performed on cooperative associations between leguminous plants and nitrogen-fixing bacteria (Fisher and Long, 1992; Werner et al., 1994), plants and mycorrhizal fungi (Smith and Read, 1997), and a sepiolid squid *Euprymna scolopes* and a luminous bacterium *Vibrio fischeri* (Ruby, 1996; McFall-Ngai, 1998). These studies demonstrate the potential for important and exciting breakthroughs in the understanding of how cnidarians and their algal symbionts regulate their complex association.

An excellent model for the study of cnidarian-algal symbiosis is the temperate North American, Pacific Coast sea anemone, *Anthopleura elegantissima*, that harbors a zooxanthella *Symbiodinium californium*. This species differs from reef-building corals and other tropical symbiotic cnidarians in that it can occur naturally in the symbiotic or non-symbiotic (aposymbiotic) state (Buchsbaum, 1968). In low light habitats, such as in caves or rock crevices, clones of *A. elegantissima* occur symbiont-free. In contrast, most reef-building corals are obligately symbiotic and cannot survive for long periods without zooxanthellae. The facultative nature of the *A. elegantissima* symbiosis allows for comparisons between aposymbiotic and symbiotic individuals, leading to the identification of proteins and genes that are differentially expressed as a function of symbiotic state.

Adding to the attraction of *A. elegantissima* as a model for the study of animal-algal interactions is its ability to harbor algae from two different taxa (Muscatine, 1971). At latitudes above 43°, (Secord, 1995) *A. elegantissima* can harbor either or both zooxanthellae and a green alga, that has not been extensively described taxonomically, in the genus *Chlorella* (referred to as zoochlorellae). This ability to harbor two distantly related symbionts provides additional experimental fodder for addressing questions of host-symbiont genetic regulation.

We have previously documented, using comparative two-dimensional protein profiles, numerous proteins that are present in *A. elegantissima* containing zooxanthellae but absent, or nearly absent, from aposymbiotic anemones (Weis and Levine, 1996). We have also described symbiosis-enhanced expression and synthesis of carbonic anhydrase (Weis and Reynolds, 1999), an enzyme now known to function in inorganic carbon transport in cnidarian-algal mutualisms (Weis et al., 1989; Goiran et al., 1996). The objective of this study was to identify and characterize the most abundantly produced symbiosis-enhanced protein, sym32. We provide evidence that both the expression of the *sym32* gene and the synthesis of the sym32 protein are influenced by host symbiotic state. We characterize the full-length cDNA and report that sym32 shares significant homology to the Fas I family of cell adhesion proteins, suggesting that sym32 is involved in regulation of the symbiosis by mediating cell-cell interactions.

2. Materials and methods

2.1. Animal collection

Specimens of *Anthopleura elegantissima* were collected at several sites on the Oregon Coast: Seal Rock, Boiler Bay or Neptune Beach. Animals were placed in Ziplock bags, and brought back in a cooler to the laboratory at Oregon State University in Corvallis. Some animals were kept for a short time (less than 1 week) in a recirculating aquarium containing seawater at 12°C before being frozen in liquid nitrogen and stored at −80°C until processing. Other animals were immediately frozen and stored. Animals symbiotic with zooxanthellae (referred to as zooxanthellate animals) were collected from sun-exposed locations. Animals symbiotic with zoochlorellae (referred to as zoochlorellate animals) are extremely rare in Oregon (Secord, 1995) and were collected from a single clone at Boiler Bay. Aposymbiotic

animals were collected from several rock crevices at all three sites.

2.2. Preparation of animal protein homogenates and polyacrylamide gel electrophoresis

The preparation of protein homogenates and resolution of the resulting proteins using one- (1D) and two-dimensional (2D) polyacrylamide electrophoresis (PAGE) is described in detail by Weis and Levine (1996). Protein homogenates have been shown to be free of algal protein contamination by immunoscreening for algal-specific proteins (Weis and Levine, 1996). Animal homogenates were resolved in one dimension using 12.5% polyacrylamide gels with sodium dodecyl sulfate (Laemmli, 1970). Two-dimensional PAGE was performed on the Multiphor II System from Pharmacia, using precast gels, according to the manufacturer's instructions.

2.3. Purification of sym32 protein and protein microsequencing

One of the most abundant proteins in symbiotic *A. elegantissima* 2D protein profiles was a 32 kD, 7.9 p*I* protein (Weis and Levine, 1996) we have designated sym32. In order to purify sym32, we generated three 2D gels of symbiotic host tissue, transferred the proteins to a membrane, and excised the sym32 spot. Three hundred μg of protein homogenate was loaded onto each 180×150 mm gel. The proteins were electrophoretically transferred from unstained gels onto Immobilon PVDF membrane (Millipore) using Pharmacia's Novablot semidry transfer apparatus, according to the manufacturer's instructions. Proteins were visualized on the membrane by staining for 2 min in 0.025% Coomassie R250 in 40% MeOH and brief destaining in 50% MeOH. The sym32 spots were excised with a razor blade and stored at -80°C until processing. The three spots were subjected to N-terminal sequence analysis using a PE-ABD Procise 494 sequencer (Patterson et al., 1996).

2.4. RNA isolation and cDNA synthesis

RNA isolation and cDNA synthesis were performed using methods described in detail in Weis and Reynolds (1999) except where differences are noted below. When isolating symbiotic anemone RNA, special steps were taken to remove symbiotic algae by centrifugation before extraction of RNA to prevent contamination with algal RNA (see Weis and Reynolds, 1999). For cDNA synthesis, total RNA was reverse transcribed using 0.5 μg oligo $(dT)_{12\text{-}18}$ and the Superscript Preamplification System (Life Technologies).

2.5. PCR amplification and cloning of sym32 cDNA

For the PCR amplification of *sym32*, cDNA was synthesized from 5 μg total RNA from symbiotic anemones, using a modified oligo (dT) primer, 5′-CTCTAGAACTAGTCT$_{18}$-3′, at a final concentration of 0.75 μM. An initial PCR was performed on 1μl of this cDNA reaction using a degenerate forward primer, 5′-GGIAA-YYTIGTIGARGTYGCYAA-3′, designed from the sym32 peptide sequence (Fig. 1), and a reverse primer, 5′-CTCTAGAACTAGTAT$_5$-3′, that anneals to the 3′ end of the above modified oligo (dT) primer sequence, at final concentrations of 3 and 2 μM, respectively. A second PCR was performed using a nested degenerate forward primer, 5′-GGNYTIACIGAYYTNAT-3′, again designed from the sym32 peptide sequence, and the same reverse primer, at final concentrations of 5.5 and 1 μM, respectively. Reactions were cycled 28 times using an annealing temperature of 45.5°C. Two microliters of the nested reaction were ligated into the pCR2.1 plasmid vector (Invitrogen) according to the manufacturer's instructions and subsequently used to transform INVαF' *Escherichia coli*.

2.6. 5′ Rapid amplification of cDNA ends (5′-RACE)

Total RNA was reverse transcribed using a reverse primer, 5′-GATTTCTTTCAGCTTT-TCGGCAG-3′, that corresponds to nucleotides 690–669 of the sym32 cDNA sequence (Fig. 1), at final concentration of 50 nM. Multiple dGTPs were then added to the 5′ end using terminal deoxynucleotidyl transferase (Frohman et al., 1988; Weis and Reynolds, 1999). The 5′ end of the *sym32* cDNA was amplified using a modified oligo (dC) forward primer, 5′-TGTCGACGAATTC$_{12}$-3′ and a nested reverse primer, 5′-ATCTTACCACCACCGTACACA-3′,

corresponding to nucleotides 425–405 of the *sym32* cDNA sequence, at final concentrations of 0.6 and 1.2 μM respectively. Reactions were cycled 40 times using an annealing temperature of 59°C. The products of the nested reaction were cloned as described above.

```
      -65  attagcatccgtgctgttgaagtagcaagtactaaagactttgggagtaaatacattttgtcact    0
 1    atgaagtgcatcttgctggccagtgttgttgctgttctcatcgtttcctcttacagtttgccattattggag   72
 1    M  K  C  I  L  L  A  S  V  V  A  V  L  I  V  S  S  Y  S  L  P  L  L  E   24
73    ctcaagcctgcagtcaagcttcccaaaccaagccaaaaccttggaaattttgtagaagttgccaattcccttt  144
25    L  K  P  A  V  K  L  P  K  P  S  Q  N  L  G  N  F  V  E  V  A  N  S  L   48
145   ggcttgaccgacttgataagcgctgccaaggcagccggtatcgctcaatttctggtgatgggaaagtccttg  216
49    G  L  T  D  L  I  S  A  A  K  A  A  G  I  A  Q  F  L  V  M  G  K  S  L   72
217   actttattcggccccaccaacgaagcattcgataccattcccgaagcctacaaacccataaactcaaccttc  288
73    T  L  F  G  P  T  N  E  A  F  D  T  I  P  E  A  Y  K  P  I  N  S  T  F   97
289   ttgaaggaggtgttgttgttccatgtcataaagtcagtagtgtacgctaatgccatcaagaatgaacttttg  360
98    L  K  E  V  L  L  F  H  V  I  K  S  V  V  Y  A  N  A  I  K  N  E  L  L  121
361   gtaccaagcatcttggagatgcccaaaaaggacatacgattcaatgtgtacggtggtggtaagatcgtcacc  432
122   V  P  S  I  L  E  M  P  K  K  D  I  R  F  N  V  Y  G  G  G  K  I  V  T  145
433   gcacagtgtagtccaattatcaaagtcaatcagaatgcttcaaatggcgtgatccacgtggtcagtagagtc  504
146   A  Q  C  S  P  I  I  K  V  N  Q  N  A  S  N  G  V  I  H  V  V  S  R  V  169
505   atgatcccaccattcggcaccgttacagatgttgtcgccatggagaagcaatacttcagtaccctcctgaca  576
170   M  I  P  P  F  G  T  V  T  D  V  V  A  M  E  K  Q  Y  F  S  T  L  L  T  193
577   gctgtgctcgctgctaaattacagggtgtattggcaggacctgggcctttcactgtatttgcgccaaccaat  648
194   A  V  L  A  A  K  L  Q  G  V  L  A  G  P  G  P  F  T  V  F  A  P  T  N  217
649   gaagcttttgccaagattcctgccgaaaagctgaaagaaatcctcaaaaatatcccactgttgaccaagatc  720
218   E  A  F  A  K  I  P  A  E  K  L  K  E  I  L  K  N  I  P  L  L  T  K  I  241
721   ctcaaatatcacgtggtatccgggaccttctgctccgcgggtctgacaaacggagcgactgtgcccaccta  792
242   L  K  Y  H  V  V  S  G  T  F  C  S  A  G  L  T  N  G  A  T  V  P  T  L  265
793   gaggggagtgatgtcactgttcacatatcgggcggatcagtgactgtaaacaatgctgttgttgtttttgtt  864
266   E  G  S  D  V  T  V  H  I  S  G  G  S  V  T  V  N  N  A  V  V  V  F  V  289
865   gatatacctgtcaccaatggcgttgttcatgttattgacactgttttgattcctaaagacgttgaggtttaa  936
290   D  I  P  V  T  N  G  V  V  H  V  I  D  T  V  L  I  P  K  D  V  E  V  *  313
937   attatcttcactaacgtgttaaaaatggaaaaatgtcttcagtcaaaaaagtgaacgaggtgaaaaatcttc 1008
1009  atgtagttgttggactattttcaaatttaaaattgatatagcttatagttaaaatattttgaaagaaaacct 1080
1081  ggcataaaaatttgtaaaaattaaatggtgtaagggtagaaagatatacaataaaaaataataatt  1146
```

Fig. 1. Nucleotide and deduced amino acid sequence of *sym32* cDNA. The N-terminal sequence, derived from Edman degradation, is shown in purple, except amino acids 41 and 46 that in the peptide sequence were leucine (L) and lysine (K), respectively. A putative signal sequence, consisting of 38 amino acids, occurs upstream of the N-terminus. The FRa, H-box, and FRb regions, conserved regions within the Fas I family of cell adhesion proteins, are shown in blue, pink, and green, respectively. Potential N-linked glycosylation and myristylation sites are underlined (see text for details). This sequence has been deposited with GenBank under the accession number AF229054.

2.7. Nucleotide sequencing and sequence alignments

Plasmid DNA from individual colonies was purified using a standard small scale protocol (Sambrook et al., 1989). The plasmid DNA was then sequenced using the Applied Biosystems Taq DyeDeoxy(TM) Terminator Cycle Sequencing Kit, and the reaction product was analyzed on an Applied Biosystems Model 373 DNA Sequencer. A single contiguous sequence was generated using the Staden Package software. Characteristics of the sym32 protein, based on the deduced amino acid sequence, were identified using the Scan-Prosite program (website: www.expasy.ch) and the SignalP program (website: www.cbs.dtu.dk/services/signalP/). Related amino acid sequences were identified using the BLAST algorithm in the GenBank database (Altschul et al., 1990). The predicted anemone sym32 amino acid sequence was aligned with other Fas I sequences using MacDNASIS Pro V3.6 (Hitachi Software, Ltd). The Higgins-Sharp algorithm was used and minimal adjustments were made by sight.

2.8. PCR amplification of sym32 from genomic DNA of aposymbiotic anemones and freshly isolated algae

To determine whether *sym32* is animal or algal in origin, PCR was performed on genomic DNA obtained from aposymbiotic host tissue and from zooxanthellae freshly isolated from a symbiotic host. Genomic DNA from host and algae was obtained by methods modified from Rowan and Powers (1991). Algae were removed from host tissues as described in Rowan and Powers, but were then resuspended in filtered seawater and incubated at 13°C for 72 h before starting the DNA extraction, in an attempt to destroy any host DNA present. For PCR, two *sym32* primer combinations were used. The first combination consisted of the forward primer 5′-TGCCCAAAAAGGACRTACGA-3′ (corresponding to nucleotides 380–399 of the *sym32* sequence; Fig. 1), and the reverse primer 5′-GATTTCTTTCAGCTTTTCGGCAG-3′ (corresponding to nucleotides 690-669 of the *sym32* sequence; Fig. 1). The second combination consisted of the same forward primer and the reverse primer 5′-GGAGCAGAAGGTCCCGGATAC-3' (corresponding to nucleotides 756–735 of the *sym32* sequence; Fig. 1).

To test for DNA contamination from the other symbiotic partner, PCR was also performed using primers to both host-specific and algal-specific genes. The host-specific gene carbonic anhydrase (CA), described in detail in Weis and Reynolds (1999) was amplified using primers 7-for (5′-CTTCATCTTGTTCACTGGA-3′) and 5-rev (5′-AAGCCTTCATCTCGGCTAC-3′) with a predicted product size of 543 bp. For the dinoflagellate-specific gene, a forward primer, 5′-AATGGGCTGTTTCTCCARGC-3′ and a reverse primer, 5′-CCGTTCACCGAGGACTTCAT-3′ were designed from a consensus sequence of peridinin-chlorophyll protein (PCP) sequences in GenBank, to amplify a product with a predicted size of 630 bp. Fifty nanograms of template were used for each reaction and all reactions were cycled 30 times with an annealing temperature of 54°C.

2.9. Semi-quantitative PCR

To compare expression of *sym32* in symbiotic vs. aposymbiotic *A. elegantissima*, semi-quantitative PCR was performed as described in Weis and Reynolds (1999), using *sym32* primers. To control for the potential of differential RNA degradation and cDNA synthesis efficiency, we also amplified actin, which should have approximately equal expression in both symbiotic and aposymbiotic animals. Two μg of total RNA were reverse transcribed as described above. *Sym32* and actin fragments were amplified in separate reactions. *Sym32* fragments were amplified in a 50 μl reaction containing 5% of the cDNA, 2 μM of the forward primer 5′-TGCCCAAAAAGGACRTACGA-3′ (corresponding to nucleotides 380–399 of the *sym32* sequence; Fig. 1), 2 μM of the reverse primer 5′-GATTTCTTTCAGCTTTTCGGCAG-3′ (corresponding to nucleotides 690–669 of the *sym32* sequence; Fig. 1), and dNTPs having 1% of the dTTP replaced with digoxigenin-11-dUTP. Twelve microliters of the reaction were removed after 17 and 20 cycles and the remainder was cycled 24 times. Actin was amplified in a separate reaction under the same conditions except using the actin forward primer, 5′-TCCCTTGAGAAGAGCTACGA-3′, and reverse primer, 5′-AATGGATGGGCCGGACTCAT-3′. Five μl of the samples were run on a 1.5% agarose gel, blotted onto positively charged nylon membrane (Roche) overnight, and detected with anti-digoxigenin-al-

kaline phosphatase, Fab fragments and the chemiluminescence substrate CSPD® (Tropix).

2.10. Fusion protein and antibody production

To determine whether the *sym32* cDNA sequence encodes the 32 kD, 7.9 pI protein so abundant in symbiotic anemones, a polyclonal antiserum was produced against a fusion protein generated from a fragment of the *sym32* cDNA sequence. The ability of the resulting antibody to recognize the sym32 protein was determined using immunoblot analyses (see below).

PCR was used to amplify a cDNA sequence coding for amino acids 21–252 of the *sym32* cDNA (Fig. 1). The resulting product was cloned into the plasmid pProEX HTc (Life Technologies) that contains a 6X histidine affinity tag. A selected clone was sequenced to confirm the reading frame. The protein was then expressed in *E. coli*, according to the manufacturer's instructions. The expressed protein (referred to as symF) was purified using a Ni-NTA resin (Qiagen). Centrifuged cells were resuspended in a lysis buffer containing 6M guanidine-HCl, 0.1 M NaH_2PO_4, 0.01 M Tris, 1% Triton X-100, pH 8.0 and lysed by vortexing. After centrifugation, the supernatant was loaded onto Ni-NTA resin that had been pre-equilibrated in lysis buffer. The column was washed with approximately ten column volumes of a wash buffer, 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0. A second wash series was performed with the same buffer at pH 6.3 and a third at pH 5.9. The symF protein was then eluted in the same buffer at pH 4.5. A polyclonal antiserum to symF was produced by immunizing a rabbit with 500 μg of symF, followed by two 250 μg boosters. Immunizations and antiserum preparation were performed by Covance.

2.11. Immunoblots

For immunoblotting, proteins were resolved in one or two dimensions by gel electrophoresis, as described above, and then electrophoretically transferred from unstained gels onto nitrocellulose membranes using Pharmacia's Novablot semi-dry transfer according to the manufacturer's instructions. Immunoblots were developed with a chemiluminescence detection system (Renaissance Kit, Dupont NEN Research Products), and all incubations were carried out on a rotating table. Membranes were first blocked for 1 h at 37°C in 20 mM Tris, 0.5 M NaCl, 0.1% Tween 20, pH 7.5 (TTBS), containing 5% powdered milk. Following this, they were incubated in a 1:1500 dilution of anti-symF antiserum, in TTBS for 1.5 h at rt, rinsed in 20 mM Tris, 0.5 M NaCl, pH 7.5 (TBS), and subsequently incubated in a 1: 5000 dilution of secondary antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase (Renaissance kit, NEN), in TBS. Following a rinse in TBS, the immunoblots were developed according to the manufacturer's directions.

Densitometry was performed on the immunoblots to quantify band intensities. Immunoblot films were scanned on a Molecular Dynamics Personal Densitometer and analyzed using ImageQuaNT 5.0 software. Intensities of whole bands were quantified and reported in relative optical density units (OD).

3. Results

3.1. Characteristics of the sym32 cDNA and sym32 predicted amino acid sequence

We purified the sym32 protein, originally described in Weis and Levine as an abundant symbiosis-enhanced 32 kD, 7.9 p*I* protein (Weis and Levine, 1996), from symbiotic anemone homogenates by 2D PAGE and obtained, by protein microsequencing, the N-terminal sequence: GNLVEVAKSLGLTDLISA. This sequence did not align strongly with any sequences in GenBank, as determined by BLAST searches. We used this sequence to design degenerate primers for amplifying *sym32* cDNA using RT-PCR, and we performed 5′ RACE to obtain the sequence upstream of the N-terminus. Three separate 5′- and 3′ end cDNA clones of *sym32* were sequenced. Where discrepancies occurred between the three, we report the most common sequence.

The nucleotide and deduced amino acid sequences and their characteristics are shown in Fig. 1. The complete *sym32* cDNA sequence consists of a 1211-nucleotide cDNA with a 936-nucleotide open reading frame. A poly-A signal (AATAAA) occurs 192 nucleotides downstream from the in-frame stop codon and 11 nucleotides upstream from the poly-A tail. The likely start codon is 65 nucleotides downstream from the 5′ end.

	FRa	D	H-box	D	FRb	D
sym32 (sea anemone)	TVFAPTNEAF	2	ILKYHVV	2	DIPVTNGVVHVIDTVLIP	2
β-igh3 (mouse)	TVFAPTNEAF	4	ILKYHIG	4	DIMATNGVVYAINTVLQP	4
RGD-CAP (chicken)	TVFAPTNEAF	4	ILKFHMA	4	DIMATNGVIHAVSSVLQP	4
OSF-2 (human)	TLFAPTNEAF	2	LMKYHMV	2	DIVTNNGVIHLIDQVLIP	2
Fas (*Drosophila*)	TIFVPTNEAF	1	HVLYHIT	1	DVAQTNGYVHIIDHYLGV	3
Mfas (*Drosophila*)	TIFVPTDAAF	2	ILKNHLL	2	DIMGTNGVLHVIDTILPT	2
EBP-α (sea urchin)	TVLAPTNAAF		VLRYHMI		DIPTTNGVIQVVDRVVLP	
(*Caenorhabditis*)	TFFAPWNEAF	3	ILKLHIV	3	NVAATNGIVHYIERVLGV	3
Algal-CAM (*Volvox*)	TIFVPTDEAF	2	ALSYHVL	1	DVAAGASCINVVDTVLQY	1
MPB70 (*Synechocystis*)	TVFAPTDTAF		ILTYHVV		GVSTANATVYMIDSVLMP	

Fig. 2. Alignment of highly conserved regions of *Anthopleura elegantissima* sym32 predicted amino acid sequence and sequences from the Fas I family of cell adhesion proteins. The homologous regions FRa, H-box, and FRb of the Fas I sequences are displayed from the most closely aligning domain of each sequence. Domain number for each sequence is listed to the right of each region, except in the cases of sea urchin EBP-α and Synechocystis MPB70, both of which have a single domain. Residues identical to anemone sequence are shaded. Percent identities with *A. elegantissima* sequence ranged from 70–100% for FRa, 30–86% for H-box, and 33–72% for FRb.

The N-terminus of the deduced amino acid sequence occurs 38 amino acids downstream from the start codon (atg). Characteristics of the sequence upstream of the N-terminus indicate that it is a signal sequence, suggesting that the protein is exported out of the cytoplasm. The peptide sequence of the N-terminus, obtained by microsequencing, differs slightly from the deduced amino acid sequence obtained from PCR of the cDNA. Amino acids 41 and 46 in the peptide sequence are leucine (L) and lysine (K), whereas in the deduced amino acid sequence they are phenylalanine (F) and asparagine (N), respectively. The deduced amino acid sequence contains two repeating domains, each of which each contains three conserved motifs. In the first domain these are: TLFGPTNEAF (amino acids 73–82), VLLFHVI (100–106), and VNQNASNGVIHVVSRVMIP (153–171), and in the second domain they are: TVFAPTNEAF (210–219), ILKYHVV (240–246), and DIPVTNGVVHVIDTVLIP (289–306). The sequence outside of these three motifs is poorly conserved between the two domains. The overall sequence has a net basic charge, with a predicted isoelectric point of 8.0 (MacDNAsis), very similar to the measured isoelectric point of 7.9 for the protein (Weis and Levine, 1996).

In addition to the presence of a signal sequence, there are several other characteristics of the sym32 predicted amino acid sequence which indicate that it is an external membrane-associated protein. There are two potential N-glycosylation sites (NSTF and NASN, at amino acids 93–96 and 156–159) suggesting that the protein is secreted, and four potential myristylation sites (GTFCSA, GLTNGA, GSDVTV, and GGSVTV, amino acids at 248–253, 254–259, 266–271, and 275–280) suggesting that the protein may be attached to a membrane with myristyl anchors. The sym32 protein contains 72% hydrophobic residues but lacks a clear transmembrane region.

3.2. Alignments of sym32 with Fas I proteins from other organisms

Insight into the possible function of *A. elegantissima* sym32 was revealed by its homology to the Fas I family of cell adhesion proteins, which occur in organisms ranging from prokaryotes to humans. In total, this gene family is loosely conserved, with sequences having approximately 25% identity and 50% similarity to one another throughout the family. The general structure of proteins in this family consists of one, two or four repeating domains 130–150 amino acids in length, that contain three moderately to highly conserved regions, the FRa motif, H-box, and the FRb motif (Hu et al., 1998). The three motifs in the anemone sequence that are highly conserved between its two domains (see above) correspond to the FRa, H-box, and FRb motifs in the Fas I family. Fig. 2 illustrates these three homologous regions in *A. elegantissima* sym32 and Fas I proteins from other organisms. These are mouse β-igh3 (accession #: AAC37658), chicken RGD-CAP (BAA21479), human OSF-2 (S36111), *Drosophila* Fas I (P10674) and Mfas (AF038842), sea urchin EBP-α (BAA82956), an unnamed gene from *Caenorhabditis* (CAB03000), *Volvox* algal-CAM (S51614), and *Synechocystis* MPB70 (BAA17432). The anemone sym32 sequence is from 70 to 100% identical to other sequences in the FRa region, 30–86% identical in the H-box, and 33–72% identical in the FRb region.

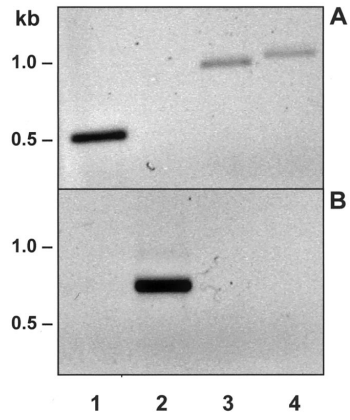

Fig. 3. PCR of genomic DNA from aposymbiotic host and freshly isolated zooxanthellae, using CA, PCP and *sym32* primers. Panels A and B show products amplified from host DNA and zooxanthellae DNA respectively. For both panels, lane 1, products amplified with host-specific CA primers; lane 2, products amplified with algal-specific PCP primers; lanes 3 and 4, products amplified with two different sets of *sym32* primers.

3.3. Origin of sym32 gene in the symbiosis

Despite our efforts to prevent contamination of algal nucleic acids during extraction of host nucleic acids (see Section 2), we were concerned that some transcript from the zooxanthellae could be included in the host transcript population. There remained a remote possibility that *sym32* could originate from algal tissues. We were interested, therefore, in determining the origin of the *sym32* gene within the anemone-algal symbiosis. We performed PCR using *sym32* primers on genomic DNA extracted from an aposymbiotic host and from zooxanthellae, freshly isolated from a symbiotic host. To control for cross-contamination of DNA from the opposing symbiotic partner, we also performed PCR using primers to a host-specific gene, carbonic anhydrase (CA), and an algal-specific gene, peridinin-chlorophyll protein (PCP). From host DNA, products were present using the CA primers and both sets of the *sym32* primers, but no products were present using the PCP primers (Fig. 3A). In contrast, only the PCP primers produced a product from the algal DNA (Fig. 3B). These results indicate 1) that both the host and algal DNA were free of contamination from the opposing partner and 2) that the *sym32* gene resides within the host, not the algal, genome.

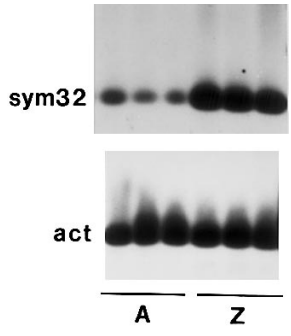

Fig. 4. Estimation of *sym32* and actin mRNA levels in aposymbiotic and zooxanthellate *Anthopleura elegantissima* using semi-quantitative RT-PCR. The top and bottom panels display products generated using *sym32* primers and actin primers respectively (see Section 2 for sequences). The first three lanes of both panels contain products from cDNA from three different aposymbiotic anemones (A) and the last three from zooxanthellate anemones (Z). The *sym32* product is more highly amplified in zooxanthellate animals than in aposymbiotic animals whereas the actin product is approximately equally expressed in both sets of animals.

3.4. Influence of symbiotic state on sym32 gene expression

To determine if *sym32* is differentially expressed as a function of symbiotic state, semi-quantitative PCR was performed using specific primers designed from the *sym32* cDNA sequence (Fig. 4). Actin, a presumed housekeeping gene, was also amplified to control for differential RNA degradation and cDNA synthesis efficiency. From all samples, the *sym32* primers amplified a 310 bp band, however, bands amplified from zooxanthellate host cDNA were much darker than those amplified from aposymbiotic cDNA. In contrast, bands amplified using the actin primers were of approximately equal intensity in all samples. These data indicate that *sym32* is more highly expressed in zooxanthellate animals than in aposymbiotic animals.

3.5. Influence of symbiotic state on sym32 synthesis

In order to monitor the presence of the sym32 protein in *A. elegantissima*, we developed an immuno-probe to a sym32 fusion protein. The *sym32* cDNA was cloned into an expression vector, the fusion protein symF, was expressed in bacteria and injected into a rabbit for production of an anti-symF antibody. Surprisingly, the resulting antiserum labeled two 32 kD spots in zooxan-

thellate protein homogenates (Fig. 4A and B). The larger spot had a p*I* of 7.9, the same pI as the spot purified to derive the sym32 N-terminus (see above), and the smaller spot had a p*I* of 8.2. No crossreactivity was detected on any other area of the 2D gel (data not shown). Crossreactivity with two 32 kD spots could indicate that sym32 is post-translationally modified with different types of sugars, which might slightly alter the p*I* of the protein. The crossreactivity of the anti-symF antiserum with the sym32 spots confirms that the *sym32* cDNA sequence encodes the sym32 protein extracted from symbiotic *A. elegantissima*.

To determine if sym32 abundance is influenced by the symbiotic state of the host, we performed anti-symF immunoblots of zooxanthellate, zoochlorellate, and aposymbiotic host soluble protein homogenates. Fig. 4B and C demonstrate that the abundance of sym32 is high in zooxanthellate anemones, relatively low in zoochlorellate anemones, and extremely low in aposymbiotic anemones. The bands in zooxanthellate, zoochlorellate and aposymbiotic lanes had total ODs of 3598, 2097, and 1219, respectively. These data indicate, therefore, that both the presence of algae and the type of the algae have a direct influence on sym32 synthesis in *A. elegantissima*.

4. Discussion

4.1. Sym32 shares sequence homology and biochemical characteristics with Fas I proteins

The sym32 predicted amino acid sequence and biochemical characteristics strongly suggest that sym32 is a member of the fasciclin I (*fasI*) gene family. *FasI* genes encode membrane-associated proteins or secreted proteins in a diverse array of organisms (Kreis and Vale, 1993). All Fas I proteins, including sym32, have one, two or four repeating domains, approximately 150 amino acids in length, and all have three homologous regions in each domain, the FRa motif, H-box and FRb motif (Hu et al., 1998). In addition, sym32 has a signal sequence, potential glycosylation sites, and no conclusive transmembrane region, characteristics identical to the best described of the Fas I proteins, fasciclin I (Snow et al., 1988; Zinn et al., 1988) and midline fasciclin from insects (Hu et al., 1998).

Sym32 is present in large quantities in the soluble fraction of symbiotic *A. elegantissima* protein homogenates (Weis and Levine, 1996). In addition, anti-symF immunoblots of anemone insoluble fraction (extracted with SDS) labeled a 32 kD band (data not shown), indicating that some sym32 remains attached to membranes. In *Drosophila*, Fas I also occurs in both soluble and membrane fractions (Hortsch and Goodman, 1990). Fasciclin I can be attached to the plasma membrane by a glycosyl-phosphotidylinositol (GPI) anchor, as evidenced by the presence of 15–20 hydrophobic residues at the carboxyl terminus, that are cleaved after attachment of the anchor, and experimental cleavage of the GPI anchor with a bacterial phospholipase C (Hortsch and Goodman, 1990). Fas I can also occur free in the soluble fraction apparently after cleavage of the GPI anchor (Hortsch and Goodman, 1990). The relative proportion of membrane-associated versus soluble Fas I changes depending on developmental state of the animal (Hortsch and Goodman, 1990) . The carboxyl terminus of the sym32 deduced amino acid sequence fits the criteria for the GPI anchor sequence (Ferguson and Williams, 1988), suggesting that sym32 could be GPI-linked, and it also contains four potential myristylation sites (Fig. 1) that could serve to anchor the protein to a membrane. More detailed biochemical studies are needed to definitively establish the presence of the putative membrane anchors and determine the relative proportions of membrane-associated and soluble forms of sym32 in *A. elegantissima*.

4.2. Fas I protein functions and implications for sym32 in A. elegantissima symbiosis

The Fas I gene family is extremely diverse phylogenetically. To date, members of the family have been described in mycobacteria, algae, nematodes, insects, sea urchins and mammals. All encode proteins that are either membrane-associated or secreted. Several members have been implicated in a variety of cell-cell interactions. Fas I in insects has been shown to play a role in cell adhesion, cell signaling and cell proliferation during morphogenesis (Elkins et al., 1990a,b; McAllister et al., 1992) . β-igh3 in mammals has been shown to have an anti-adhesion role in preventing formation of tumors (Skonier et al., 1994, 1992). *Beps* genes from sea urchins (Brennan and

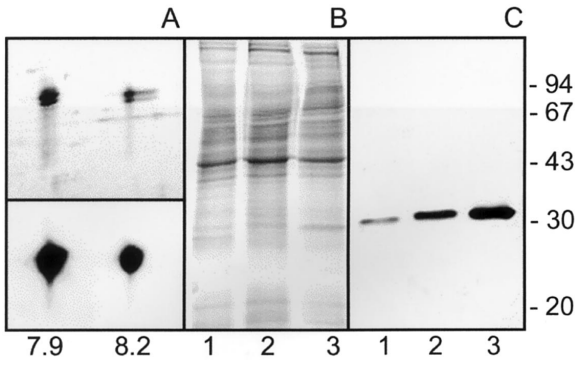

Fig. 5. SDS-PAGE and anti-symF immunoblots comparing homogenates of aposymbiotic, zoochlorellate and zooxanthellate *Anthopleura elegantissima*. Panel A shows a 2D gel and immunoblot of sym32 in zooxanthellate animal homogenate. Both gels contained 50 μg of protein. The top panel is a portion of a silver-stained gel showing a strongly staining 32 kD sym32 spot at 7.9 p*I*, and a smaller spot at 8.2. The bottom panel is an anti-symF immunoblot of the same zooxanthellate anemone homogenate showing labeling of the two spots. Panels B and C show Coomassie-stained 1D gels and anti-symF immunoblots respectively of aposymbiotic (lane 1), zoochlorellate (lane 2) and zooxanthellate (lane 3) anemone homogenates. All lanes contained 15 μg of protein. Molecular weights are shown on the right in kD. Panel C shows low labeling (1219 ODs) of a band at 32 kD in aposymbiotic homogenates, moderate labeling (2097 ODs) in zoochlorellate homogenates and strong labeling (3598 ODs) in zooxanthellate homogenates.

Robinson, 1994; DiCarlo et al., 1990; Romancino et al., 1992) and the algal-CAM gene in *Volvox* (Huber and Sumper, 1994) are both thought to play a role in cell adhesion. These purported roles for Fas I proteins in cell-cell interactions provide exciting insight into a role for sym32 in the anemone-algal symbiosis. Interpartner signaling and communication must be in place in these associations to regulate a variety of parameters including symbiont specificity, host-symbiont biomass ratios, and movement of metabolic products between partners. It is possible that sym32 is in some way mediating this contact.

4.3. Expression and synthesis of sym32 are influenced by symbiotic state

This study supports the hypothesis that the presence of algae in *A. elegantissima* tissue has a direct effect on gene expression and protein synthesis in the host. Previously we have reported on descriptive differences in patterns of protein synthesis between zooxanthellate and aposymbiotic anemones (Weis and Levine, 1996) and expression and synthesis of the enzyme carbonic anhydrase in zooxanthellate anemones (Weis and Reynolds, 1999). This study extends symbiosis-specific expression to a new gene in cnidarians.

A. elegantissima is of particular interest in the study of invertebrate/algal symbioses because of its ability to remain aposymbiotic and its ability to harbor two vastly different symbionts in its tissues, either or both zooxanthellae or zoochlorellae (Muscatine, 1971). In this study we have the first evidence of biochemical differences between zooxanthellate and zoochlorellate animals (Fig. 5). Therefore it is worth briefly introducing the ecophysiological differences between aposymbiotic, zooxanthellate and zoochlorellate anemones.

The three different anemones types are differentially distributed along a light and latitudinal gradient in their natural environment. Zooxanthellate animals occur in high light, warm water locations, zoochlorellate animals occur in relatively low light, cool water locations, and aposymbiotic animals occur in extremely low light locations, such as in caves and rock crevices (Saunders and Muller-Parker, 1997). In addition to different distributions in the field, the two symbioses differ markedly in their physiology, apparently due to the two symbiont types making very different energetic contributions to the host (Verde and McCloskey, 1996a,b). Zooxanthellae have low division rates and very high photosynthetic rates. They therefore transport a majority of their photosynthetically fixed carbon to the host. In contrast, zoochlorellae have high division rates and low photosynthetic rates, thereby contributing relatively little to host nutrition.

We provide the first evidence that synthesis of a "symbiosis" protein is differentially regulated depending on the type of symbiont that the host contains. Anemones containing the more productive zooxanthellae have considerably more sym32 than those that contain the less productive zoochlorellae, while aposymbiotic anemones contain very little sym32 (Fig. 5). These results raise an interesting question about how the symbiosis is regulated. What is the signal from the symbiotic algae that turns on sym32 expression? If equal synthesis of sym32 in zooxanthellate and zoochlorellate animals were observed, one might argue that it is the physical presence of the algae that affects gene expression. However the graded

response of sym32 synthesis observed in our results, that is correlated with symbiont productivity: more for zooxanthellae, less for zoochlorellae, none in the case of aposymbiotic animals, suggests that symbiont productivity in some way controls host gene expression.

Acknowledgements

We thank S. Patterson for technical support with peptide sequencing, J. Smythe for help with the engineering and expression of the fusion protein, and A. Pribyl for assistance with the immunoblots. This work was supported by grants from the National Science Foundation (9728405-IBN) and from the Office of Naval Research (N0001 49710101) to V.M.W.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Brennan, C., Robinson, J.J., 1994. Cloning and characterization of HLC-32, a 32-kDa protein component of the sea urchin extraembryonic matrix, the hyaline layer. Dev. Biol. 165, 556–565.

Buchsbaum, V.M., 1968. Behavioral and physiological response to light in the sea anemone *Anthopleura elegantissima* as related to its algal symbionts. Ph.D. Stanford University, Stanford, CA, 123 pp.

DiCarlo, M., Montant, G., Bonura, A., 1990. Analysis of the sequence and expression during sea urchin development of two members of a multigenic family, coding for butanol-extractable proteins. Mol. Reprod. Dev. 25, 28–36.

Douglas, A.E., 1994. Symbiotic Interactions. Oxford Science Publications, Oxford.

Dubinsky, Z., 1990. Coral Reefs. Elsevier, New York.

Elkins, T., Hortsch, M., Bieber, A.J., Snow, P.M., Goodman, C.S., 1990a. *Drosophila fasciclin* I is a novel homophilic adhesion molecule that along with fasciclin III can mediate cell sorting. J. Cell Biol. 110, 1825–1832.

Elkins, T., Zinn, K., McAllister, L., Hoffmann, F.M., Goodman, C.S., 1990b. Genetic analysis of a *Drosophila* neural cell adhesion molecule: Interaction of fasciclin I and abelson tyrosine kinase mutations. Cell 60, 565–575.

Falkowski, P.G., Dubinsky, Z., Muscatine, L., Porter, J., 1984. Light and the bioenergetics of a symbiotic coral. Bioscience 34, 705–709.

Ferguson, M.A.J., Williams, A.F., 1988. Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol structures. Ann. Rev. Biochem. 57, 285–320.

Fisher, R.F., Long, S.R., 1992. Rhizobium-plant signal exchange. Nature 357, 655–660.

Frohman, M.A., Dush, M.K., Martin, G.R., 1988. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. 85, 8998–9002.

Furla, P., Benazet-Tambutte, S., Allemand, D., 1998. Functional polarity of the tentacle of the sea anemone *Anemonia viridis*: role in inorganic carbon acquisition. Am. J. Physiol. 274, R303–R310.

Goiran, C., Al-Moghrabi, S., Allemand, D., Jaubert, J., 1996. Inorganic carbon uptake for photosynthesis by the symbiotic coral/dinoflagellate association I. Photosynthetic performances of symbionts and dependence on sea water bicarbonate. J. Exp. Mar. Biol. Ecol. 199, 207–225.

Hatcher, B.G., 1988. The primary productivity of coral reefs: a beggar's banquet. Trends Ecol. Evol. 3, 106–111.

Hatcher, B.G., 1996. Organic production and decomposition. In: Birkeland, C. (Ed.), Life and Death of Coral Reefs. New York, Chapman Hall, pp. 140–174.

Hortsch, M., Goodman, C.S., 1990. *Drosophila* fasciclin I, a neural cell adhesion molecule, has a phosphatidylinositol lipid membrane anchor that is developmentally regulated. J. Biol. Chem. 265, 15104–15109.

Hu, S., Sonnenfeld, M., Stahl, S., Crews, S.T., 1998. Midline fasciclin: A *Drosophila* fasciclin-I-related membrane protein localized to the CNS midline cells and trachea. J. Neurobiol. 35, 77–93.

Huber, O., Sumper, M., 1994. Alga-CAMs: isoforms of a cell adhesion molecule in embryos of the algal *Volvox* with homolgy to *Drosophila* fasciclin I. EMBO J 13, 4212–4222.

Kreis, T., Vale, R., 1993. Guidebook to the Extracellular Matrix and Adhesion Proteins. Oxford University Press, Oxford.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

McAllister, L., Goodman, C.S., Zinn, K., 1992. Dynamic expression of the cell adhesion molecule fasciclin I during embryonic development in *Drosophila*. Development 115, 267–276.

McFall-Ngai, M.J., 1998. The development of cooperative associations between animals and bacteria: Establishing detente among domains. Am. Zool. 38, 593–608.

Muscatine, L., 1971. Experiments on green algae coexistent with zooxanthellae in sea anemones. Pac. Sci. 25, 13–21.

Muscatine, L., 1990. The role of symbiotic algae in carbon and energy flux in reef corals. In: Dubinsky, Z. (Ed.), Coral Reefs. Elsevier, Amsterdam, pp. 75–87.

Muscatine, L., Weis, V.M., 1992. Productivity of zooxanthellae and biogeochemical cycles. In: Falkowski, P.G., Woodhead, A. (Eds.), Primary Productivity in the Sea. Plenum Press, New York, pp. 257–272.

Patterson, S.D., Thomas, D., Bradshaw, R.A., 1996. Application of combined mass spectrometry and partial amino acid sequence to the identification of gel-separated proteins. Electrophoresis 17, 877–891.

Romancino, D.P., Ghersi, G., Montana, G., Bonura, A., Perriera, S., DiCarlo, M., 1992. Characterization of bep1 and bep4 antigens involved in cell interactions during *Paracentrotus lividus* development. Differentiation 50, 67–74.

Rowan, R., Powers, D., 1991. Molecular genetic identification of symbiotic dinoflagellates (zooxanthellae). Mar. Ecol. Prog. Ser. 71, 65–73.

Ruby, E.G., 1996. Lessons from a cooperative bacterial-animal association: The *Vibrio fischeri-Euprymna scolopes* light organ symbiosis. Ann. Rev. Microbiol. 50, 591–624.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Plainview, New York.

Saunders, B.K., Muller-Parker, G., 1997. The effects of temperature and light on two algal populations in the temperate sea anemone *Anthopleura elegantissima* (Brandt, 1835). J. Exp. Mar. Biol. Ecol. 211, 213–224.

Secord, D.L., 1995. Host specificity and symbiotic interactions in sea anemones. Ph.D. University of Washington, Seattle, 89 pp.

Skonier, J., Bennett, K., Rothwell, V., Kosowski, S., Plowman, G., Wallace, P., Edelhoff, S., Disteche, C., Neubauer, M., Marquardt, H., Rodgers, J., Purchio, A.F., 1994. βig-h3: a transforming growth factor-β-responsive gene encoding a secreted protein that inhibits cells attachment in vitro and suppresses the growth of CHO cell in nude mice. DNA Cell Biol. 13, 571–584.

Skonier, J., Neubauer, M., Madisen, L., Bennett, K., Plowman, G.D., Purchio, A.F., 1992. cDNA cloning and sequence analysis of βig-h3, a novel gene induced in a human adenocarcinoma cell line after treatment with a transforming growth factor-β. DNA Cell Biol. 11, 511–522.

Smith, D.C., Douglas, A.E., 1987. The Biology of Symbiosis. Edward Arnold, London.

Smith, S.E., Read, D.J., 1997. Mycorrhizal Symbiosis. Academic Press, San Diego.

Snow, P.M., Zinn, K., Harrison, A.L., McAllister, L., Schilling, J., Bastiani, M.J., Mark, G., Goodman, C.S., 1988. Characterization and cloning of fasciclin I and fasciclin II glycoproteins in the grasshopper. Proc. Natl. Acad. Sci. 85, 5291–5295.

Verde, E.A., McCloskey, L.R., 1996a. Carbon budget studies of symbiotic cnidarian anemones-evidence in support of some assumptions. J. Exp. Mar. Biol. Ecol. 195, 161–171.

Verde, E.A., McCloskey, L.R., 1996b. Photosynthesis and respiration of two species of algal symbionts in the anemone *Anthopleura elegantissima* (Brandt) (Cnidaria: Anthozoa). J. Exp. Mar. Biol. Ecol. 195, 187–202.

Wang, J.-T., Douglas, A.E., 1998. Nitrogen recycling or nitrogen conservation in an alga-invertebrate symbiosis? J. Exp. Biol. 201, 2445–2453.

Weis, V.M., 1993. Effect of dissolved inorganic carbon concentration on the photosynthesis of the symbiotic sea anemone *Aiptasia pulchella* Carlgren: Role of carbonic anhydrase. J. Exp. Mar. Biol. Ecol. 174, 209–225.

Weis, V.M., Levine, R.P., 1996. Differential protein profiles reflect the different lifestyles of symbiotic and aposymbiotic *Anthopleura elegantissima*, a sea anemone from temperate waters. J. Exp. Biol. 199, 883–892.

Weis, V.M., Reynolds, W.S., 1999. Carbonic anhydrase expression and synthesis in the sea anemone *Anthopleura elegantissima* are enhanced by the presence of dinoflagellate symbionts. Physiol. Zool. 72, 307–316.

Weis, V.M., Smith, G.J., Muscatine, L., 1989. A "CO_2 supply" mechanism in zooxanthellate cnidarians: role of carbonic anhydrase. Mar. Biol. 100, 195–202.

Werner, D., Ahlborn, B., Antoun, H., Bernard, S., Bolanos, C., Cooper, J.E., Gorge, E., Jacobi, A., Kalliopi, P., Kape, R., Katinakis, P., Kosch, K., Muller, P., Parniske, M., Schmidt, P., Streit, W., Wetzel, A., 1994. Communication and signal exchange in the *Rhizobium*/*Bradyrhizobium*-legume system. Endocyto. Cell Res. 10, 5–16.

Zinn, K., McAllister, L., Goodman, C.S., 1988. Sequence analysis and neuronal expression of fasciclin I in grasshopper and *Drosophila*. Cell 53, 577–587.