Evidence for the Participation of the Proteasome in Symbiont-Induced Tissue Morphogenesis

JENNIFER R. KIMBELL, TANYA A. KOROPATNICK, AND MARGARET J. MCFALL-NGAI*

Pacific Biomedical Research Center, Kewalo Marine Laboratory, University of Hawaii, Honolulu, Hawaii

The mediation of remote events in host tissues by a bacterial partner is a phenomenon reported in both plant and animal symbioses (1,2). Underlying these events are complex cellular and molecular dialogues orchestrating the indirect communication between the symbionts and host cells. One such example is the morphogenesis of the light organ of juveniles of the squid Euprymna scolopes*, a process that is induced by symbionts residing deep within these host tissues (1). The most dramatic consequence of this process is the loss of the light organ's superficial ciliated epithelium, resulting in a remodeling of the organ accompanied by hemocyte infiltration into this tissue and apoptosis of associated cells. The host genes regulated by the symbionts to mediate this morphogenesis remain to be determined. Here we provide evidence that the symbionts induce an increase in host mRNA coding for the C8α subunit of the proteasome, a highly regulated complex that degrades proteins. C8α subunit mRNA localized to the hemocytes infiltrating the tissue undergoing morphogenesis. Experiments with inhibitors of proteasome activity suggested that these changes in gene expression are accompanied by modifications in proteasome activity. These findings support a role for the expression and activity of the proteasome in development of the host's light organ.*

In the association of the squid *Euprymna scolopes* Berry, 1913, with its bacterial symbiont *Vibrio fischeri,* a specialized host light organ is colonized anew each generation (Fig. 1A; for review, see Nyholm and McFall-Ngai, 2004 (1)). The complex architecture of the hatchling light organ facilitates inoculation by the symbiont. Each lateral surface of the bilobed organ consists of a conspicuous ciliated epithelial field with a base and two protruding appendages, which are composed of a single epithelial layer overlying a sinus. During inoculation, the cilia of these fields entrain the symbionts into the proximity of three pores at the base of each field. *V. fischeri* cells migrate through these pores and down ciliated ducts to colonize deep crypt spaces on either side of the organ. Throughout the life of the host, the extracellular symbionts reside in the crypts, in physical contact with two host cell types: the polarized epithelium that lines each crypt, and macrophage-like blood cells, or hemocytes, which are transient inhabitants of the crypt.

Approximately 12 h after inoculation, *V. fischeri* cells induce a dramatic, irreversible morphogenesis of the light organ that occurs over about 96 h (Fig. 1B). Most dramatic is the complete regression of the ciliated fields that mediate the inoculation process. The symbionts induce this developmental program remotely. Specifically, the crypts where *V. fischeri* cells reside and signal morphogenesis are several cell layers away from the ciliated fields that regress. Cellular events that correlate with the symbiont-induced regression include hemocyte infiltration into the sinuses of the appendages and apoptosis of the cells composing the superficial ciliated epithelium (3) (Fig. 1C).

We employed a subtractive hybridization method with cDNA libraries of light organs from 12-h aposymbiotic (*i.e.,* exposed to other environmental bacteria, but with no *V. fischeri* cells present) and symbiotic juvenile animals to identify candidate host genes involved in the response to the symbiont-induced signal for morphogenesis (4, 5). One candidate (GenBank accession no. AY616011), which comprised 30% of the clones identified as more abundant in the symbiotic light organs, encoded a host mRNA most similar to C8α subunits of the mammalian proteasome. The proteasome is a highly regulated enzyme complex responsible for the majority of cellular protein degradation *via* the ubiq-
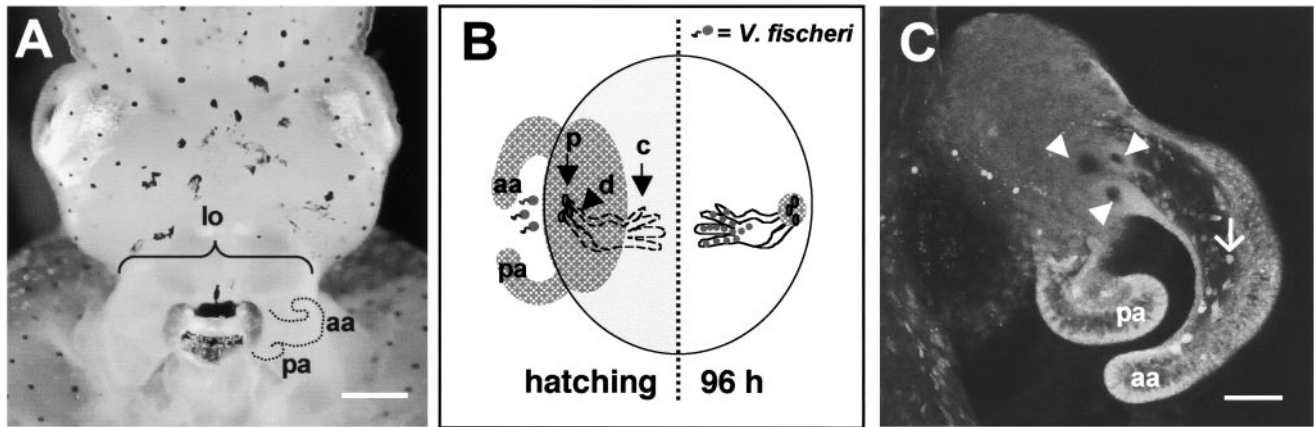
Figure 1.   Juvenile squid (*Euprymna scolopes*) were maintained in open-flow seawater tables as previously described (13). Newly hatched squid were made symbiotic by incubating them in seawater containing cells of *Vibrio fischeri* ES114 (~10^3 cells/ml) for 12 h, followed by maintenance in filter-sterilized seawater (FSSW). Aposymbiotic squid were maintained in FSSW. (**A**) A ventral dissection of a squid revealing the light organ. The epithelial fields extend from either side of the light organ. The right field is outlined to indicate location and size. aa = anterior appendage of the light organ; lo = light organ; pa = posterior appendage of the light organ. Scale bar = 200 μm. (**B**) Schematic of the light organ depicting the superficial epithelial fields (stippled gray), pores (p), ducts (d), and deep epithelium-lined crypts (c). At hatching (left of the dotted line) the fields are present. By ~96 h post-hatching (right of the dotted line), the fields of symbiotic juveniles have regressed, leaving only a small patch of cilia around the pores. The crypts are colonized by *V. fischeri*. (**C**) Confocal micrograph (Zeiss LSM 510) of one epithelial field, stained with acridine orange and LysoTracker red, showing the pores (arrowheads) and a cross section through the anterior appendage (aa) and posterior appendage (pa). The arrow points to a hemocyte within the appendage sinus. Scale bar = 50 μm.

uitin-proteasome pathway (6). In higher eukaryotes, the 28 subunits of the 20S proteasome core occur as two outer alpha and two inner beta rings. The C8α subunit is pivotal; it acts as a nucleation site for the other alpha subunits, which in turn are a scaffold for 20S proteasome assembly. The proteasome participates not only in protein turnover, but in the regulation of various signaling events and processes key to morphogenesis, such as apoptosis and tissue remodeling (7, 8). These events are hallmarks of light organ morphogenesis.

We first analyzed the characteristics of the protein encoded by the mRNA. The protein has 255 amino acids (data not shown), with characteristics similar to those of other C8α subunits; that is, high similarity occurs across the entire sequence (alignment score over the length $\geq$200) for all matches in the NCBI database. The first hits to the *E. scolopes* protein in the database are to vertebrate sequences, from fish to mammals; these sequences have 73%–74% identities and 84%–85% positives with the *E. scolopes* protein. Sequences of other invertebrates (*e.g., Strongylocentrotus purpuratus, Drosophila pseudoobscura, Anopheles gambiae*) and plant species (*e.g., Arabidopsis thaliana, Spinacia oleracea*) in the database had somewhat lower similarity, with 51%–68% identity and 72%–81% positives with the squid protein. Of note is that, in the translated C8α subunit of *E. scolopes,* an alanine and glutamine occur at positions 243 and 250, respectively. In mammalian C8α

subunits, serines occur at these two positions and are sites of phosphorylation; increased proteasome activity results from phosphorylation of these serines, which project outwardly from the alpha ring (9). The lack of serines in these positions in the squid protein suggests that a different regulation mechanism exists for the squid proteasome.

The mRNA encoding the C8α subunit in 12-h and 24-h symbiotic light organs was about 10 times more abundant than in that from aposymbiotic juveniles (Fig. 2A). By 96 h, no difference was detected between symbiotic and aposymbiotic animals. Labeling by the antisense riboprobe for C8α subunit mRNA was detectable above background only in hemocytes that had infiltrated into the sinuses of the light organ appendage of symbiotic animals (Fig. 2B–C), although not all hemocytes in these regions cross-reacted strongly with the riboprobe. In contrast, the few hemocytes present in the appendages of the ciliated field of aposymbiotic light organs did not show detectable levels of C8α subunit mRNA (Fig. 2B). Hemocytes in the gills of aposymbiotic and symbiotic animals did not show detectable levels of C8α subunit mRNA (data not shown). Incubation with the sense probe (Fig. 2B–C, insets) did not produce fluorescence above background.

We next sought to determine the effects of proteasome inhibitors (lactacystin, MG-132, and proteasome inhibitor I) on light organ morphogenesis and on the abundance of C8α subunit mRNA. Hatchling squid were subjected to either

A

B

apo

C

sym

aa

aa
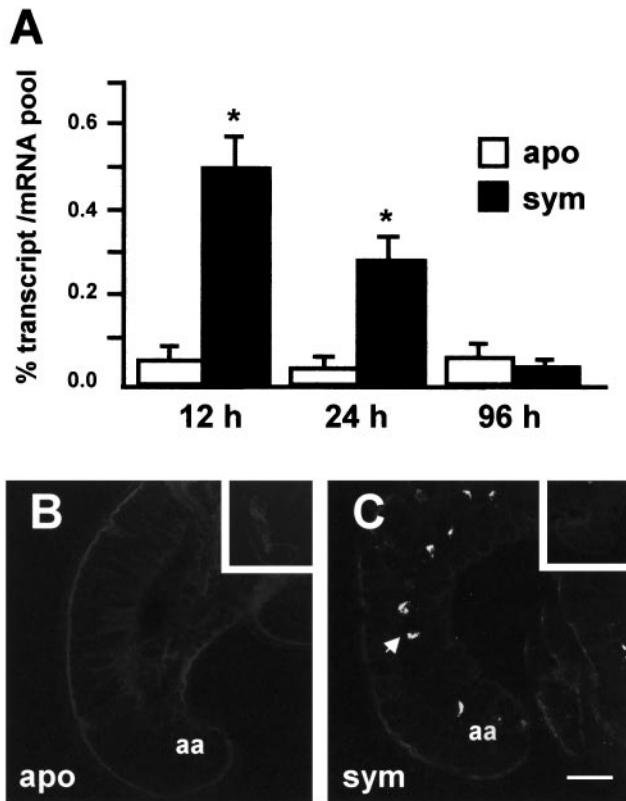
Figure 2. **(A)** A representative graph showing C8α subunit mRNA abundance (average $\pm$ s.e.m.) in the light organ of aposymbiotic and symbiotic squid at 12, 24, and 96-h post-hatching as determined by real-time reverse-transcriptase (RT)-PCR (iCycler, Bio-Rad). mRNA extraction, quantification, and quality control have previously been described (25). For a control, actin mRNA abundance was determined using the same mRNA pools. No significant differences in actin mRNA abundance were detected at these time points (data not shown). Procedures for RT-PCR experimental and control reactions were as previously described (25) using sequence-specific reverse primers for the actin and C8α transcripts. Conditions for amplifying the C8α subunit cDNA were as follows: 10-μl reactions contained SYBR Green core reagents (Perkin Elmer Biosystems), 0.2 μM of C8α-specific forward and reverse primers, and 5 mM MgCl$_2$. PCR cycles were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 5 s, and 72 °C for 10 s. The following C8α subunit primers were used: forward 5′-GAGGTCGCCAAAATAATCC-3′, reverse 5′-GGTCTTCTTCATCGTCTG-3′. Conditions for amplifying actin cDNA were as previously reported (25). Reactions were run in triplicate. Asterisks represent significant differences between aposymbiotic and symbiotic light organs at a given time point (Student's *t*-test, $P < 0.05$). **(B–C)** Confocal micrographs showing the localization of C8α subunit mRNA by *in situ* hybridization in the sinus of the anterior appendage (aa) of the light organ in 12-h aposymbiotic animals (**B**) and 12-h symbiotic animals (**C**). Insets show corresponding control groups treated with sense probe. Scale bar = 30 μm. *In situ* hybridization was performed as previously reported (25), with the following modification. For detection, specimens were incubated in Fab fragments from anti-digoxigenin sheep antibodies conjugated to fluorescein Roche at a ratio of 1:1000 overnight at 4 °C. Arrow points to a cross-reactive hemocyte.

proteasome inhibitors or protease inhibitors (ALLN, ALLM, and calpeptin) not specific to the proteasome to determine whether the effect was specific to the proteasome. Working concentrations for each inhibitor were 5 μM lactacystin, 5 μM MG-132, 1 μM PSI, 30 μM ALLN, 10 μM ALLM, and 1 μM calpeptin, as reported in previous cell culture studies (10–12).

The effect that each inhibitor had on epithelial field regression was determined in aposymbiotic and symbiotic animals by using confocal microscopy. Epithelial fields of control 48-h aposymbiotic light organs did not regress, and the fields of the control 48-h symbiotic animals were about halfway through the regression process. Degree of regression was assessed as previously described (13). The three proteasome-specific inhibitors each had a similar effect on field regression at 48 h, either inducing or expediting field regression of aposymbiotic and symbiotic light organs, respectively. In contrast, regression in the epithelial fields of squid incubated with the non-proteasome-specific protease inhibitors was not different from that in the control groups. At the concentrations used, none of these inhibitors had apparent detrimental effects on the health of the animals or on the growth rate of *V. fischeri* in liquid culture.

Because lactacystin is reported to be a very specific proteasome inhibitor (6) and induces morphological responses similar to those triggered by symbiosis, we used this inhibitor for further analyses. Specifically, we reasoned that if this inhibitor was affecting the proteasome in the same way as symbiosis, we should see similar phenotypes; conversely, we might see different phenotypes, suggesting that this pharmacological agent was acting in a way that would mimic the symbiosis by different means. Thus, aposymbiotic and symbiotic animals were treated with 5 μM lactacystin, and localization of C8α subunit mRNA in the light organ was determined by *in situ* hybridization. Examination of the appendage sinuses of the light organ by confocal microscopy revealed that lactacystin mimicked three symbiosis phenotypes. First, in treated 12-h juvenile squid, we observed symbiotic-like expression patterns in C8α subunit transcript in hemocytes present in the appendage sinuses of not only symbiotic animals, but also aposymbiotic animals (Fig. 3A). Second, in aposymbiotic animals exposed to lactacystin, hemocyte infiltration into the sinuses was induced by 24 h to levels characteristic of the symbiosis (Fig. 3B). Twelve-hour symbiotic animals treated with lactacystin had levels of hemocyte infiltration significantly higher than those of untreated symbiotic animals—that is, the symbiotic phenotype was enhanced. Third, at 24 h post-hatching, the number of apoptotic cells in aposymbiotic animals treated with lactacystin was significantly higher than in the control group, with counts similar to those observed in symbiotic controls (Fig. 3C). The addition of lactacystin to symbiotic animals accelerated regression so that morphogenesis was complete by 24 h.

The patterns of gene expression of the proteasome, as well as the response to inhibitors, that we observed in the

squid-vibrio association have precedence in other systems. Numerous studies focusing on changes of host gene expression in response to either pathogenic (14) or beneficial (15) bacteria have reported increases in the expression of host genes encoding proteasome subunits. Additionally, studies

have revealed that some bacterial pathogens directly or indirectly target proteasome activity as a part of their virulence repertoire (16–17). In both vertebrate and plant host cells, interactions with bacteria induce the incorporation of particular subunits into a defense-specific proteasome (18,
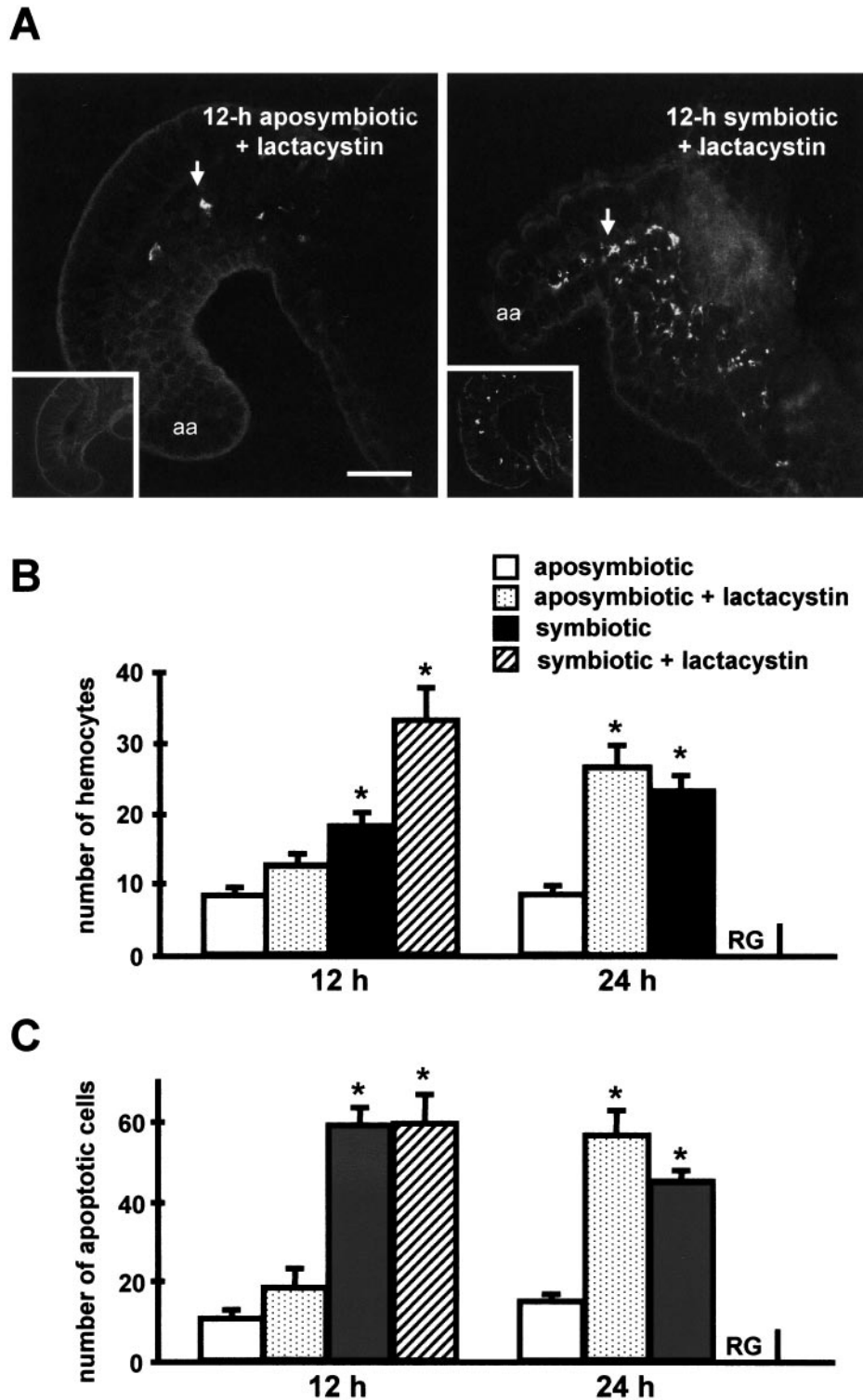
19). Also, the activation of the NF-κB pathway, a conserved response to bacterial molecules, depends on proteasome-mediated degradation of its inhibitor, I-κB (20). The finding that inhibitors of proteasome activity up-regulated expression of a proteasome subunit suggests that a positive feedback mechanism may exist, as has been previously reported (11). In addition, changes in proteasome gene expression have been implicated in development and tissue remodeling in other animals (8, 21).

The influence of bacteria on the mediation of remote events in host tissues is not unique to the squid-vibrio symbiosis. This phenomenon is best understood in the legume-rhizobia associations in which, during the onset of an infection, the bacterial cells interacting with the root surface induce division of host cells deep in the root cortex (22). In vertebrates, native microbiota colonizing the intestinal mucosa interact with components of the innate immune system to direct the maturation of the adaptive immune system (23). Extensive research on the legume-rhizobia symbioses and more recent studies of vertebrate systems (24) have revealed that fundamental to these events is a highly regulated communication between the symbiont and host, often involving changes in gene expression.

The results of this study, while providing a piece of a complex puzzle, introduce a myriad of further questions about the reciprocal signaling between *E. scolopes* and *V. fischeri* during the onset of the symbiosis, and about the involvement of the proteasome in this process. A better understanding of the role of the proteasome in light organ morphogenesis should be afforded by in-depth analyses of the patterns of hemocyte infiltration into organ tissues over the trajectory of the early development of the symbiosis. Not only should such studies add to our understanding of this complex binary symbiosis, but they also promise to provide insight into the role of the proteasome in more complex beneficial and pathogenic host-bacteria interactions.

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Figure 3. To determine the effects of a proteasome inhibitor on hemocyte infiltration into the epithelial fields, aposymbiotic and symbiotic squid were incubated with or without 5 μM lactacystin. (A) Confocal micrographs showing the localization of C8α subunit mRNA by *in situ* hybridization in the sinus of the anterior appendage (aa) of the light organ in animals treated with lactacystin for 12 h. Insets show the corresponding control groups of animals not treated with lactacystin. Arrow points to a hemocyte; $n = 6$ for each sample. Scale bar = 30 μm. Experiments were done in triplicate. (**B–C**) Representative graphs showing the number of hemocytes (**B**) and apoptotic cells (**C**) in one epithelial field per light organ under different conditions. It was not possible to count hemocytes or apoptotic cells in fully regressed epithelial fields (RG). * $P < 0.001$ by one-way ANOVA followed by Tukey's pairwise comparisons (95% confidence interval) to aposymbiotic control; $n = 8$ per treatment. Animals were co-stained with 0.001% acridine orange and 0.008% LysoTracker Red in seawater for 30 min, then anesthetized with 2% ethanol in seawater so that ventral dissections could be performed. For hemocyte quantification, confocal microscopy was used to count hemocytes within appendage sinuses of one epithelial field per animal. Fluorescence microscopy was used for apoptotic cell quantification. Pycnotic nuclei were counted within one epithelial field per light organ as previously described (26). Replicate experiments were performed.

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