Knockdown of Actin and Caspase Gene Expression by RNA Interference in the Symbiotic Anemone Aiptasia pallida

SIMON R. DUNN1,*, WENDY S. PHILLIPS1, DOUGLAS R. GREEN2, AND VIRGINIA M. WEIS1

1Department of Zoology, Oregon State University, Corvallis, Oregon 97331; and 2Department of Immunology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105

Abstract. Since the discovery of the ancient eukaryotic process of RNA-mediated gene silencing, the reverse-genetics technique RNA interference (RNAi) has increasingly been used to examine gene function in vertebrate and invertebrate systems. In this study, we report on the use of RNAi, adapted from studies on animal model systems, to manipulate gene expression in a symbiotic marine cnidarian. We describe gene knockdown of actin and of acasp—a cysteine protease, or caspase—in the symbiotic sea anemone Aiptasia pallida. Knockdown was assessed qualitatively with in situ hybridizations for both genes. Quantitative PCR and caspase activity assays were used as a quantitative measure of knockdown for acasp.

Introduction

RNA interference (RNAi) was introduced by Fire et al. (1998) after experimental injection of double-stranded RNA (dsRNA) into the nematode Caenorhabditis elegans resulted in specific silencing or “interference” of genes homologous to the dsRNA. Discovery of this mechanism of gene regulation earned Fire and Mello the Nobel prize in 2006. RNAi has since been shown to exist in plants, fungi, other invertebrates, and vertebrates, indicating that it is a highly conserved process. RNAi can control gene expression and act as a molecular immune system against viral dsRNA, transposons, retrotransposons, and retrovirus infection within eukaryotes (Hamilton and Baulcombe, 1999; Plasterk, 2002; Bagasra and Prilliman, 2004).

Since the discovery of RNAi, its functions and potential applications have been widely explored. The use of reverse genetics is now established in functional investigations in plants (Kusaba, 2004), invertebrates (Lamitina, 2006), and vertebrates (Silva et al., 2004). It is being used in the treatment of diseases such as cancer (Gartel and Kandel, 2006), neurological disorders (Boudreau and Davidson, 2006), and viral infections (Tan and Yin, 2004). We were interested in developing RNAi in the model marine symbiotic cnidarian Aiptasia pallida. Knockdown was assessed qualitatively with in situ hybridizations for both genes. Quantitative PCR and caspase activity assays were used as a quantitative measure of knockdown for acasp.

Received 11 September 2006; accepted 16 February 2007.
* To whom correspondence should be addressed. E-mail dunns@science.oregonstate.edu

Abbreviations: dsRNA, double-stranded RNA; RNAi, RNA interference.

bleaching of symbiotic *A. pallida* (Dunn et al., 2002, 2004). However, the evolutionary origin of these pathways remains elusive. Recent bioinformatics evidence of two apoptotic genes suggests that cnidarian apoptosis is closely linked to vertebrate apoptosis (Dunn et al., 2006). We were interested in developing reverse-genetics techniques as a part of an effort to understand the apoptotic role of caspases in the symbiotic *A. pallida*. In this study we report on the development of RNAi in *A. pallida* and on successful knockdown of both actin and acasp, a caspase. This is the first study to knock down a gene associated with cnidarian apoptosis.

**Materials and Methods**

**Synthesis of double-stranded RNA**

Double-stranded RNA (dsRNA) was synthesized for both the caspase, *acasp*, and β actin from *Aiptasia pallida*. For this process, PCR products were generated for specific regions of *acasp* and actin, using primers tagged with T7 sequences for dsRNA synthesis. The *acasp* forward primer (5′-TAATAGCAGTCATATGGTCTACATCCAAGGTCTCGTGA-3′) and reverse primer (5′-TAATAGCAGTCATATGGCTTAGGTCACTAGTTGC-3′) generated a 600-bp product. The β actin forward primer (5′-TAATAGCAGTCATATAGGAGTCTAGCTACCA-3′) and reverse primer (5′-TAATAGCAGTCATATAGGTCTAGGTGCATCAGTTGC-3′) generated a 340-bp product. The PCR products were cleaned with Montage filters (Millipore), 1:1 phenol (pH 7.8)/chloroform, followed by chloroform only with Phase Lock Gel tubes (PLG, Eppendorf) and centrifuged at 12,000 × *g* for 30 min. The supernatants were precipitated using 2.2 volume of 100% EtOH and 0.1 volume of 3 mol l⁻¹ NaAc treated with dimethyl pyrocarbonate (DMPC). Samples were centrifuged at 12,000 × *g* for 10 min; pellets were washed with DMPC-treated 70% EtOH, re-centrifuged, and then air-dried. DNA pellets were resuspended in DMPC-H₂O and quantified using a Beckman DU530 spectrophotometer. The dsRNA synthesis mixture was adapted from the T7 RNA polymerase protocol (MBI Fermentas). A master mix consisted of 3 µl of PCR product, 90 µl of 5× transcription buffer (MBI Fermentas), 90 µl of 10 mmol l⁻¹ nucleotide triphosphates (MBI Fermentas), 12 µl of RNAsin (Promega), 15 µl of T7 RNA polymerase (MBI Fermentas), and distilled water (dH₂O) to a final volume of 450 µl. The mix was subdivided into three aliquots and incubated at 37 °C for 2 h. Afterward, 1 µl of DNAse (Gibco) was added to each aliquot and incubated at 37 °C for 15 min. Aliquots of dsRNA were then placed in PLG tubes and cleaned with 1:1 phenol (pH 4.5)/chloroform, followed by chloroform only. The dsRNA was precipitated, washed with 70% EtOH, resuspended in 10 µl of dH₂O, measured using an ND1000 spectrophotometer (NanoDrop), and adjusted with sterile filtered seawater to the required concentration. In initial trials, several concentrations (0.5 µg/µl, 0.75 µg/µl, 1 µg/µl, and 1.25 µg/µl) of both *acasp* and actin were applied to anemones over 24- and 48-h periods. After these optimization trials, concentrations of 0.8 µg/µl for *acasp* and 1 µg/µl for β actin were used.

**Incubation of anemones in double-stranded RNA**

Anemones about 1 cm in height were removed from batch culture (26 ± 0.5 °C, practical salinity 34, photon flux 50 µmol quanta m⁻² s⁻¹) and placed in individual wells of a 24-well plate (Falcon Becton Dickinson) with 100 µl of sterile seawater. The liposomal compound 1,2-dimyristoyl-sn-glycero-3-phosphocholine ethyl ammonium bromide linked to cholesterol (DMRIE-C; Invitrogen/Gibco) was used as an RNA transfection agent. Once the anemones had reacclimatized, the sterile seawater was removed and replaced with between 50 and 70 µl of a dsRNA/DMRIE-C (0.15 µl per 50 µl) seawater solution to a level that allowed the anemone to remain submerged. After 24 and 48 h, 100 µl of sterile seawater was added to each well.

After 72 h of incubation, anemones were removed and processed for tissue sectioning and *in situ* hybridization or for the caspase activity assay. Control treatments were (1) no dsRNA; (2) a non-reversible caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (ZVAD-fmk, Enzyme Systems Products, final concentrations: 12.5 µmol l⁻¹ and 25 µmol l⁻¹ in 0.2% DMSO); and (3) DMRIE-C only, in seawater. For a positive control, dsRNA-treated and non-dsRNA-treated anemones were incubated at a hyperthermic stress of 33–34 °C, which has previously been shown to induce apoptosis (Dunn et al., 2004).

**Synthesis of digoxigenin RNA probe**

To observe localization and treatment knockdown of *acasp* and actin, digoxigenin (DIG)-labeled RNA probes were synthesized and used in *in situ* hybridizations of *A. pallida* tissue sections.

Sense and antisense DIG probes were synthesized from PCR products generated from *A. pallida* cDNA. The *acasp* sense PCR product was generated using a T7-tagged forward primer (5′-TAATAGCAGTCATATGGTCTACATCCAAGGTCTCGTGA-3′) and a reverse primer (5′-CTGGAGGTGCATCAGTTGC-3′). For the *acasp* antisense probe, the forward primer (5′-CTGGAGGTGCATCAGTTGC-3′) and a T7-tagged reverse primer (5′-TAATAGCAGTCATATGGTCTACATCCAAGGTCTCGTGA-3′) and a T7-tagged reverse primer (5′-TAATAGCAGTCATATGGTCTACATCCAAGGTCTCGTGA-3′) and a T7-tagged reverse primer (5′-TAATAGCAGTCATATGGTCTACATCCAAGGTCTCGTGA-3′) and a T7-tagged reverse primer (5′-TAATAGCAGTCATATGGTCTACATCCAAGGTCTCGTGA-3′) and a T7-tagged reverse primer (5′-TAATAGCAGTCATATGGTCTACATCCAAGGTCTCGTGA-3′).
CAGGATCTTCA-3') were used. The PCR products were cleaned as above. DIG RNA was synthesized using a mix of 300 ng of DNA template, T7 reaction buffer (Fermentas), T7 RNA polymerase (Fermentas), DIG RNA label mix (Enzo, Roche), RNase out (Promega), and DMPC H2O to a final volume of 20 µl. Reactions were incubated at 37 °C for 2 h, then 1000 units of DNase I (Gibco) was added to each preparation and incubated at 37 °C for 15 min. DIG RNA preparation quality was visualized by resolving a subsample on an agarose gel. The remainder of each DIG RNA preparation was precipitated using 0.2 volume of 0.5 M LiCl and re-centrifuged prior to air drying

Samples were thawed and centrifuged at 12,000 rpm for 10 min, and then given two 5-min PBST rinses.

For pre-hybridization, sections were incubated in hybridization buffer (100 mmol l−1 NaCl, 10 mmol l−1 Tris pH 8) for 30 min. Slides were rinsed twice in 0.1× SSC at 37 °C for 5 min, followed by two 10-min rinses in Buffer 1 (100 mmol l−1 Tris, 150 mmol l−1 NaCl, pH 7.5). Slides were then incubated in blocking solution (0.1% Triton X-100, 2% sheep serum in Buffer 1) for 45 min at room temperature. The blocking solution was replaced with anti-DIG antibody solution (0.1% Triton X-100, 1% sheep serum, 0.001× Anti-Dig Digoxigenin-AP FAB conjugate, Enzo-Roche, in Buffer 1) and incubated for 1.25 h. Slides were removed from the chamber and rinsed twice in Buffer 1 for 10 min, followed by a rinse in NTMT buffer (0.58 mmol l−1 NaCl, 0.05 mmol l−1 MgCl2, 1.21 mmol l−1 Tris, 0.1% Triton X-100, pH 9.5) for 10 min. Slides were incubated in detection buffer (1.2 BCIP:NBT, Calbiochem, 1 mmol l−1 levamisole in NTMT) until color precipitation was observed. Staining was stopped by rinsing in dH2O prior to mounting with glycerol mountant. Sections were viewed using an Olympus Vanox-T AH2 microscope and Sony DKL 5000 digital camera.

Sectioning and in situ hybridization of tissue

Whole anemones were embedded in TBS tissue freezing medium (Triangle Biomedical Sciences) and stored at −80 °C. Tissue sections (20–25 µm) were produced using a 2800 Frigocut N cryostat microtome (Reichert-Jung), mounted within a PAP pen (Daido Sangyo Co, Ltd) restricted area of poly-l-lysine coated slides (LabScientific, Inc), and returned to −80 °C. Slides were later removed from the −80 °C, allowed to briefly thaw, and then placed into a Coplin jar containing 4% paraformaldehyde in phosphate buffered saline (PBS, 2 mmol l−1 NaH2PO4, 7.7 mmol l−1 Na2HPO4, 0.14 mol l−1 NaCl in DMPC-H2O, pH 7.0) for 45 min at room temperature. Slides were washed twice in PBST (1% Triton X-100 in PBS) for 5 min before a MeOH dehydration series (25%, 50%, 75%, 100%, 75%, 50%, 25%) in PBST for 2 min. Slides were rinsed in PBST twice for 5 min and once in 0.1 mol l−1 TEA (1.33% triethanolamine, 0.3% HCl) for 5 min, incubated in acetylation buffer (0.25% acetic anhydride in 0.1 mol l−1 TEA) for 10 min, and then given two 5-min PBST rinses.

For pre-hybridization, sections were incubated in hybridization buffer (50% formamide, 10% 2× SSC [0.3 mol l−1 NaCl, 30 mmol l−1 sodium citrate, pH 7.0]), 2.5% tRNA 10 mg ml−1, 2% 50× Denhardt’s solution, 10% dextran sulfate, 0.25% 10 mg ml−1 denatured salmon sperm) in a humid chamber at 37 °C for 45 min. During the pre-hybridization, antisense and sense probes in hybridization buffer (final concentration 0.5 µg ml−1) were heated at 80 °C for 10 min and then placed briefly on ice prior to application. The pre-hybridization solution was removed and replaced with the probe in hybridization buffer and incubated within a humid chamber at 55 °C overnight. The following morning, the probe was removed and the slides were placed in a Coplin jar containing 2× SSC and 50% formamide for 5 min at 52 °C. This was followed by two 15-min rinses of 2× SSC at 37 °C, followed by two 15-min rinses of 1× SSC at 37 °C. Slides were then placed in a separate Coplin jar containing a 37 °C pre-warmed RNase solution (2% RNase A 10 µg ml−1 [Promega], 0.2% RNase T [Sigma] in NTE [0.5 mol l−1 NaCl, 10 mmol l−1 Tris pH 8]) for 30 min. Slides were rinsed twice in 0.1× SSC at 37 °C for 5 min, followed by two 10-min rinses in Buffer 1 (100 mmol l−1 Tris, 150 mmol l−1 NaCl, pH 7.5). Slides were then incubated in blocking solution (0.1% Triton X-100, 2% sheep serum in Buffer 1) for 45 min at room temperature. The blocking solution was replaced with anti-DIG antibody solution (0.1% Triton X-100, 1% sheep serum, 0.001× Anti-Dig Digoxigenin-AP FAB conjugate, Enzo-Roche, in Buffer 1) and incubated for 1.25 h. Slides were removed from the chamber and rinsed twice in Buffer 1 for 10 min, followed by a rinse in NTMT buffer (0.58 mmol l−1 NaCl, 0.05 mmol l−1 MgCl2, 1.21 mmol l−1 Tris, 0.1% Triton X-100, pH 9.5) for 10 min. Slides were incubated in detection buffer (1.2 BCIP:NBT, Calbiochem, 1 mmol l−1 levamisole in NTMT) until color precipitation was observed. Staining was stopped by rinsing in dH2O prior to mounting with glycerol mountant. Sections were viewed using an Olympus Vanox-T AH2 microscope and Sony DKL 5000 digital camera.

Quantitative real-time-PCR

To quantify changes in transcript level as a function of incubation in acasp dsRNA, quantitative real-time-PCR (QPCR) was performed on control and acasp dsRNA-incubated animals. Three anemones were incubated per treatment for 48 h. Specific primers for acasp and β actin were designed for real-time PCR amplification. The acasp forward primer (5′-GAATGGGCCATGGTATCCA-3′) and reverse primer (5′-TCGGTGCCACGGGATGCCTCAC-3′) and the β actin forward (5′-CTCAGATGAGCTAGAGGATG-3′) and reverse (5′-CTCGTGGATACCGAGGATG-3′) primers resulted in products of approximately 100 bp in length. Total RNA was extracted and 500 ng per sample was used for cDNA synthesis. One microliter of cDNA was used with 5 µmol l−1 of the above primers, dH2O, and iQ SYBR Green Supermix (BioRad) and ROX (Invitrogen) in a 20-µl reaction amplified using an ABI Prism 7000 sequence detection system (Applied Biosystems). Reactions were performed in triplicate. PCR efficiency was determined using LinReg PCR (Ramakers et al., 2003) and normalized using beta Q of acasp and beta actin, a housekeeping gene, as previously described by Rodriguez-Lanetty et al. (2006). Log10-transformed data were expressed as a ratio of acasp to actin quantity. Statistical
analysis using a paired t-test was undertaken with Minitab (ver. 12) statistical software (Minitab Inc).

Assay of caspase activity

The caspase acasp from A. pallida is homologous in deduced amino acid sequence and secondary structure to vertebrate executioner caspase 3 (Dunn et al., 2006). Therefore, to investigate knockdown in acasp, a caspase 3 activity assay (ApoAlert, BD Bioscience Clontech) was used on anemone extracts. After experimental incubation, individual anemones were homogenized in 150 μl of cell lysis buffer according to the manufacturer’s instructions. For the remainder of the protocol, the sample and working volumes were reduced by half. The remaining homogenate was frozen for protein measurement. Caspase activity was measured in units of fluorescence (FU), using a SpectraMax Gemini XS fluorometer and SOFTmax Pro (ver. 4) software (Molecular Devices). The total protein concentration of each sample was measured using a Micro BCA protein assay kit (Pierce), a BSA protein standard (Pierce), and a Vmax Kinetic spectrophotometer microplate reader and SOFTMax software (ver. 2.34, Molecular Devices). Caspase activity therefore was expressed as FU · mg anemone protein⁻¹. Data from the caspase activity assay were tested for normality and heteroscedasticity using Minitab (ver. 12) statistical software (Minitab Inc.). Log10-transformed data were analyzed using one-way ANOVA and Tukey post hoc pairwise comparison.

Results

Optimization of acasp and β actin double-stranded RNA concentration and phenotypic effects

In initial trials, anemones treated with either dsRNA at concentrations of 1.25 μg/μl died within 24 h. Anemones incubated in 1.25 μg/μl β actin dsRNA displayed a shrunken morphology followed by fatal widespread tissue disassociation during the incubation period. Anemones incubated in 1.0 or 1.25 μg/μl of acasp dsRNA displayed the same shrunken morphology followed by widespread loss of algae and mortality. Anemones incubated in 0.5 and 0.75 μg/μl of β actin dsRNA and 0.5 μg/μl acasp dsRNA showed no phenotypic change, and any effect of RNAi was undetectable. At maximum concentrations of 0.8 and 1.0 μg/μl for acasp and β actin, respectively, anemones displayed the shrunken “stress” morphology but then recovered after 48 h. These concentrations of dsRNA were used in future assays.

Acasp and actin in situ hybridization, localization, and knockdown

Knockdown of acasp transcript in Aiptasia pallida (n = 3) tissues was observed by in situ hybridization. Hybridizations with acasp antisense probes of all control anemone sections strongly labeled endoderm in both column and tentacles (Fig. 1A, B). No labeling was seen in sections hybridized with negative control acasp sense probes (Fig. 1C). In contrast to the controls, sections from all acasp dsRNA-incubated animals showed greatly reduced hybridization with the antisense acasp (Fig. 1D). Knockdown of actin transcript was also demonstrated. Whereas control animals that were hybridized with actin antisense probes labeled strongly in the mesenteries, which are rich in epitheliomuscular cells (Fig. 1E), actin dsRNA-incubated animals showed almost no hybridization with actin antisense probes (Fig. 1F).

Quantitative PCR

The average ratio (n = 8) of acasp to β actin transcript quantity was 0.17 in untreated controls compared to 0.51 in DMRIE-C controls. These ratios were not significantly different (P = 0.068). However, the ratio of transcripts in acasp dsRNA-treated anemones was 0.05, significantly lower than untreated controls (P = 0.02; Fig. 2) and DMRIE-C controls (P = 0.012) (data not shown). Three of the 8 anemones incubated in dsRNA were within ± 1 standard deviation of the control mean.

Assay of acasp caspase activity

The fluorometric assay of caspase 3 activity, which detects activated caspase 3, showed that there was a significant difference in activity between controls and treatments (one-way ANOVA (6) P < 0.0001). Caspase activity in control animals averaged 44.4 ± 1.7 FU·mg protein⁻¹ (Table 1, Fig. 3). In contrast, anemones subjected to a hyperthermic stress had a significantly higher caspase activity of 64.4 ± 4.7 FU·mg protein⁻¹, a 45% increase over controls. Caspase activity was reduced in all anemones incubated in dsRNA when compared to individuals from the control. Anemones treated with the caspase inhibitor ZVAD-fmk exhibited a significant dose-dependent decrease in activity. Activities of those treated with 12.5 μmol l⁻¹ ZVAD-fmk decreased by 35%, to 28.7 ± 3.1 FU·mg protein⁻¹, and those treated with 25 μmol l⁻¹ ZVAD-fmk decreased by 87%, to 5.5 ± 0.0 FU·mg protein⁻¹. RNAi using acasp dsRNA was effective at knocking down caspase activity, as indicated by a significant 30% decrease in activity, to 31.1 ± 1.1 FU·mg protein⁻¹, in acasp dsRNA-incubated animals. Acasp RNAi was even effective in animals subjected to a hyperthermic stress. The mean activity in these animals of 30.1 ± 1.7 was 31% lower than in controls and 53% lower than in hyperthermic-stressed animals. Caspase activities in animals incubated in 12.5 μmol l⁻¹ ZVAD-fmk and those incubated with acasp dsRNA were not significantly different, suggesting that
reduced caspase activity from RNAi knockdown was comparable to that at the low concentration of inhibitor. Finally, the mean caspase activity of anemones incubated with actin dsRNA (46.5 ± 3.5 FU · mg protein⁻¹) was not significantly different from activity in control anemones, indicating that β actin dsRNA did not have a nonspecific effect of silencing caspase activity.

In this study we report the successful use of an RNAi technique for gene knockdown in the symbiotic sea anemone Aiptasia pallida. Specifically, dsRNA synthesized from acasp, a caspase with homology to vertebrate apoptotic executioner caspase 3 (Dunn et al., 2006), achieved RNAi

Discussion

In this study we report the successful use of an RNAi technique for gene knockdown in the symbiotic sea anemone Aiptasia pallida. Specifically, dsRNA synthesized from acasp, a caspase with homology to vertebrate apoptotic executioner caspase 3 (Dunn et al., 2006), achieved RNAi
of caspase 3 activity, using an optimized chemical transfection delivery. The advantage of this method is that it overcomes the problems associated with electroporation in high-saline media. This success enables future in-depth studies of gene function in cellular and physiological processes in *A. pallida* and other marine cnidarians.

During optimization of dsRNA, initial trials of both *acasp* and */H9252* actin dsRNA showed no significant change in phenotype or successful knockdown at the lowest concentrations, but deleterious phenotypic effects—including cell death, tissue degradation, and animal mortality—were seen at the highest concentrations. Low dsRNA concentrations likely failed to achieve a successful transformation or a strong enough gene-targeting response. At high dsRNA concentrations, although the target gene may have been silenced, additional and possibly deleterious antiviral responses and pathways may also have been initiated (Wang and Carmichael, 2004). Optimal dsRNA concentrations were ultimately determined by observing animal phenotype. Optimal dsRNA concentration resulted in initial phenotypic change to a shrunken “stress” morphology that would disappear after 48 h. Animals incubated in these optimal concentrations for 72 h and afterward removed to sterile seawater were still alive after 2 weeks. The working concentrations of dsRNA in this study are in the same range as those used in invertebrate cell line transfection (Gesellchen et al., 2005) but higher than in vertebrate microinjection (Wargelius et al., 1999) or freshwater cnidarian electroporation (Lohmann et al., 1999). Finally, the fact that the optimal concentrations were different for the two genes examined suggests that these optima are gene-specific and must be determined empirically for each dsRNA, as indicated by Steele (2002).

The template lengths for *acasp* and */H9252* actin dsRNAs were designed in accordance with other invertebrate studies. The length of dsRNA has previously been shown to be an important factor in obtaining efficient gene-specific RNAi. For example, in *Drosophila melanogaster* S2 cells transfected with *cyclin E*, both 400-bp and 540-bp dsRNAs were effective. However, 200-bp and 300-bp dsRNAs were less effective, and 50–100-bp dsRNAs were ineffective (Hammond et al., 2000). Likewise, Elbashir et al. (2001) reported efficient RNAi with 39–50-bp dsRNA but not with dsRNA of 29–36-bp in size.

*Acasp* was more abundant in endodermal than ectodermal cells. The observed distribution of *acasp* may indicate a tissue-specific *acasp* function in processes associated with the endoderm such as digestion, symbiosis, immunity, and recognition. This is a topic for future studies. The observed reduction of *acasp* DIG RNA staining within tissues of *acasp* dsRNA-treated anemones indicated a significant reduction of available transcripts and therefore successful *acasp* RNAi. The successful *in situ* labeling of actin and the reduced labeling after delivery of actin dsRNA indicate that this RNAi technique is effective for different genes. These results were corroborated by QPCR data that indicated a

![Figure 2.](image-url)

**Figure 2.** The mean ratio of *acasp* to actin transcripts in untreated controls and dsRNA treated anemones, measured by QPCR. *n* = 8 anemones.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature stress</th>
<th>12.5 μmol l⁻¹ ZVAD-fmk</th>
<th>25 μmol l⁻¹ ZVAD-fmk</th>
<th><em>acasp</em> dsRNA temperature stress</th>
<th>Actin dsRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Temperature stress</td>
<td>&lt;0.05</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
<td>&lt;0.001</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>12.5 μmol l⁻¹ ZVAD-fmk</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&gt;0.05</td>
<td>&lt;0.001</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>25 μmol l⁻¹ ZVAD-fmk</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><em>acasp</em> dsRNA</td>
<td>&gt;0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>acasp</em> dsRNA temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stress</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Summary of probabilities determined by one-way ANOVA and post hoc Tukey pairwise comparison of caspase activity assay.
significant reduction of acasp transcripts with dsRNA incubation (Fig. 2).

High caspase 3 activity was observed in control animals (Fig. 3). Similarly high activities have been observed in the larvae of Hydractinia echinata (Seipp et al., 2006) and in the anemone Anemonia viridis (Richier et al., 2006). Such high activity could indicate a high cell turnover during development, metamorphosis, and tissue homeostasis in cnidarians. Successful gene knockdown with RNAi was also demonstrated with the caspase 3 activity assay, which showed the gene-specific phenotypic knockdown of acasp. Anemones treated with acasp dsRNA had significantly less caspase 3 activity than controls. This confirms that the function of acasp is associated with the sequence and structure homology with vertebrate caspase 3 that was previously shown by Dunn et al. (2006). Hyperthermic stress, previously shown to increase apoptosis in Aiptasia sp. (Dunn et al., 2004), induced increased caspase 3 activity in A. pallida. In contrast, pretreatment with acasp dsRNA prevented the increase in caspase 3 activity in anemones treated with the same hyperthermic stress. In future studies, prevention of caspase-mediated apoptosis will be a valuable tool in the examination of physiological responses to stress induced by environmental stimuli.

Delivery of acasp dsRNA did not achieve 100% knockdown; such failures are common in initial transfection. Large-scale RNAi of caspase activity may require repeated inoculations, as shown with other systems (Zender et al., 2003). However, the aim of this study was to develop a method for knockdown of specific genes in a marine cnidarian for the purpose of gene-manipulation experiments. Further studies may determine the longevity of gene knockdown and whether the effect could be incorporated into progeny or enhanced by multiple applications. Evidence from the inhibitor treatments indicates that caspase activity could be inhibited beyond the knockdown achieved by a single acasp RNAi treatment. There was no significant difference in caspase activity between anemones treated with 12.5 μmol l⁻¹ ZVAD-fmk and acasp dsRNA, but anemones treated with 25 μmol l⁻¹ ZVAD-fmk did exhibit significantly lower activities than RNAi-treated animals. However, the further decrease in activity with the higher inhibitor concentration may not reflect inefficient acasp RNAi but instead may indicate the inhibition of different caspase isoforms that are not targeted by the acasp dsRNA. The successful delivery of actin dsRNA and resulting RNAi of actin, shown by in situ hybridization, had no significant effect on caspase 3 activity. This illustrates the specificity of acasp dsRNA delivery and RNAi.

There is increasing use of RNAi in familiar higher inver-
tebrate model systems to identify key molecular pathways (Boutros et al., 2004; Agaisse et al., 2005; Hamilton et al., 2005; Philips et al., 2005). The development and use of RNAi techniques in basal metazoan model systems such as cnidarians will help to determine the evolutionary lineage and complexity of homologous pathways such as apoptosis in higher metazoans (Golstein et al., 2003; Zender and Kubicka, 2004; Gesellchen et al., 2005). The use of acasp RNAi will also enable us to answer key questions about the roles of cnidarian apoptosis in the onset and breakdown of symbiosis. This technique has been used in recently completed large-scale experiments in which manipulation of apoptosis resulted in a marked effect on symbiosis stability (Dunn et al., unpubl.). Further, using RNAi in cnidarians could help answer key questions about characteristic attributes of indeterminate growth and longevity.

Acknowledgments

We gratefully thank members of the Weis lab for their comments on this manuscript. Supported by a grant from the National Science Foundation (02372230-MCB) to V. W. and D. G.

Literature Cited


