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Localization of a Symbiosis-Related Protein, Sym32, in the *Anthopleura elegantissima–Symbiodinium muscatinei* Association

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Abstract. Cnidarian–dinoflagellate symbioses are widespread in the marine environment. Growing concern over the health of coral reef ecosystems has revealed a fundamental lack of knowledge of how cnidarian–algal associations are regulated at the cellular and molecular level. We are interested in identifying genes that mediate interactions between the partners, and we are using the temperate sea anemone *Anthopleura elegantissima* as a model. We previously described a host gene, *sym32,* encoding a fasciclin domain protein, that is differentially expressed in symbiotic and aposymbiotic *A. elegantissima.* Here, we describe the subcellular localization of the sym32 protein. In aposymbiotic (symbiont-free) hosts, sym32 was located in vesicles that occur along the apical edges of gastrodermal cells. In symbiotic hosts, such vesicles were absent, but sym32 was present within the symbiosome membranes. Sym32 (or a cross-reactive protein) was also present in the accumulation bodies of the symbionts. Although the anti-sym32 antiserum was not sufficiently specific to detect the target protein in cultured *Symbiodinium bermudense* cells, Western blots of proteins from two *Symbiodinium* species revealed a protein doublet of 45 and 48 kDa, suggesting that the symbionts may also produce a fasciclin domain protein. We suggest that host sym32 is relocated from gastrodermal vesicles to the symbiosome membrane when symbionts are taken into host cells by phagocytosis.

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Introduction

Intracellular associations between eukaryotic microorganisms and animal hosts encompass a wide range of interactions ranging from parasitic to mutualistic. Most eukaryotic microbes that infect animals reside within a membrane-bound vacuole within the cytoplasm of host cells (Hackstadt, 2000). The formation of the vacuole is a dynamic process that is initiated either by active invasion of the host cell by the microbe—for example, the parasite *Toxoplasma* (Dobrowolski and Sibley, 1996)—or by phagocytic uptake of the microbe by the host cell—for example, the parasite *Leishmania* (Courret *et al.,* 2002) and the symbiont *Symbiodinium* (Fitt and Trench, 1983).

Studies on eukaryotic microbial parasites show that, in the vast majority of cases, the nascent vacuole that contains the microbe fails to continue through the complete endocytic pathway that culminates in acidification of the vacuole and fusion with lysosomes. Instead, the microbial inhabitant actively interferes with one or more steps in the normal process of fusion between the vacuole and endocytic organelles that direct the vacuole through the phagocytic process (Mellman, 1996). The result is that the microbe successfully transforms what would have been a phagolysosome into a compartment that is hospitable for growth, replication, or differentiation into various life-history stages (Hackstadt, 2000; and for examples, see Mukkada *et al.,* 1985; Sinai and Joiner, 1997).

In the marine environment, associations between animals and eukaryotic microbes include mutualistic as well as parasitic interactions. One prevalent mutualistic association occurs between cnidarians (most commonly sea anemones and corals) and photosynthetic dinoflagellates (usually *Symbiodinium* spp.). These associations are characterized by reciprocal nutritional interactions between host and

symbiont (Falkowski *et al.,* 1984; Muller-Parker and D'Elia, 1998). The photosynthetic symbionts contribute glycerol and other organic compounds to host metabolism (Lewis and Smith, 1971; Battey and Patton, 1987; Muscatine, 1990), and the host contributes nitrogen (Wang and Douglas, 1998, 1999) and carbon dioxide (Weis, 1993) for algal photosynthesis. These associations occur in the photic zones of both temperate and tropical benthic habitats, and they thrive in nutrient-poor tropical marine environments because they conserve and recycle nutrients.

In its associations with dinoflagellates, the host cnidarian most commonly houses symbionts within gastrodermal cells, in a vacuole of phagosomal origin. The initial infection, during which the dinoflagellate symbionts are internalized, typically occurs when dinoflagellates that enter the host's mouth are taken into phagocytic gastrodermal cells that line the gastric cavity (Colley and Trench, 1983; Fitt and Trench, 1983; Schwarz *et al.,* 1999, 2002). The phagosome, through unknown mechanisms, fails to fuse with lysosomes (Fitt and Trench, 1983), and the dinoflagellates remain undigested within the vacuole. Ultimately, the dinoflagellates reside within a compartment delineated by multiple membranes (Taylor, 1968, 1987; Wakefield *et al.,* 2000). Historically, the origin of the multiple membranes that surround the symbiont has been uncertain. Recently, immunolocalization studies using host-specific and dinoflagellate-specific monoclonal antibodies suggest that only the outermost membrane originates from the host, and all of the inner membranes originate from the dinoflagellates, perhaps accumulating from repeated cycles of ecdysis within the host vacuole (Wakefield and Kempf, 2001).

We have been interested in identifying host genes that play roles in symbiotic interactions in cnidarian–dinoflagellate symbioses (Weis and Levine, 1996; Weis and Reynolds, 1999; Reynolds *et al.,* 2000). We have used as a model system the temperate symbiotic sea anemone *Anthopleura elegantissima* (Brandt, 1835), which is abundant in the intertidal region of the eastern Pacific, from Mexico through Alaska. This species is able to form associations with two species of dinoflagellates, *Symbiodinium californium* and *S. muscatinei.* The presence of one or more of the symbionts within a particular host depends upon microhabitat differences that are created along temperature and light gradients that occur along latitudinal and intertidal ranges. Along the Oregon coast, most hosts contain only a single symbiont, *S. muscatinei* (LaJeunesse and Trench, 2000).

Using biochemical and molecular approaches, we have identified a number of host genes that are likely to function in mediating host-symbiont interactions (Reynolds *et al.,* 2000; Weis and Levine, 1996; Weis and Reynolds, 1999). One of these, *sym32,* encodes a protein that belongs to a class of cell adhesion proteins called fasciclin domain proteins (Reynolds *et al.,* 2000). Fasciclin domain proteins share low overall sequence identity, but all possess between one and four repeats of three highly conserved "fasciclin" domains that are believed to function as adhesive domains in cell–cell and cell–extracellular matrix interactions (Hu *et al.,* 1998). Members of this family have been identified in organisms as diverse as mycobacteria (Harboe and Nagai, 1984; Harboe *et al.,* 1995; Terasaka *et al.,* 1989), *Volvox* (Huber and Sumper, 1994), insects (Zinn *et al.,* 1988), sea urchins (Brennan and Robinson, 1994), and humans (Skonier *et al.,* 1992; Takeshita *et al.,* 1993).

To further investigate the role played by *sym32* in symbiotic interactions between cnidarian hosts and dinoflagellate symbionts, we used immunocytochemical techniques to examine the distribution of the sym32 protein within the *A. elegantissima–S. muscatinei* association. In this paper we demonstrate that sym32 protein is differentially distributed in symbiotic and aposymbiotic *A. elegantissima* both at the cellular and subcellular level. Of particular interest, sym32 antiserum labels the multiple layers of membrane that surround the symbiont within the host cell. We also show that anti-sym32 antiserum specifically labels the accumulation body of dinoflagellates residing within host gastroderm. Western blots of proteins from two *Symbiodinium* species revealed a protein doublet of 45 and 48 kDa (45/48 kDa) that cross reacts with the sym32 antiserum; but in immunolocalization studies, the antiserum was insufficiently specific to detect the target protein in cultured specimens of *S. bermudense.* Possibly, both the host and symbiont produce fasciclin domain proteins that interact *via* the fasciclin adhesive domain.

Materials and Methods

Animal maintenance

Symbiotic and aposymbiotic specimens of *A. elegantissima* were collected at low tide from the intertidal zone at Seal Rock, Oregon. Aposymbiotic anemones were taken from under rock overhangs or crevices where there was little or no light to support the growth of symbiotic dinoflagellates. Symbiotic anemones were taken from the open rock benches that are exposed to light. Anemones were transported to the laboratory, where they were maintained in an 11 °C recirculating seawater aquarium on a 12:12 h light:dark cycle. Anemones were fed previously frozen adult brine shrimp about once a week.

Light-level immunocytochemistry

Anemones. Tentacles from both symbiotic and aposymbiotic specimens of *A. elegantissima* were clipped and immediately transferred to tissue freezing medium (Triangle Biomedical Sciences) and frozen at −80 °C. Tentacles were cryosectioned at −20 °C on a Reichert-Jung cryostat (20-μm sections) and placed on polylysine slides, then immersed in 4% paraformaldehyde fixative in phosphate

buffered saline (PBS: 10 m*M* phosphate buffer, pH 7.2, + 150 m*M* NaCl) for 1.5 h. Sections on slides were rinsed three times, 5 min each time, in PBS with 0.5% BSA; dehydrated in a methanol series (25%, 50%, 75%, 100%, 75%, 50%, 25%); and rinsed again in PBS/BSA. Sections were incubated in a blocking solution of 1:200 dilution of goat serum:PBS/BSA for 30 min at room temperature and then rinsed three times, 5 min each time, in PBS/BSA. Slides were incubated for 1 h in either a 1:2000 dilution of sym32 antiserum from rabbit (antibody development described in Reynolds *et al.*, 2000) in PBS/BSA or in a 1:2000 dilution of preimmune serum from the same rabbit. Sections were rinsed three times, 5 min each time, in PBS/BSA and then incubated for 1 h in a 1:200 dilution of goat anti-rabbit IgG–5-nm colloidal gold conjugate (Ted Pella). Slides were rinsed as above. Gold particle labeling was silver-enhanced using a silver enhancement kit (Ted Pella). To stop color development, slides were washed in ePure water. Coverslips were affixed with a glycerol mount and sealed with fingernail polish.

Cultured Symbiodinium bermudense *cells.* Immunofluorescence was used to investigate whether the symbiont-produced 45/48 kDa cross-reactive protein (identified from Western blots, described below) could be localized to symbionts free from host cells. Cells of *S. bermudense* (CCMP830) were grown in sterile filtered seawater enriched with f/2-Si media at about 100 μE light on a cycle of 12 h light to 12 h dark at 25 °C. Cells were collected by centrifugation from liquid media and resuspended into 3% paraformaldehyde in PBS. After fixation for 30 min, cells were given two 10-min washes in PBS and transferred to PBS. Cells were incubated 30 min in blocking solution (PBS + 3% BSA); 30 min in PBS/BSA/0.2% Triton X-100; 1 h in preimmune serum or anti-sym32 antiserum diluted with PBS/BSA/Triton X to 1:50, 1:200, 1:2000; 5 min in PBS, repeated five times; 1 h in 1:200 Alexa Fluor 488 goat anti-rabbit IgG in PBS/BSA; and 5 min in PBS, repeated five times. After incubation, the cells were viewed under an Olympus BX-60 fluorescence microscope.

EM-level immunocytochemistry

Anemones. Immunocytochemistry was performed on three occasions using symbiotic anemones collected at different times and on one aposymbiotic anemone. Tentacles from aposymbiotic and symbiotic anemones were clipped and immersed in 1% paraformaldehyde, 1% glutaraldehyde fixative in PBS for 1.5 h. Tentacles were rinsed three times, 10 min each time, in PBS and then dehydrated for 15 min in each concentration of a methanol (MeOH) series (15%, 30%, 50%, 85%, 95%, 100%, 100%). Tentacles were infiltrated with LR White resin on a rotating table in a series of MeOH dilutions (1:3 LR White:MeOH overnight, 1:1 overnight, 100% LR White for 3 h), and then placed in gelatin capsules in fresh LR White. LR White was allowed to polymerize at 52 °C for 2 days.

Ultra-thin, gold–silver sections were cut with a diamond knife and placed onto Formvar-coated nickel grids. These grids were processed for immunocytochemistry as follows: they were immersed in blocking solution (PBS + 5% BSA) for 15 min, incubated in a 1:1000 dilution of sym32 antiserum or a 1:1000 dilution of preimmune serum in PBS for 1.5 h, rinsed 3 times, 10 min each time, in PBS/BSA + 0.1% Tween 20, incubated in a 1:75 dilution of EM grade goat anti-rabbit IgG–15-nm colloidal gold (Ted Pella) in PBS for 1 h, rinsed as above, rinsed in ePure water for 5 min, and then allowed to dry. Grids were stained in 2% uranyl acetate for 5 min, rinsed by dipping in water 10 times each in three changes of water, then immediately stained in 0.4% lead acetate for 3 min, with water rinses as above, and then air dried. Between 5 and 10 grids of each type of anemone were viewed under 60 kV using a CM-12 Phillips transmission electron microscope.

Cultured Symbiodinium bermudense *cells.* Cells were collected as described in the light microscopy section, fixed as described for anemone tentacles, and processed essentially as described for anemone tentacles. Several dilutions of anti-sym32 antiserum and preimmune serum were tested (1:10, 1:50, 1:200, 1:500, 1:1000, 1:2000), as well as various dilutions of either Tween-20 or Triton X (0%, 0.1%, 1%) in the following solutions: blocking solution, primary antibody solution, wash solution.

Preparation of anemone and dinoflagellate proteins: one- and two-dimensional SDS-PAGE and Western analysis

Proteins were isolated from the host as follows: a host anemone was removed from an 11 °C recirculating aquarium and flash-frozen in liquid nitrogen. The anemone was minced with a razor blade, and placed into a glass grinder with a Teflon pestle driven by a hand drill in four volumes ice-cold grinding buffer (100 m*M* Tris, 100 m*M* NaCl, 10 m*M* EDTA) with protease inhibitors (Sigma: 5 μl per 10 ml buffer). The homogenate was placed into a centrifuge tube and the grinder was rinsed with two volumes of buffer (v:w anemone tissue), which was added to the tube and mixed well. The remaining homogenate was centrifuged at 16,000 $\times$ *g* for 10 min at 4 °C to remove algal cells and host cell debris and membranes from the homogenized anemone tissue. The supernatant was removed to a new tube and centrifuged again. Protein concentration was determined on this cleared homogenate using the Bradford assay (Pierce Coomassie reagent). Host proteins prepared according to this protocol are free from contamination by symbiont proteins (Weis and Levine, 1996).

Proteins were isolated from symbionts that had been either continuously maintained in culture with no host contact for many generations or freshly isolated from an

A. elegantissima host. Many species of *Symbiodinium* can be isolated from their hosts and brought into culture in nutrient-supplemented seawater. These symbionts are therefore free from any host cell contact. We obtained frozen pelleted symbionts from cultures of *S. bermudense,* which was originally isolated from the tropical sea anemone *Aiptasia pallida.* A chunk of the pelleted symbionts (about 100 μl in volume) was briefly ground in a glass tissue grinder with a Teflon pestle in an equal volume of grinding buffer with protease inhibitor cocktail. Examination under a light microscope revealed that nearly all symbionts were still intact after this step. An equal volume of acid-rinsed glass beads (Sigma: 425–600 μm) was added and the mixture alternately vortexed for 15–30 s and placed on ice for about 30 s, for a total of 20 times. With repeated vortexing, the homogenate became intensely orange, likely indicating the release of the major water-soluble accessory pigment, peridinin-chlorophyll protein. By this means, at least 75% of symbionts were broken open, as determined by light microscopy. With a syringe and a 24-gauge needle, the homogenate was removed from the glass beads and centrifuged at 16,000 $\times$ *g* for 10 min at 4 °C to remove cellular debris. The supernatant was placed into a new tube and again centrifuged. This cleared supernatant fraction was then assayed for protein concentration, as described above, and then prepared for one-dimensional or two-dimensional SDS-PAGE, as described below.

Proteins from freshly isolated symbionts were prepared by isolating symbionts from a host and then extracting proteins using glass beads to fracture the symbiont cell wall and release the contents of the cytoplasm. A host anemone weighing about 2 g was removed from an 11 °C recirculating aquarium and flash-frozen in liquid nitrogen. The anemone was minced with a razor blade and placed into a glass grinder with 3 ml of grinding buffer plus protease inhibitor. All subsequent steps were performed on ice. The anemone was ground with a Teflon pestle instead of a ground glass pestle to homogenize the animal tissues without shearing the cell walls of the symbionts. This homogenate was centrifuged at 2000 $\times$ *g* for 10 min to pellet the symbionts. The pellet, about 300 μl in volume, was partially cleaned of anemone debris by regrinding the pellet in grinding buffer using a glass tissue grinder with a Teflon pestle (this regrinding step was sufficient to break up the pellet, but not break open the symbionts) and reconcentrating the symbionts by centrifugation. The partially cleaned pellet was ground a final time before adding 500 μl of buffer with protease inhibitor cocktail and an equal volume of prerinsed glass beads. This resuspended pellet contained a significant amount of host tissue, as revealed by the presence of numerous nematocysts. We then followed the same vortexing and centrifugation protocol as described above. The cleared supernatant fraction (intensely orange in color) was used for one-dimensional SDS-PAGE, as described below.

One-dimensional SDS-PAGE with 10% Nu-PAGE Bis-Tris gels (Invitrogen) was performed on proteins from host tissue and from freshly isolated, and cultured symbiont proteins. Samples were denatured and prepared for electrophoresis using LDS buffer + DTT (Invitrogen) according to manufacturer's instructions; 10 μg of protein was loaded for each sample. Electrophoresis was performed in MOPS buffer according to the manufacturer's instructions. Gels were transferred to 12.5 m*M* Tris, 100 m*M* glycine, 10% MeOH for 20 min, and proteins were electrophoretically transferred onto nitrocellulose membrane for 1.25 h at 100 V in a BioRad chamber.

Two-dimensional SDS-PAGE was performed using proteins extracted from freshly isolated symbionts. Proteins were extracted as described above, except that no NaCl was used in the buffer, as salt interferes with isoelectric focusing. The SDS-PAGE was carried out on a Multiphor II system (Amersham Pharmacia), according to the manufacturer's instructions and as described in Reynolds *et al.* (2000). Thirty microliters of symbiont homogenate containing 60 μg of protein was used for isoelectric focusing on an 180-mm IPG strip, pH 3–10. After isoelectric focusing, the IPG strip was equilibrated and placed on a 12% ExcelGel; electrophoresis was performed according to manufacturer's instructions. After electrophoresis, the gel was placed into transfer buffer (50 m*M* Tris, 40 m*M* glycine, 0.04% SDS, 20% methanol) for 20 min. The gel was removed from the plastic backing, and proteins were transferred to a nitrocellulose membrane under a discontinuous buffer system (Multiphor II system from Amersham).

For Western analysis, the membranes from one- and two-dimensional SDS-PAGE were incubated at 4 °C overnight in blocking buffer (TBS: 20 m*M* Tris, 500 m*M* NaCl, Ph 7.5, + 5% powdered milk + 0.1% Tween-20). The following morning, the membrane was washed for 15 min in TBS + 0.5% Tween-20 (TBST); incubated for 45 min in a 1:1500 dilution of anti-sym32 antiserum:block buffer; rinsed 10 min each in TBS, TBST, TBS; incubated 45 min in a 1:5000 dilution of HRP-antirabbit IgG (Amersham Pharmacia); and washed as before. Sym32 protein was detected by chemiluminescence using ECL detection reagents (Amersham Pharmacia) and exposing membranes to film for 1 min.

Results

Microscopy of anemone tentacles

Cryosectioned tentacles. Cryosectioned tentacles of aposymbiotic and symbiotic anemones were incubated with (1) preimmune serum as a negative control for endogenous staining or (2) sym32 antiserum. Staining for sym32 in aposymbiotic tentacles was distinct from that in symbiotic tentacles, relative to preimmune controls (Fig. 1). In both symbiotic and aposymbiotic tentacles, preimmune controls

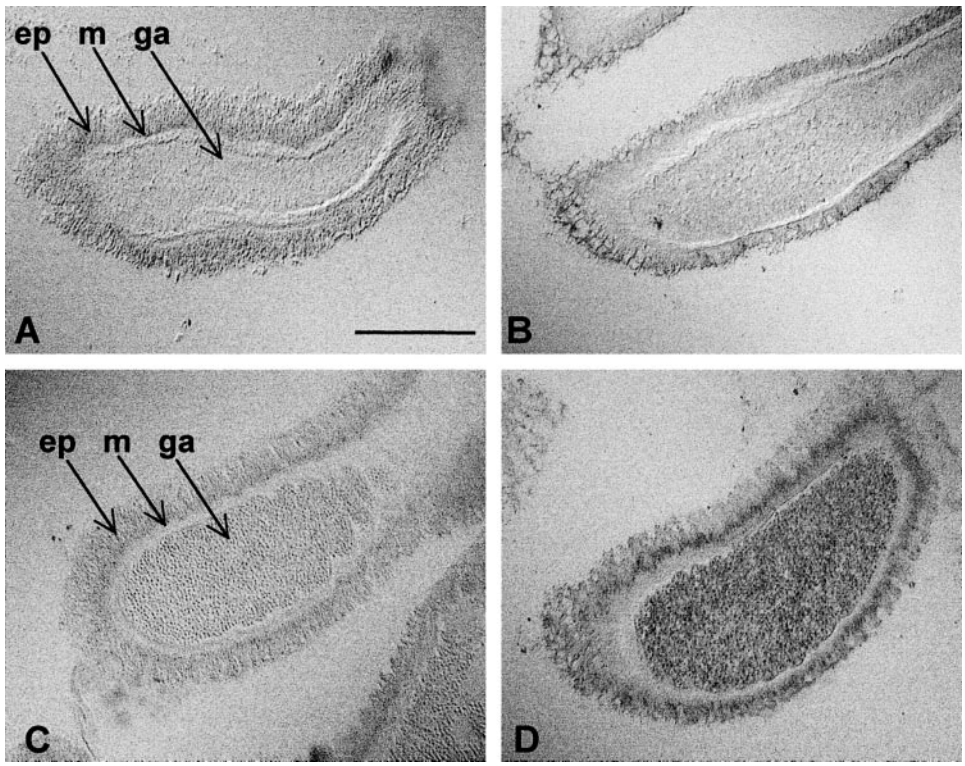

Figure 1. Light micrographs showing immunolocalization of sym32 protein within cryosections of tentacles from aposymbiotic (A and B) and symbiotic (C and D) *Anthopleura elegantissima.* The spherical symbionts can be clearly seen within the gastroderm of symbiotic tentacles. Sections were incubated in either preimmune serum (A and C) or sym32 anti-serum (B and D), and sym32 was visualized using silver enhancement of colloidal gold labeling. Sym32 levels are high in the gastrodermis of symbiotic anemones, low in the gastrodermis of aposymbiotic anemones, faint in the epidermis of both types of anemones, and absent in the mesoglea of both types of anemones. Host tissue layers are marked as ep = epidermis, ga = gastrodermis, m = mesoglea. Scale bar = 0.3 mm for all panels.

showed light brown staining in epidermal and gastrodermal tissues, and no staining in the mesoglea. In aposymbiotic tentacles incubated in sym32 antiserum, staining in the epidermal and gastrodermal layers was slightly darker than in the preimmune controls. In symbiotic tentacles, staining in the epidermis was also slightly darker than in the preimmune controls, and in the gastrodermis, where dinoflagellates are housed, it was significantly darker. The mesogleal layer of both aposymbiotic and symbiotic tentacles remained unstained.

Electron microscopy. To further examine the location of sym32 within the host–symbiont association, we performed EM-level colloidal gold immunocytochemistry using sym32 antiserum to label the sym32 protein within thin sections of resin-embedded tentacles from one aposymbiotic and three symbiotic anemones (Figs. 2–4). We examined between 15 and 25 sections from each anemone. We also performed negative controls with preimmune serum to check for nonspecific labeling of the tissues. In all cases, the preimmune controls were almost completely free of gold-sphere labeling (data not shown).

In aposymbiotic tentacles, sym32 gold-sphere labeling was associated exclusively with medium-density vesicles located within both epidermal and gastrodermal cells (Fig. 2). There was no evidence of any sym32 label within the mesogleal layer, and the pattern of distribution of the sym32-containing vesicles in the epidermal cells was distinct from that in the gastrodermis. The vesicles were relatively uncommon in the epidermis (Fig. 2A, B) but more abundant in the gastrodermis, where they were concentrated along the apical end of the gastroderm, near the interface between the gastroderm and the gastric cavity (Fig. 2C, D).

In symbiotic tentacles, the pattern of distribution in the epidermis was the same as in aposymbiotic tentacles; sym32 gold-sphere labeling was contained within vesicles that were relatively sparsely distributed, most commonly occurring near nematocysts. In contrast, the distribution of sym32 within the gastrodermis was dramatically different. The sym32-containing vesicles that were so abundant in aposymbiotic gastroderm were not present in symbiotic gastroderm. Instead, the sym32

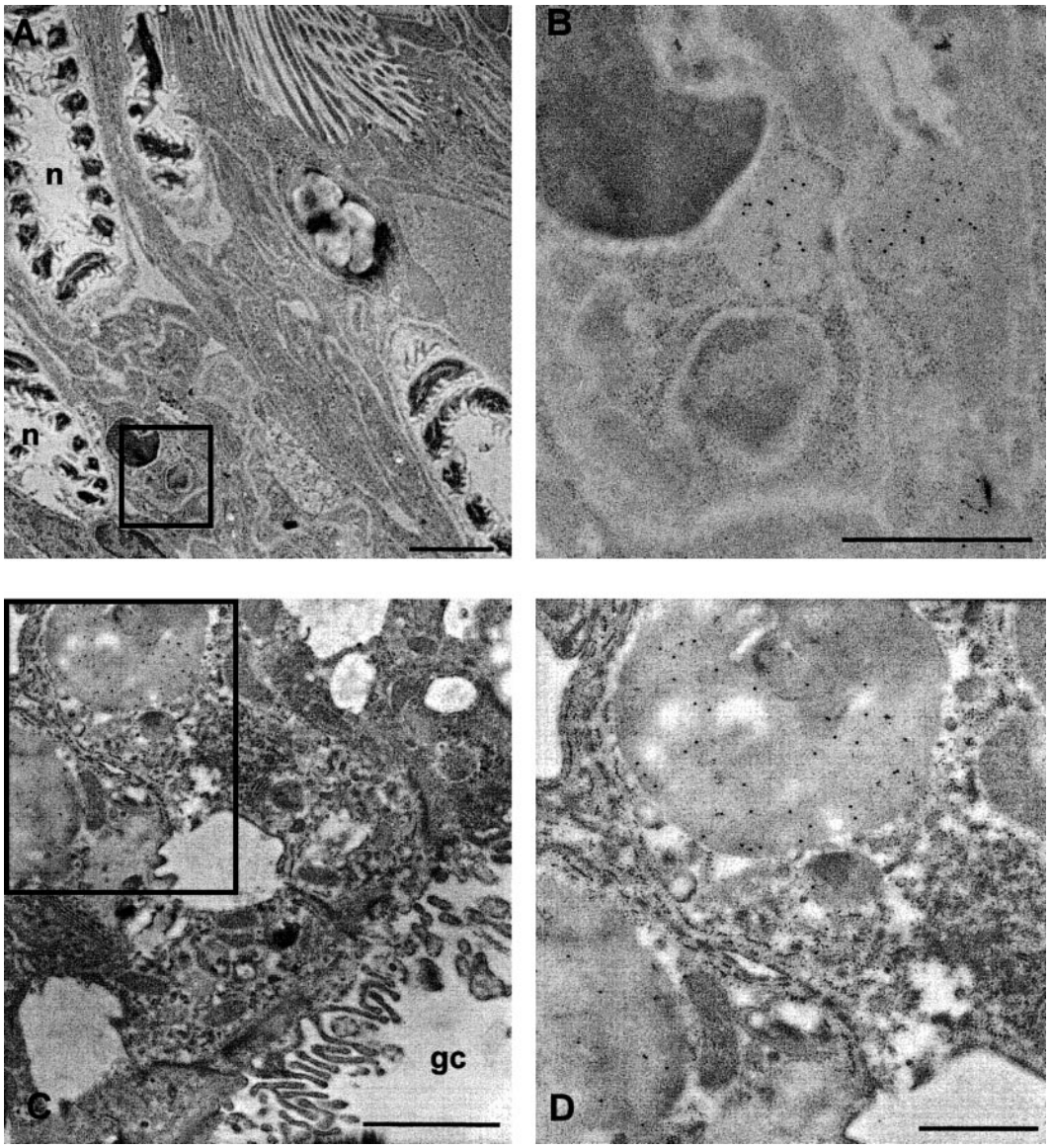

Figure 2. Transmission electron micrographs of immunogold-labeled sections from tentacles of aposymbiotic anemones. Gold spheres, visible as black dots, indicate the presence of sym32. (A) Epidermal cells with nematocysts (n). (B) Enlargement of the boxed section of A, with gold spheres labeling vesicles located near nematocysts. (C) Gastrodermal cells adjacent to the gastric cavity (gc). (D) Enlargement of the boxed section of C, showing gold spheres within vesicles in the gastrodermal cells. Scale bars = 2 μm (A, C) and 1 μm (B, D).

label was associated with the multiple membranes that enclose the dinoflagellate symbiont within the host cell (Fig. 3A, B). Gold spheres were diffusely arranged within these membranes, not clearly associated with any single membrane layer. To confirm that this labeling was specific to the membranous layers, we quantified the staining relative to areas outside the membranes. The membranous layers contained an average of 12.2 gold spheres $\pm$ 4.65 (SD), $n = 19$, while equivalent areas outside the membranous layers contained an average of 1.0 $\pm$ 1.1, $n = 19$).

Symbiodinium *within host cells.* In addition to the sym32 labeling within the membranes that surround the symbionts, there was a significant amount of labeling within the symbionts themselves (Fig. 4). Specifically, gold spheres were located within the accumulation body, a poorly described organelle that is believed to function in the endocytic pathways of dinoflagellates. The density of labeling within the accumulation bodies was highly variable: some contained only a few gold spheres, while others contained hundreds (average = 56.4 $\pm$ 83.2 [SD] gold spheres/μm^2, $n = 18$).

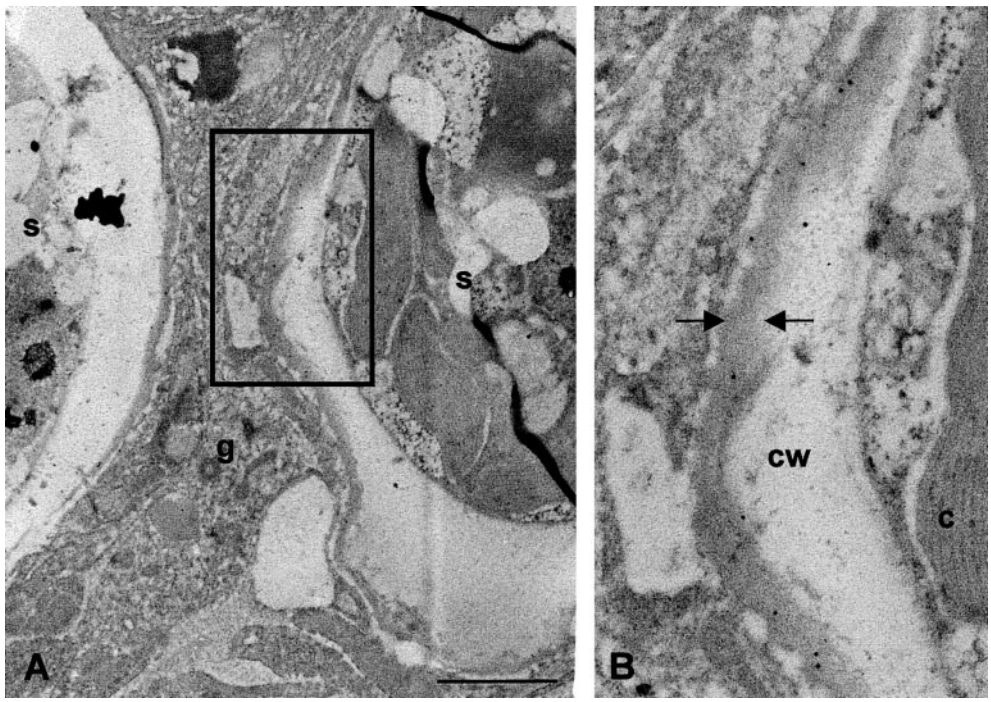

Figure 3. Transmission electron micrographs of immunogold-labeled sections of tentacles from symbiotic *Anthopleura elegantissima.* (A) Illustrates the presence of dinoflagellates within host gastrodermal cells. (B) Enlargement of boxed area in A, showing gold spheres associated with the multiple layers of membrane that surround the dinoflagellates. The arrows delineate the margins of the multiple membranes surrounding the dinoflagellate. Preimmune controls showed virtually no gold-sphere labeling (not shown). c = symbiont chloroplast, cw = symbiont cell wall, g = gastrodermal cell of the host, s = dinoflagellate symbiont. Scale bar = 2 μm.

Western blots

The presence of gold-sphere label within the accumulation bodies of the symbionts suggested that the symbionts were producing a sym32 homolog. We performed Western analysis using anti-sym32 antiserum to look for cross-reactive proteins in homogenates from symbionts freshly removed from a host anemone and from cultured symbionts that were not in contact with host cells. Anti-sym32 Western blots of one-dimensional gels from homogenates of freshly isolated *Symbiodinium muscatinei* revealed three bands (Fig. 5A, lane 2). The 32-kDa band, identical in size to a band in host-only homogenate (lane 1), is probably due to contamination from host sym32. This was expected, as protein preparations of the symbionts are invariably contaminated by host proteins (Weis *et al.*, 1998). A 48-kDa band and a faint 45-kDa band below it suggest the presence of cross-reactive proteins that are produced by the symbionts. Homogenates of cultured algae (*Symbiodinium bermudense*) that were never in contact with host tissues (lane 3) also contained the same 45/48-kDa doublet but lacked the 32-kDa host band. Western blots of two-dimensional gels of freshly isolated *S. muscatinei* homogenates revealed two spots with distinctly different molecular weights and isoelectric points (pI) (Fig. 5B). A cross-reactive spot at 32 kDa, 8.2 pI, corresponds exactly with host sym32 (Reynolds *et al.*, 2000), and again is probably due to host contamination. In addition there was a 48-kDa spot with a pI range of 4.3 to 4.5. The 45/48-kDa protein doublet that is present in cultured *S. bermudense* and in *S. muscatinei* freshly harvested from a host, and is also faintly visible in the host lane, therefore represents a protein produced by *Symbiodinium* both when it is in symbiosis with a host, and when it is free-living.

Microscopy of cultured Symbiodinium bermudense *cells*

We were interested in determining the location of the symbiont-produced 45/48-kDa protein doublet. We therefore used the anti-sym32 antiserum (which was developed against recombinant host sym32 protein), to localize the target protein in cultured specimens of *S. bermudense.* We used two immunolocalization methods. For intact cells, we used immunofluorescence, in which a fluorescent secondary antibody is detected by fluorescence microscopy. For sectioned cells, immunoelectron microscopy allowed us to detect any patterns in staining

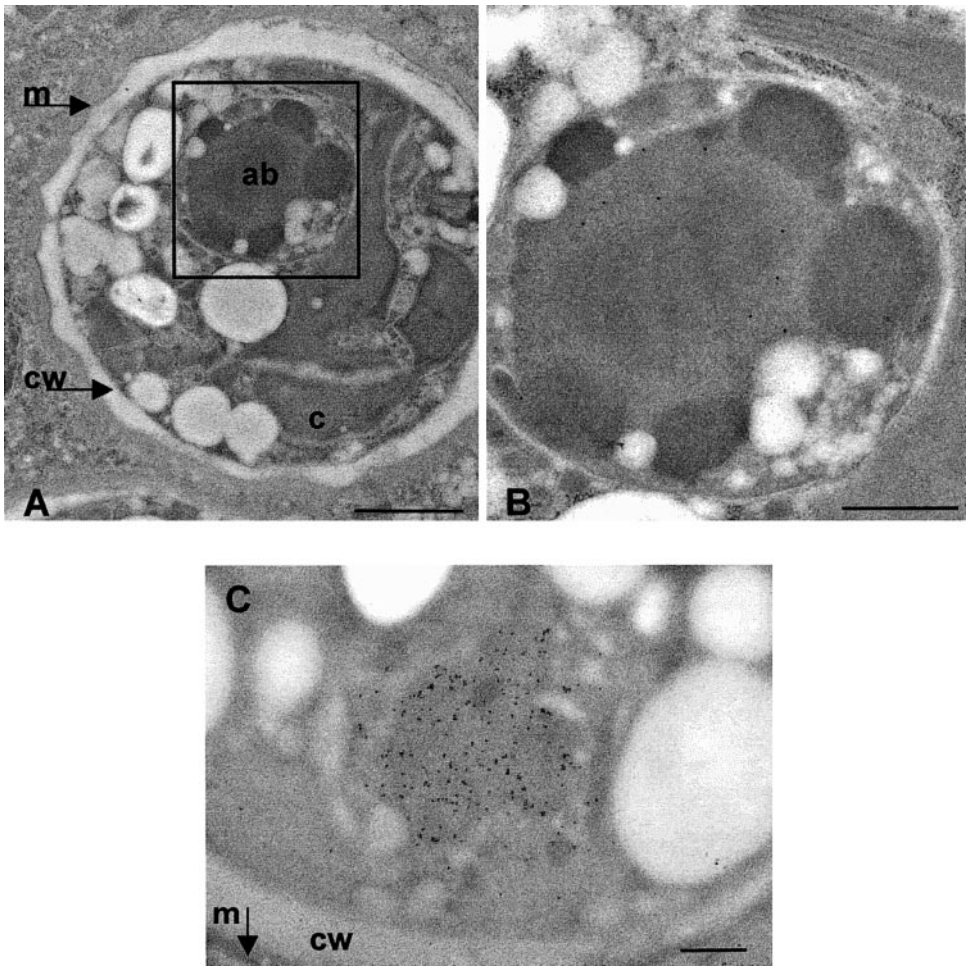

Figure 4. Transmission electron micrographs of immunogold-labeled sections of dinoflagellate symbionts contained within host gastrodermal cells. (A) Dinoflagellate contained within a host gastrodermal cell. (B) Enlargement of the accumulation body, shown boxed in A, illustrating sparse gold labeling of the dinoflagellate accumulation body. (C) Region around the accumulation body of another symbiont (section not counterstained) showing intense labeling specific to the accumulation body. The cell wall (cw) and membrane layers (m) are visible as concentric gray rings around the symbiont. Sections incubated with preimmune serum showed virtually no gold-sphere labeling (data not shown). m = membranes surrounding the dinoflagellate, cw = dinoflagellate cell wall, c = dinoflagellate chloroplast, ab = accumulation body of the dinoflagellate. Scale bar = 2.5 μm (A), 1.0 μm (B, C).

at the subcellular level. Despite testing many concentrations of antiserum and buffer solutions, we were unable to detect a pattern of staining of the target protein in cultured cells of *S. bermudense*. With the immunofluorescence method, preimmune controls looked identical to antiserum-incubated specimens at all dilutions of serum that we tested. With immunoelectron microscopy, we could detect no specific staining to any particular compartment within the cell, even though the background staining varied from almost no gold-sphere labeling at the 1:2000 dilutions to heavy labeling at the 1:10 dilutions. In the preimmune controls, labeling was restricted to a few, widely scattered spheres.

Discussion

The sym32 protein is distributed among different subcellular compartments in symbiotic and aposymbiotic anemones. Most notably, the sym32-containing vesicles that are so abundant in the gastroderm of aposymbiotic hosts are absent from symbiotic hosts; instead, sym32 localizes to the symbiosome membranes. This suggests that the internalization of symbionts is accompanied by a transfer of sym32 from gastrodermal vesicles to the symbiosome membranes. This would most likely occur during phagocytic uptake of the symbiont, by fusion of the sym32 vesicles with the phagosome.

The presence of sym32 within the dinoflagellates them-

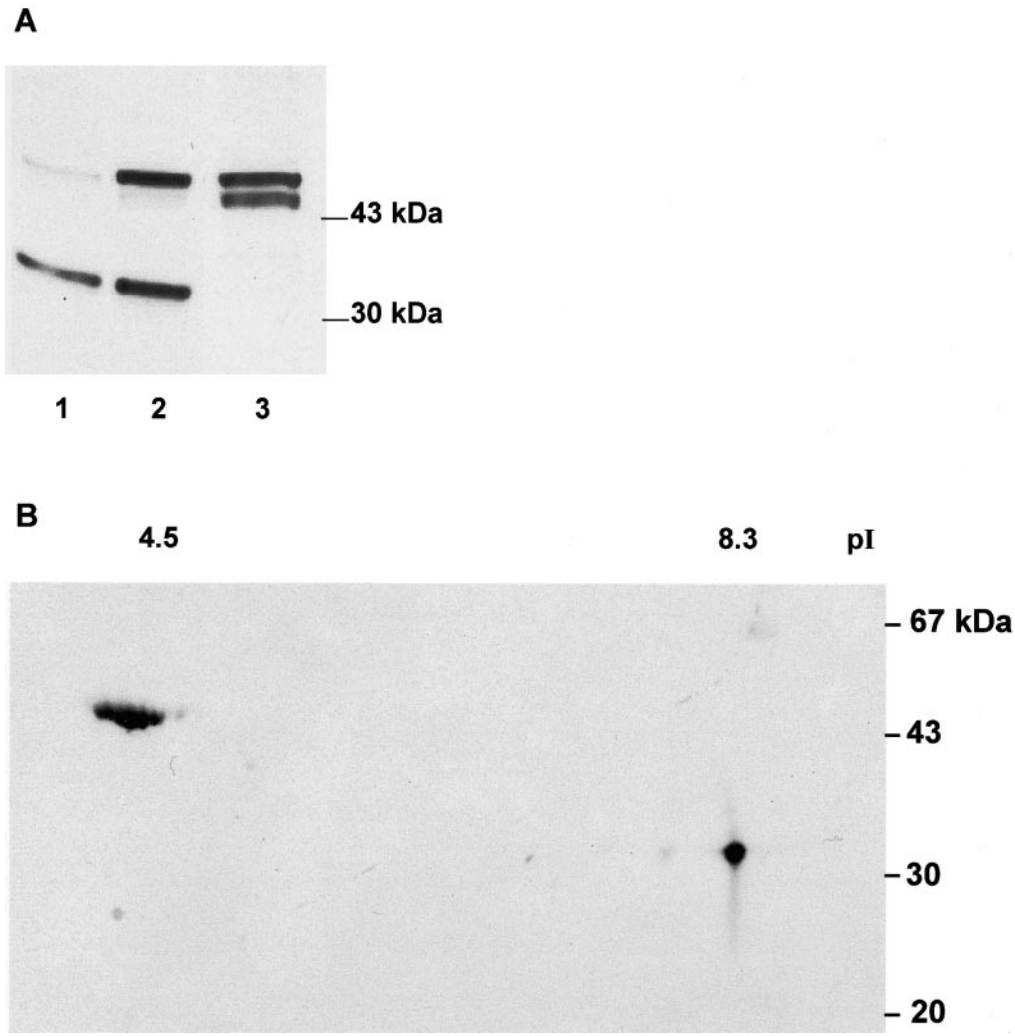

Figure 5. Anti-sym32 Western blots of protein homogenates from *Symbiodinium.* (A) Western blot of a one-dimensional gel. All lanes contained 10 μg of soluble protein. Lane 1, host-only proteins, shows a 32-kDa band and a faint 48-kDa band. Lane 2, *Symbiodinium muscatinei* freshly harvested from a host anemone (and therefore contaminated with host tissues), shows three bands: a strongly staining 32-kDa band, a strongly staining 48-kDa band, and a faint 45-kDa band. Lane 3, cultured *Symbiodinium bermudense,* contains two equal intensity bands at 45-kDa and 48-kDa. (B) Western blot of a two-dimensional gel of protein homogenate (60 μg) from *S. muscatinei* freshly harvested from an anemone host. Two spots are present: a 32-kDa, pI 8.2, spot, identifiable as sym32, presumably from contaminating host tissue, and a 48-kDa spot, pI range 4.3–4.5.

selves complicates the picture and adds a new dimension to our studies of sym32. The accumulation body in dinoflagellates is postulated to function as a lysosome, although this organelle has not been studied in many species, and its function has not been examined in *Symbiodinium.* The free-living dinoflagellate *Prorocentrum* has multiple accumulation bodies with features characteristic of eukaryotic lysosomes. These bodies contain electron-dense material, fibrous material, and membranous material, and they possess acid phosphatase activity, react positively with the periodic acid/Schiff reagent, and stain with acridine orange (Zhou and Fritz, 1994). *Symbiodinium* has a single accumulation body that varies in size and is postulated to function as a molecular "trash dump" (Taylor, 1987; Wakefield *et al.,* 2000). We observed that the accumulation body is invariably located adjacent to the nucleus, often appearing to displace the edge of the nucleus. If the accumulation body is a lysosome or a trash dump, host sym32 may be transported from the vacuolar membranes, across the dinoflagellate cell wall, and into a degradative pathway within the dinoflagellate. This ability to transport molecules from the host cell, across the vacuolar membrane, into the cytosol or organelles of an intracellular inhabitant is common in parasitic protozoans, and there are many mechanisms by which this occurs (Schwab *et al.,* 1994; Raibaud *et al.,* 2001; Goodyer *et al.,* 1997).

It is also possible, however, that the protein detected in the accumulation body is not a host protein but a symbiont protein. To determine whether the symbionts also produce a sym32 cross-reactive protein, we performed anti-sym32 Western analysis on symbiont proteins separated by one- or two-dimensional SDS-PAGE. Anti-sym32 antiserum labeled a 45/48-kDa cross-reactive protein doublet in both cultured *S. bermudense* (free from any host cell contact) and *S. muscatinei* that we removed from host cells (Fig. 5). The presence of a 32-kDa band in the lane containing *S. muscatinei* proteins almost certainly results from the presence of contaminating host proteins. It is highly likely that the symbiont 45/48-kDa protein is, in fact, a sym32 homolog. Its size is consistent with the fasciclin domain proteins, which consist of between one and four repeats of an approximately 15-kDa domain (thus symbiont p45/48 might consist of three repeats of the 15-kDa-domain). Furthermore, fasciclin domain proteins are diversely distributed and have been identified in bacteria (Paulsrud and Lindblad, 2002; Terasaka *et al.,* 1989), photosynthetic algae (Huber and Sumper, 1994), invertebrate animals (Bastiani *et al.,* 1987; Zinn *et al.,* 1988; Brennan and Robinson, 1994; Bostic and Strand, 1996; Reynolds *et al.,* 2000), and humans (Skonier *et al.,* 1992). To confirm that symbiont p45/48 is in fact a fasciclin domain protein, we are attempting to sequence this gene.

We attempted to immunolocalize the 45/48-kDa symbiont protein in fixed specimens from cultures of *S. bermudense,* by using the anti-sym32 antiserum to detect the 45/48-kDa target protein. Two methods (immunofluorescence to examine intact cells, and immunoelectron microscopy to examine sectioned cells) failed to detect any pattern of staining. This suggests that the anti-sym32 antiserum, although able to recognize epitopes in denatured symbiont proteins on a Western blot (Fig. 4), was not specific enough to detect epitopes in the native protein within *S. bermudense* (data not shown). Occasionally, antibodies can work in Western analysis but not immunolocalization, especially when the antibody is being used to detect a protein other than that to which it was developed (Harlow and Lane, 1999). These results strengthen the conclusion that the protein that we observed within the symbiosome membranes and the symbiont accumulation body is the host-derived protein and not the symbiont-derived protein. If true, this indicates that the symbiont has an active role in modifying the structure or design of the symbiosome membranes. However, this possibility will remain untested until antibodies that can distinguish between the host and symbiont proteins are developed.

Interest in fasciclin domain proteins appears to be gaining momentum judging from the many recent reports that describe the functions or structures of these proteins in diverse organisms (for example, Kim *et al.,* 2002; Tamura *et al.,* 2002; Carr *et al.,* 2003; Clout *et al.,* 2003). These reports greatly expand upon the initial description of the fasciclin I protein as a homophilic cell adhesion molecule in insects (Elkins *et al.,* 1990). It is now known that, while all fasciclin domain proteins contain at least one 15-kDa fasciclin binding domain, they may also contain a variety of other functional domains that lend diversity to their functions. All, however, share the common role, *via* the fasciclin domain, of mediating recognition and specificity events in cell-cell or cell-extracellular matrix interaction. The mechanisms by which they do so have yet to be elucidated, but clues may be provided by their structures, as in the insect fasciclin I protein (domains 3 and 4) and the *Mycobacteria tuberculosis* complex MBP70 protein (Carr *et al.,* 2003; Clout *et al.,* 2003). Although the mycobacterial MBP70 protein consists of a single fasciclin domain, and the insect fasciclin I protein contains four domains, the structures of the individual domains are strikingly similar. Each domain appears to fold to produce two functional faces on opposite sides of the protein, each of which probably binds independently to other molecules (Carr *et al.,* 2003). Mutations within these functional faces in the human protein βig-h3 are known to be associated with corneal dystrophy, suggesting that specificity in binding of these molecules to their targets is mediated by small changes in amino acid composition along the functional faces (Carr *et al.,* 2003). This has important implications for the possible role of a fasciclin domain protein in a symbiosis, where specificity in molecular interactions between host and symbiont likely mediates the establishment and regulation of the partnership.

Recent evidence suggests that fasciclin domain proteins function in mediating symbiotic interactions in other associations. In both the rhizobium–plant association and the cyanobacterial–fungal lichen association, homologs have been identified from the symbiont genomes, and in plants, deletion of this gene reduces its ability to fix nitrogen (Oke and Long, 1999; Paulsrud and Lindblad, 2002). This is the first report to suggest that both partners in a symbiosis may produce fasciclin domain proteins.

The sym32 story is complex. The sym32 protein apparently has functions in multiple biological processes within the host; both in symbiotic interactions with dinoflagellates (as evidenced by the presence of sym32 within the membranes surrounding the dinoflagellates), and in other non-symbiosis-related processes (as evidenced by the presence of sym32 in the epidermis of both aposymbiotic and symbiotic anemones). Furthermore, the presence of a cross-reactive protein in both free-living *Symbiodinium* and *Symbiodinium* freshly isolated from a host suggests that the symbionts possess a sym32 homolog. Still to be elucidated are the degree to which the host and symbiont proteins interact and the roles that each plays in the biology of each partner separately and of the partners in symbiosis.

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