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Cyclophilin and the Regulation of Symbiosis in *Aiptasia pallida*

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Abstract. The sea anemone *Aiptasia pallida,* symbiotic with intracellular dinoflagellates, expresses a peptydyl-prolyl *cis-trans* isomerase (PPIase) belonging to the conserved family of cytosolic cyclophilins (ApCypA). Protein extracts from *A. pallida* exhibited PPIase activity. Given the high degree of conservation of ApCypA and its known function in the cellular stress response, we hypothesized that it plays a similar role in the cnidarian-dinoflagellate symbiosis. To explore its role, we inhibited the activity of cyclophilin with cyclosporin A (CsA). CsA effectively inhibited the PPIase activity of protein extracts from symbiotic *A. pallida*. CsA also induced the dose-dependent release of symbiotic algae from host tissues (bleaching). Laser scanning confocal microscopy using superoxide and nitric oxide-sensitive fluorescent dyes on live specimens of *A. pallida* revealed that CsA strongly induced the production of these known mediators of bleaching. We tested whether the CsA-sensitive isomerase activity is important for maintaining the activity of the antioxidant enzyme superoxide dismutase (SOD). SOD activity of protein extracts was not affected by pre-incubation with CsA *in vitro*.

Introduction

Many cnidarian species, such as sea anemones and corals, are symbiotic with intracellular dinoflagellates of the genus *Symbiodinium*. There is little understanding of the molecular and cellular interactions governing these intimate associations. Comparing the gene expression patterns of symbiotic *versus* aposymbiotic (those lacking symbionts) individuals has been a fruitful approach in resolving some of the cellular mechanisms behind these ecologically important symbioses (Weis and Levine, 1996; Kuo *et al.*, 2004; Barneah *et al.*, 2006; Rodriguez-Lanetty *et al.*, 2006; deBoer *et al.*, 2007). In a recent study using the symbiotic tropical sea anemone *Aiptasia pulchella*, one of the genes that was found highly expressed in symbiotic anemones was identified as a peptydyl-prolyl *cis-trans* isomerase (PPIase) of the cyclophilin family (Kuo *et al.*, 2004). PPIases, also known as rotamases or foldases, catalyze the isomerization of peptide bonds in which one of the adjacent amino acids is proline (Scholz *et al.*, 1997; Fanghanel and Fischer, 2004). This isomerase activity has been hypothesized to be important during the stress response of organisms. For example, cyclophilin mRNA levels increase in response to temperature stress as well as to stress induced by pathogens (Hacker and Fischer, 1993; Sykes *et al.*, 1993; Chou and Gasser, 1997). In symbiotic cnidarians, one well-known stress response to elevated temperature and ultraviolet irradiance involves the loss of algal symbionts from host tissues. This response, known as cnidarian bleaching, results through mechanisms dependent on host cell death pathways triggered in part by cellular oxidative and nitrosative stress (Lesser, 1996, 2006; Perez *et al.*, 2001; Dunn *et al.*, 2004; Franklin *et al.*, 2004; Perez and Weis, 2006). Cnidarian bleaching of scleractinian corals has had deleterious ecological effects on tropical coral reef ecosystems worldwide (Hoegh-Guldberg, 1999). Given that cyclophilins may be involved in cellular stress pathways, we studied the role of cyclophilin in the symbiotic sea anemone *A. pallida.*

The cyclophilins constitute a large family of well-conserved genes, some of which are also known as immunophi-

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Abbreviations: ApCypA, *Aiptasia pallida* cyclophilin A; CsA, cyclosporine A; DAF-FM-DA, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; DHEt, dihydroethidium; DTT, dithiothreitol; Et, ethidium; PPIase, peptydyl-prolyl *cis-trans* isomerase; SOD, superoxide dismutase; TBP, *tert*-butyl hydroperoxide.

lins due to the fact that these proteins are the targets of the potent immunosuppressive drug cyclosporin A (CsA) (Liu *et al.*, 1991; Fruman *et al.*, 1992). CsA binds specifically to the active site of cyclophilin and inhibits its PPIase activity (Schreiber, 1991). CsA binds with greatest affinity to the prototypical cytosolic cyclophilin isoform (Fruman *et al.*, 1994; Gothel and Marahiel, 1999). The isomerase activity is believed to catalyze the proper folding of certain proteins (Kofron *et al.*, 1991; Galat, 1993). The physiological roles of immunophilins are varied and are beginning to be understood in greater detail. Some of these functions include cell signaling, inflammation, and cell cycle control (Barik, 2006). In addition, some of the effects of CsA are attributed to non-PPIase functions, such as the inhibition of signaling through the phosphatase calcineurin involved in the control of the immune response.

Cyclophilins are also known to play an important role in host-microbe interactions. One interesting example of this function is illustrated by the requirement for host cell cyclophilins in the infection process of apicomplexan parasites (Hoerauf *et al.*, 1997). This is particularly relevant to our study given that dinoflagellates are a sister group to the apicomplexa (Wolters, 1991). Expression of cyclophilin A (CypA; cytosolic isoform; E.C.5.2.1.8) in macrophages was critically important in the successful replication of *Leishmania major* amastigotes (Hoerauf *et al.*, 1997). In addition, CsA also inhibits the growth of *Plasmodium falciparum* and *Toxoplasma gondii* (Hoerauf *et al.*, 1997). Other studies suggest that cyclophilins play a role during oxidative stress, a function that may involve the antioxidant enzyme superoxide dismutase (SOD; Lee *et al.*, 1999; Jin *et al.*, 2000, 2004; Liao *et al.*, 2000; Santos *et al.*, 2000; Hong *et al.*, 2002; Krauskopf *et al.*, 2003; Reddy and Suleman, 2004; Boulos *et al.*, 2007).

Here we describe the *A. pallida* CypA homolog (ApCypA) and show that CsA inhibits the *in vitro* isomerase activity of protein extracts from symbiotic *A. pallida*. In addition, incubation with CsA induces production of reactive oxygen species (ROS), nitric oxide (NO), and bleaching. We tested the hypothesis that these effects could be due to an indirect inhibition of activity of the enzyme SOD, which is known to have functional importance as an antioxidant in cnidarian-algal symbioses (Lesser and Shick, 1989; Richier *et al.*, 2003, 2005).

Materials and Methods

Maintenance of anemones

Cultures of *Aiptasia pallida* (Verrill) from the Florida Keys were maintained in artificial seawater (Instant Ocean) at 25 °C and 12 h:12 h light/dark photoperiod with a light irradiance of approximately 70 μmol PAR quanta $\cdot$ m^{-2} s^{-1}. The anemones were fed to satiation twice per week with freshly hatched *Artemia salina* nauplii.

RNA extraction and cDNA synthesis

All anemones used in the experiment were starved for one week to avoid contamination from *Artemia* RNA. Twenty anemones were blotted dry and placed in 1-ml microfuge tubes that were immediately placed in liquid nitrogen and stored −80 °C. Total RNA was extracted using a modification of the acid pH guanidinium thiocyanate/phenol/chloroform method (Bird, 2005). From the RNA extracted, cDNA was synthesized using the SuperScript First-Strand Synthesis system for the RT-PCR kit (Gibco BRL, Life Technologies); the supplied oligo(dT) primer was used to hybridize to the mRNA poly(A) tails.

PCR amplification and cloning of Aiptasia pallida *cyclophilin gene*

The 3′ end of the *A. pallida* cyclophilin cDNA was amplified by PCR using a reverse primer that anneals to the poly-A tail of the cDNA (PST2: 5′-GCCGAATTCT-TTTTTTTTTTTTTTT-3′) and a forward primer based on the published cyclophilin sequence from *Aiptasia pulchella* (GenBank Accession #CK663116): (ApCyp F8: 5′-CTG-GACGTGTTGTGATGGAGCT-3′) (Kuo *et al.*, 2004). This amplified product was ligated to pGEM-T vector (Promega) and transformed into *E. coli* using MAX Efficiency DH5α (Invitrogen). After screening for transformants, the cloned inserts were PCR-amplified, and colonies with inserts of an expected size were sequenced. Colonies were screened by PCR for the correct insert size, using the vector primers M13F and M13R; those containing the correct size were sequenced. All sequencing reactions were performed on column-purified PCR products (Montage PCR centrifugal filter device) amplified using the vector primers M13F and M13R. The 5′ end sequence was amplified using ApCypA reverse primers (ApCyp R7: 5′-AACGTGCTTGTTATC-CAGCCAG-3′) with the First-Choice RLM-RACE kit (Ambion) according to the manufacturer's instructions. Sequencing was performed using the Applied Biosystems Taq DyeDeoxy 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, ver. 2003.0.

Sequence and phylogenetic analysis

After vector and adaptor sequences were identified and removed, the resulting 3′ and 5′ sequence ends were aligned and the contiguous sequence obtained was input into ORF (open reading frame) finder (http://www.ncbi.nlm.nih.gov). Identified ORFs were input into translated BLAST search

tools (BLASTX and TBLASTX). The predicted protein sequence was inferred using the standard genetic code used by ORF finder. The isoelectric point and molecular weight of the predicted protein were obtained using primary structure tools available through the ExPASy (expertprotein analysis system) Proteomics Tools server of the Swiss Institute of Bioinformatics (http://ca.expasy.org).

The sequences were aligned with the CLUSTAL ver. 1.8 multiple alignment application using the slow-accurate algorithm (BLOSUM matrix, GAP penalty = 7, GAP extension penalty = 0.5; delay divergent = 30%). Distance methods were used in PAUP ver. 4.0 to build neighbor-joining trees and to calculate bootstrap values (1000 replicates).

Protein extracts

To obtain a crude protein extract from symbiotic *A. pallida* for use in the PPIase and SOD enzymatic assays, each of three replicate groups of 30 medium anemones each were quickly rinsed in extraction buffer (50 mmol l^{-1} K_2HPO_4; 0.1 mmol l^{-1} EDTA; pH 7.8) at 4 °C and then homogenized in 25 ml of extraction buffer in a glass-and-Teflon pestle homogenizer on ice. The homogenate was then sonicated for 20 s and centrifuged at 12,000 × *g* for 10 min at 4 °C. This extract contains both algal and animal proteins. The supernatant was transferred to microfuge tubes and flash-frozen in liquid nitrogen. The extracts were stored at −80 °C. Protein concentration was measured using a Coomassie (Bradford) assay kit (Pierce, Rockford, IL) (Bradford, 1976). The average final protein concentration of the extracts was about 5 mg ml^{-1}.

Enzymatic assays

To measure PPIase activity, an *in vitro* colorimetric assay was used on protein extracts from symbiotic *A. pallida*. This method employs a synthetic peptide containing a proline that is subsequently cleaved by chymotrypsin when in the *trans* conformation, releasing the colored nitroanilide derivative (Barrett, 1981; Fischer *et al.*, 1989; Takahashi *et al.*, 1989; Harrison and Stein, 1990; Zydowsky *et al.*, 1992; Scholz *et al.*, 1997). The protein extract was pre-incubated with or without dithiothreitol (DTT; 3.3 mmol l^{-1}) and with or without CsA (3 μmol l^{-1}) or was boiled for 5 min. The CsA negative controls received CsA carrier solvent (100% ethanol). To measure PPIase activity, 20 μl of protein extract, 5 μl of the synthetic peptide substrate succinyl-ala-ala-pro-phe-p-nitroanilide (Calbiochem) dissolved in DMSO (stock 40 mmol l^{-1}) and 700 μl of reaction buffer (50 mmol l^{-1} HEPES; 100 mmol l^{-1} NaCl; 1 mmol l^{-1} EDTA; pH 8.0) were combined in a spectrophotometer cuvette and cooled to 4 °C on ice. The cuvette was quickly transferred to the spectrophotometer, the sample was set as blank, and 5 μl of 1:100 of 60 mg ml^{-1} chymotrypsin (in 1 mmol l^{-1} HCl) at 4 °C was added to start the reaction. The absorbance of the reaction was measured every 5 s at 390 nm for 1 min or until the change in absorbance reached a plateau. Control blanks (without protein extract) were included. The rate of change in absorbance was measured on the portion of the curve with the greatest initial maximal rate of change and expressed as Abs_{390} min^{-1}.

SOD activity was measured *in vitro* with protein extracts from symbiotic *A. pallida* using a commercial colorimetric SOD microplate assay kit (Dojindo Molecular Technologies, Maryland) following the manufacturer instructions (Peskin and Winterbourn, 2000; Ukeda *et al.*, 2002). The assay measures the SOD-inhibitable production of a formazan dye upon reduction of a tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2*H*-tetrazolium, monosodium salt) with a superoxide anion. SOD activity is expressed as percent inhibition of the maximal reaction obtained with no extract blanks. For each sample, reactions were performed in triplicate and averaged. The concentration of the protein extracts employed in the experimental reactions was that which produced 50% inhibition of the reaction or approximately 1 unit ml^{-1} of SOD standard (Sigma-Aldrich, USA) or 20 μl of 0.5 mg ml^{-1}. The protein extracts were pre-incubated at 4 °C for 30 min with CsA at 0 (control), 0.1, 1.0, or 100 μmol l^{-1}, the control receiving CsA carrier solvent (100% ethanol).

Expulsion of algae (bleaching)

Bleaching was quantified as previously described (Perez and Weis, 2006). Briefly, hemocytometer counts were made of algae expelled by anemones over 24 h as well as of algae remaining within host tissues, and the percent expelled algae of the total initial number of algae in the anemones was calculated.

Confocal microscopy

To measure and visualize production of nitric oxide, we employed the NO probe DAF-FM-DA (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; Molecular Probes, Eugene, OR), as described in previously published work (Nagano and Yoshimura, 2002; Perez and Weis, 2006). To measure oxidative stress, anemones were incubated with dihydroethidium (DHEt; absorbance ~355 nm). In its reduced state, cytosolic DHEt exhibits blue fluorescence (~420 nm); however, once this probe is oxidized to ethidium by superoxide anion, it intercalates within the cell's DNA, staining the nucleus a bright fluorescent red (~625 nm) (Carter *et al.*, 1994; Zhao *et al.*, 2003). To obtain a relative quantification of oxidative state, we measured the relative fluorescence (after excitation with a 405-nm laser) of the reduced and oxidized ethidium with,

respectively, a 505–550-nm and a 571–625-nm band-pass filter and calculated the ratio of the oxidized ethidium fluorescence to that of the reduced DHEt.

Anemones were placed on 5-ml glass-bottom petri dishes (MatTek, Ashland, MA) with 3 ml of either Millipore-filtered seawater alone (plus vehicle solvent for CsA, 100% ethanol), or with 75 μmol l^{-1} of the pro-oxidant compound *tert*-butyl hydroperoxide, or with 1 μmol l^{-1} CsA, and then incubated for 24 h. After the incubation, the medium was removed and replaced with 1 ml of relaxing solution (1:1, 37 mmol l^{-1} $MgCl_2$: seawater at a practical salinity of about 35) with 3 μl ml^{-1} DAF-FM-DA or DHEt, (Molecular Probes; (Nagano and Yoshimura, 2002). Anemones were incubated for 30 min in the dark, and the medium was then removed and a few drops of relaxing solution added. The samples were observed under a Zeiss LSM 510 Meta microscope (The Center for Genome Research and Biocomputing at Oregon State University) with a 40×/0.8 water objective lens with a working distance of 0.8 3.2 mm. Excitation was provided by an argon laser at 488 nm to excite the DAF-FM probe and a HeNe543 laser to excite chlorophyll autofluorescence. DAF-FM NO-dependent fluorescence was detected using a 510-53-nm filter. Each excitation wavelength (488 and 543 nm) was used separately on different scans. Before image scanning, the focal plane of the optical section was adjusted to include the gastrodermal layer of tentacles. All images were obtained with the same software scanning settings, including detector gain and laser intensity. Quantification of NO-dependent DAF-FM or DHEt/ ethidium fluorescence was achieved by first defining the gastrodermal portions as regions of interest and measuring the average pixel intensity value for that region with the LSM 5 software.

Results

Aiptasia pallida *cyclophilin A*

We cloned and sequenced a cyclophilin cDNA from *Aiptasia pallida* (GenBank Accession #:EU293738). The predicted amino acid sequence of ApCypA was identical to that of *A. pulchella* (GenBank Accession #CK663116, 99% identity at the nucleotide level) and 83% identical to the *Homo sapiens* homolog (GenBank Accession #NM_021130) (Fig. 1A). The *Aiptasia* sequences had a predicted isoelectric point and molecular weight of pH 9.1 and 17.5 kDa and contained all of the amino acids known to be involved in binding to cyclosporin A (CsA) and in the activity of peptydyl-prolyl *cis-trans* isomerase (PPIase). These include the invariant R55 known to play a critical catalytic role in the *cis-trans* isomerization reaction (Barik, 2006).

In phylogenetic analyses, the predicted amino acid sequence of ApCypA grouped with homologs from higher eukaryotes with moderate support (bootstrap value = 87%). It did not group with those of the apicomplexa, suggesting that the ApCypA sequence is host-derived and not from dinoflagellate symbionts (Fig. 1B). PCR using ApCypA-specific primers on genomic DNA from cultured *Symbiodinium* from *A. pallida* sp. as a template did not amplify any product (results not shown).

Isomerase activity in Aiptasia pallida

Protein extracts from symbiotic *A. pallida* exhibited isomerase activity inhibitable by pre-incubation with 3 μmol l^{-1} CsA (Fig. 2A). CsA inhibited the activity down to background levels (no extract) as well as the activity of the boiled extracts, suggesting that most, if not all, of the PPIase activity was due to a cyclophilin and that this concentration of CsA was enough to inhibit 100% of the activity (ANOVA $P \leq 0.001$; Student-Neuman-Keuls (SNK) multiple comparisons test $P \leq 0.05$). We do not know how much of the PPIase activity is partitioned between host and algal sources because the protein extracts contained algal as well as host-derived proteins. Removing the reducing agent dithiothreitol (DTT) from the reaction medium of controls resulted in loss of activity, so the PPIase activity was sensitive to the oxidation state of the enzyme involved.

Cyclosporin A induces bleaching, oxidative stress, and nitric oxide production

Incubating anemones at 25 °C in CsA (0–1 μmol l^{-1}) for 24 h resulted in a dose-dependent increase in the rate of bleaching (Fig. 2B). When anemones were incubated at 34 °C, CsA had a large synergistic effect on bleaching at 0.1 μmol l^{-1} but was lethal at the highest concentrations used (0.5 and 1.0 μmol l^{-1}). This lethality, as evidenced by the disintegration of tissues, occurred before most algae were released into the medium, which explains the measured decrease in the rate of bleaching.

Incubating *A. pallida* with CsA (1 μmol l^{-1}) or *tert*-butyl hydroperoxide (TBP) (75 μmol l^{-1}) as positive control for 24 h resulted in an increase in oxidative load, as evidenced by an increase in the ratio of the relative fluorescence of oxidized DNA-bound ethidium to that of reduced cytosolic dihydroethidium (Fig. 3A and 3B; ANOVA $P \leq 0.001$; SNK multiple comparisons test $P \leq 0.05$). These treatments also resulted in increased relative fluorescence of the NO-sensitive dye DAF-FM (Fig. 4C; ANOVA $P \leq 0.001$; SNK multiple comparisons test $P \leq 0.05$).

Cyclosporin A and superoxide dismutase activity

Incubating protein extracts in up to 100 μmol l^{-1} CsA did not result in any measurable inhibition of *in vitro* SOD activity (Fig. 4; ANOVA $P = 0.019$). This insensitivity is not due to SOD-independent background levels, since pre-

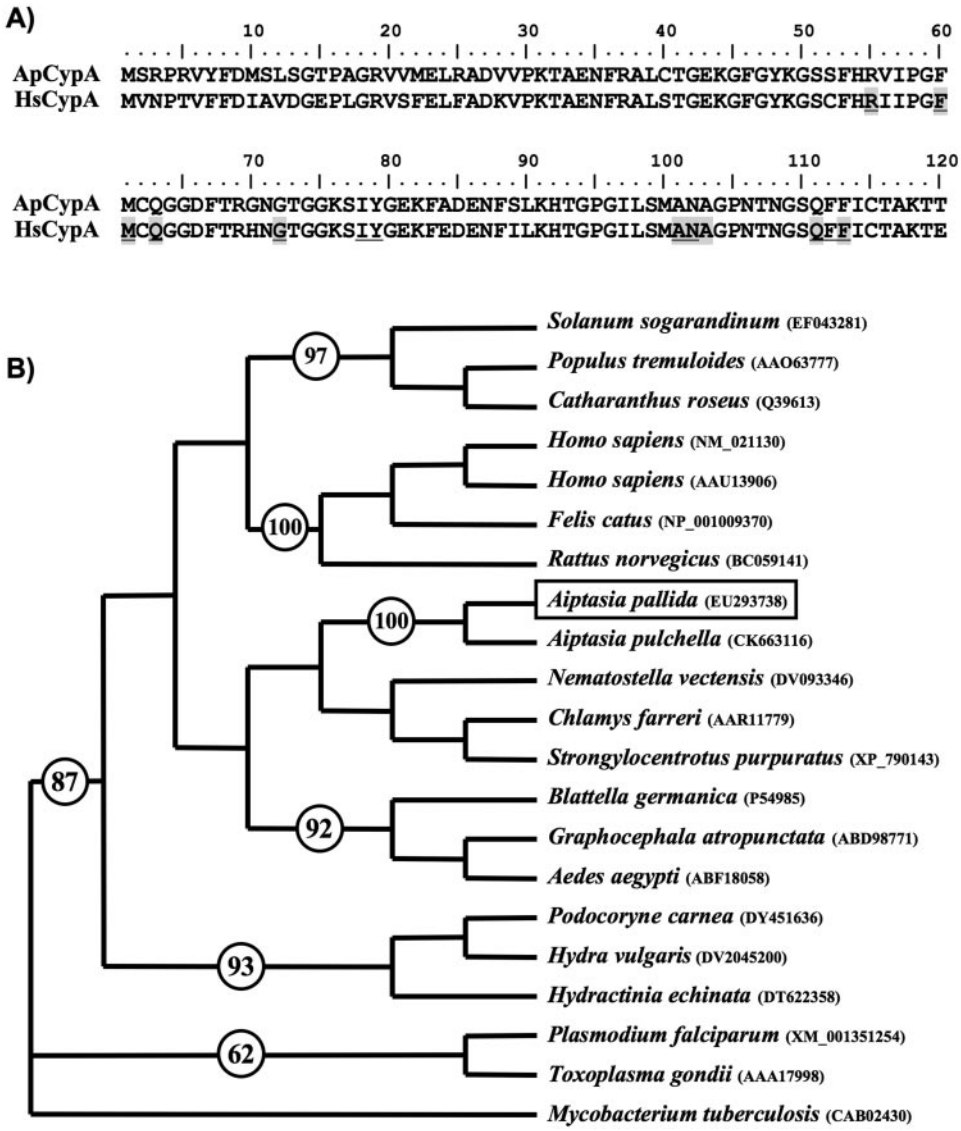

Figure 1. Sequence and phylogenetic analysis of *Aiptasia pallida* cyclophilin. (A) Alignment of the first 120 amino acids of the predicted protein sequence of cyclophilin A from *A. pallida* (ApCypA; GenBank Accession #EU293738) and *Homo sapiens* (HsCypA; GenBank Accession #1038614 and #NM_021130, respectively). Residues of the human sequence involved in binding of cyclosporin A (CsA) are shaded gray, and those involved in peptydyl-prolyl *cis-trans* isomerase (PPIase) activity are underlined. (B) Neighbor-joining phylogenetic reconstruction of cyclophilin A from selected species. Species names are followed by GenBank accession numbers in parentheses. The *A. pallida* sequence is outlined. Bootstrap percentages (>50%) are shown enclosed in circles.

vious tests showed that the SOD activity in *A. pallida* protein extracts was inhibited by boiling as well as by hydrogen peroxide (results not shown).

Discussion

This work represents the first description of a cyclophilin from a cnidarian and a first exploration of its function in the context of cnidarian-dinoflagellate symbiosis. The ApCypA sequence of *Aiptasia pallida* is likely of host origin given that, in the phylogenetic analysis, it groups with other metazoan cyclophilins and not with cyclophilins of apicomplexan origin (a close sister group to the dinoflagellates). Sequences from the more basal anthozoan genera (*Aiptasia* and *Nematostella*) do not group with those of the more derived hydrozoan genera (*Hydra, Hydractinia,* and *Podocoryne*), which reflects the long evolutionary history since

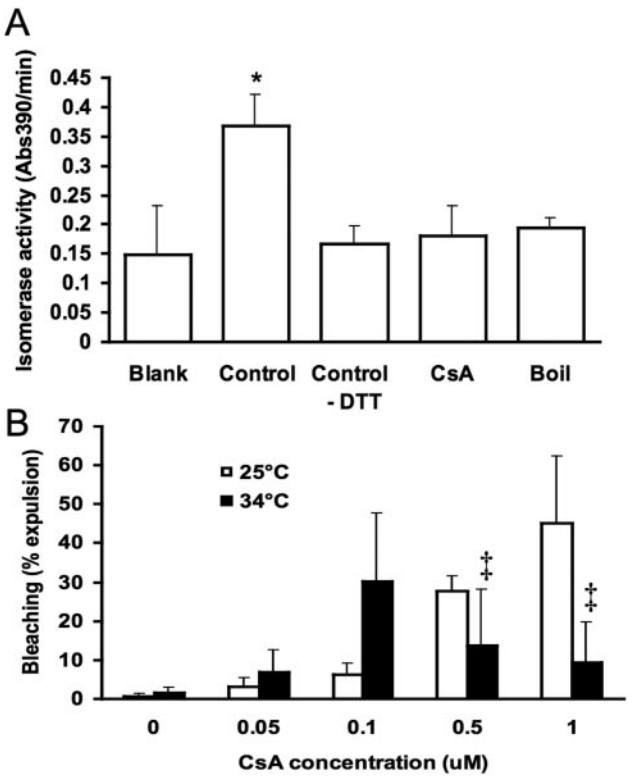

Figure 2. Cyclosporin A (CsA) inhibits peptydyl-prolyl *cis-trans* isomerase (PPIase) activity in *Aiptasia pallida* protein extracts and induces the release of symbiotic algae from host tissues (bleaching). (A) Results of PPIase colorimetric *in vitro* assay of blanks (no protein extracts), protein extracts pre-incubated with or without dithiothreitol (DTT; 3.3 mmol l^{-1}); CsA (3 μmol l^{-1}, +3.3 mmol l^{-1} DTT), or pre-boiled (+3.3 mmol l^{-1} DTT). Activity is expressed as change in absorbance at 390 nm min^{-1} (bars represent means + standard deviation; $n = 3$ extracts). Bars with an asterisk represent significantly different values (ANOVA $P \leq 0.001$; SNK multiple comparisons $P \leq .05$). (B) Expulsion of symbiotic algae (bleaching) as a function of 24-h incubation in different concentrations of CsA at 25 °C (clear bars) and 34 °C (filled bars). Bars represent the mean % expulsion + standard deviation; bars with double daggers (‡) are treatments that resulted in anemone mortality ($n = 4$).

the split of these two cnidarian clades (Bridge *et al.*, 1995). The molecular weight of ApCypA matches the molecular weight of the prototypical cytosolic human Cyp18 isoform of 17.7 kDa (Gothel and Marahiel, 1999). Given its high degree of conservation and its role in the cellular stress response of other organisms, we studied its function in *A. pallida* using a pharmacological approach.

Protein extracts from symbiotic *A. pallida* exhibited peptydyl-prolyl *cis-trans* isomerase (PPIase) activity that was inhibited by incubation with CsA. Given that the protein extracts contained both algal and host proteins, the PPIase activity could have derived from both host and algal CsA-sensitive constituents. It is unknown whether *Symbiodinium* or other dinoflagellates have or express cyclophilins. However, cyclophilins from apicomplexan parasites have been described (Berriman and Fairlamb, 1998). CsA inhibited PPIase activity down to background levels, suggesting that one or more CsA-sensitive cyclophilin isoforms were the primary source of activity. It is not known how many such isoforms are expressed in *A. pallida* or in its symbiotic algae. The PPIase activity was sensitive to the addition of the reducing agent dithiothreitol (DTT). Previous studies have shown that cyclophilin activity is regulated by redox status through the activity of thioredoxin (Motohashi *et al.*, 2001, 2003). The predicted ApCypA protein contains two pairs of cysteine groups (Cys40 and Cys62, Cys115 and Cys161), a remarkably similar arrangement to the two pairs of redox-sensitive cysteines described in the cyclophilin of *Arabidopsis thaliana* (Motohashi *et al.*, 2003). The predicted ApCypA protein sequence also contains the amino acid residues known to be involved in catalysis and binding to substrates as well as to CsA (Barik, 2006). Therefore, ApCypA is expected to bind CsA and contribute to the PPIase activity observed in protein extracts.

The PPiase activity of cyclophilins plays a role in the stress response of organisms. Although yeast mutants containing deletions of all of their known cyclophilins are viable, they are more susceptible to extreme heat stress (Sykes *et al.*, 1993; Dolinski *et al.*, 1997; Colgan *et al.*, 2004). Cyclophilin mRNA levels also increase in plant tissues in response to temperature stress as well as to stress induced by infection of pathogens. In vascular smooth muscle cells, oxidative stress leads to increased expression and secretion of CypA, and the PPIase activity of CypA is necessary for inhibiting NO-induced apoptosis and for activating extracellular signal-regulated kinase (ERK1/2) (Jin *et al.*, 2000). Therefore, cyclophilins are hypothesized to be involved in signal transduction pathways involved in stress responses, and should be important during the stress response of corals and other symbiotic cnidarians during bleaching. Furthermore, they could serve as a useful marker of environmental stress.

Incubation of *A. pallida* with CsA resulted in increased production of reactive oxygen species (ROS) and NO, and expulsion of symbionts. The fluorescent reporter system we employed is sensitive to superoxide anion (Carter *et al.*, 1994; Zhao *et al.*, 2003). TBP, a lipid-soluble pro-oxidant and activator of NFκB transcription factor, also induced NO production in *A. pallida*, suggesting that ROS are involved in similar signal transduction leading to the upregulation of nitric oxide synthase (NOS) in *A. pallida* (Lee *et al.*, 2005; Perez and Weis, 2006). Superoxide anion readily reacts with NO to produce the cytotoxic compound peroxynitrite, which is hypothesized to mediate cnidarian bleaching in *A. pallida* (Perez and Weis, 2006). Micromolar levels of CsA induced bleaching at 25 °C, which suggests that it effectively induces cellular changes such as activation of cell-death pathways, leading to an increased rate of algal release.

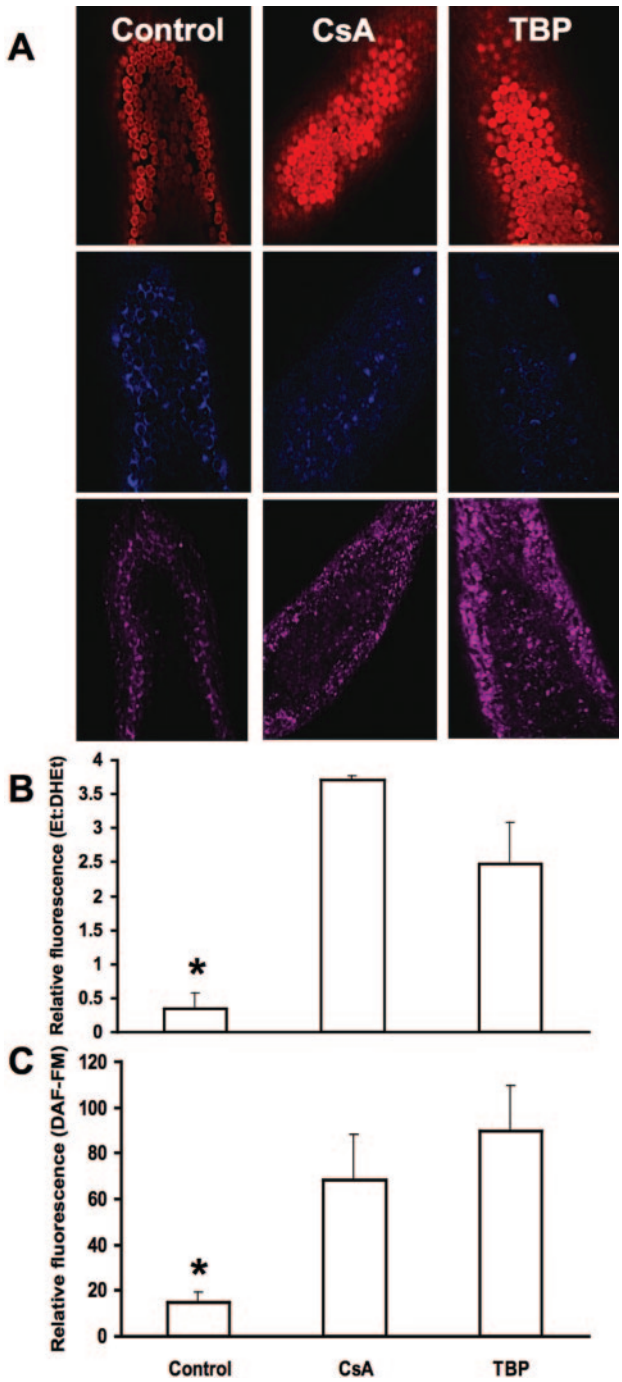

Figure 3. Cyclosporin A (CsA) induces oxidative stress and NO production in *Aiptasia pallida*. (A) False color images of optical cross-section through tentacles of symbiotic *A. pallida* incubated 24 h in CsA (1 μmol l^{-1}) or the pro-oxidant *tert*-butyl hydroperoxide (TBP; 75 mmol l^{-1}). Top pane: algal chlorophyll autofluorescence (700–750 nm); middle pane: fluorescence of reduced, cytosolic dihydroethidium (DHEt; 505–550 nm); lower pane: fluorescence of DNA-bound ethidium (Et; after oxidation of dihydroethidium; 571–625 nm). (B) Ratio of the relative fluorescence intensity of DHEt to that of Et of tentacles treated as above ($n = 4$ anemones; bars represent mean + standard deviation; bar with asterisk represents a significantly different treatment ANOVA $P \leq 0.001$; SNK $P \leq 0.05$). (C) Quantification of relative fluorescence intensity of NO-sensitive DAF-FM-DA (510–530 nm) in tentacles treated as above ($n = 4$ anemones; bars represent mean + standard deviation; ANOVA $P \leq 0.001$; SNK $P \leq 0.05$). Images of DAF-FM-DA fluorescence not shown.

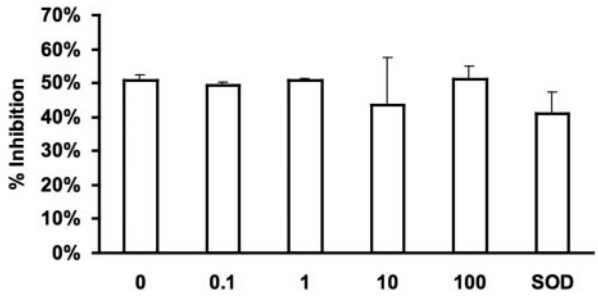

Figure 4. The *in vitro* superoxide dismutase (SOD) activity of *Aiptasia pallida* protein extracts is insensitive to cyclosporin A (CsA). Bars represent the mean + standard deviation of % inhibition of SOD-inhibitable production of colored WST-1 formazan derivative. Protein extracts (+ 3.3 mmol l^{-1} DTT) were pre-incubated 30 min in different concentrations of CsA. A 50% inhibition is approximately equal to 1 unit ml^{-1} of SOD activity; $n = 3$.

Due to algal photosynthesis, the symbiosis might impose a state of hyperoxia, requiring the sustained dependence on protective, perhaps cyclophilin-dependent or CsA-sensitive, mechanisms. In this respect, cyclophilins could play a critical role in the regulation of cnidarian-algal symbiosis. This may explain the abundance of cyclophilin transcripts in symbiotic *A. pulchella* (Kuo *et al.*, 2004). The temperature-dependence of CsA-mediated bleaching suggests that elevated temperature imposes additional oxidative stress and increased dependence on protective mechanisms mediated by cyclophilin (Lesser, 1997).

CsA has been shown to increase NO production and NOS expression through production of ROS and activation of ROS-sensitive signaling pathways (Lopez-Ongil *et al.*, 1998; Navarro-Antolin *et al.*, 1998, 2000, 2001, 2007; Navarro-Antolin and Lamas, 2001; Chen *et al.*, 2002). To date, however, the mechanism by which CsA increases ROS is unclear. Given that CsA also inhibits *in vitro* PPIase activity, it is possible that the production of ROS is mediated through alteration of one or more CsA-sensitive PPIase-dependent mechanisms.

We hypothesized that the CsA-mediated increase in ROS production was due to inhibition of the antioxidant function of SOD. In human epithelial cells, CypA binds to SOD secreted by *Mycobacterium avium* during the infection (Reddy and Suleman, 2004). However, the SOD activity of protein extracts from *A. pallida* was not sensitive to CsA treatment even when incubated at a concentration two orders of magnitude greater than necessary to inhibit PPIase activity. Given that all the measurable PPIase activity was

sensitive to CsA, it seems unlikely that CsA-insensitive sources of PPIase activity are acting to maintain SOD activity. It therefore remains unknown what specific function ApCypA may be playing. Future studies should address this important question.

CsA is also known to exert effects through mechanisms independent of inhibition of PPIase activity of CypA. The CsA-CypA complex inhibits the signaling pathways involved in immune functions that lead to gene transcription by blocking the highly conserved Ca^{2+}/calmodulin (CaM)-dependent serine/threonine protein phosphatase activity of calcineurin (also known as protein phosphatase 2B). Sequences homologous to calcineurin exist in published cnidarian expressed sequence tag and genomic databases (Putnam *et al.*, 2007). In yeast, calcineurin promotes survival during environmental stress (Cyert, 2001; Mulet *et al.*, 2006). However, CsA can induce oxidative stress and apoptosis independent of calcineurin inhibition (Alvarez-Arroyo *et al.*, 2002; Hong *et al.*, 2002).

This study represents the first description of highly conserved cyclophilin from a cnidarian. It is also the first description of the effects of CsA in a marine invertebrate symbiosis and further showcases the value of *A. pallida* as a model for the study of cnidarian-algal symbioses as well as the study of conserved cellular and molecular function. Further studies will be needed to elucidate in detail the role of cyclophilins in cnidarian-algal symbioses, but the conserved nature of ApCypA and of the cytotoxic effects of CsA suggests a conservation of function and regulation supported by studies using non-cnidarian model systems.

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