Light-stimulated dephosphorylation of the BiP-like protein, SmicHSP75 (SBiP1) from Symbiodinium microadriaticum is inhibited by elevated but not low temperature and suggests regulation of the chaperone function

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Specific phosphorylation/dephosphorylation processes are fundamental for the transduction of external stimuli into physiological responses. A few of these processes appear to be modulated by light in cultured Symbiodinium microadriaticum since the BiP-like protein SmicHSP75 undergoes threonine dephosphorylation upon light stimuli. Several isoforms of the protein are encoded in the S. microadriaticum genome and thus, we identified and heterologously expressed a specific sequence corresponding to the previously identified SmicHSP75 isoform to obtain a highly specific antibody. We then determined by western blot analysis, that the detected light-stimulated changes in SmicHSP75 threonine phosphorylation were not due to changes in the protein expression and explored further the effect of lower than normal and higher stressful temperature, on the phosphorylation levels of the protein. Upon long-term (12 h) exposure of the cells to the low temperature of 21°C under darkness, the protein was found significantly phosphorylated; however, light exposure for 30 min caused a dephosphorylation effect like the 26°C control treatment. On the other hand, in cells exposed to 32°C for 12 h under darkness, the highly Thr-phosphorylated SmicHSP75 was reverted to a low-level phosphorylated protein. Likewise, short term (30 min) exposure to 32°C under dark conditions caused dephosphorylation of the protein, similar to what was observed upon long-term exposure to 32°C and upon light stimulation of cells under the normal temperature of 26°C. These data suggested activation/inactivation of the chaperone function of SmicHSP75 by regulation of its Thr phosphorylation levels under heat stress conditions in Symbiodinium microadriaticum, independent of changes in protein expression.

Keywords: BiP-like protein; heat shock; light stimulation; phosphorylation; stress; Symbiodinium microadriaticum

Acknowledgements of Financial Support: Work financed by grants 285802 from the National Council of Science and Technology-México (CONACYT) and IN203718 from PAPIIT-UNAM. RC-M was supported by fellowship 25464 from CONACYT.

INTRODUCTION

Symbiodinium microadriaticum are photosynthetic dinoflagellates usually found in mutualistic symbiosis with the jellyfish Cassiopea xamachana. They can also live freely in the water column and therefore, are amenable for in vitro culture. Under either condition, they are subjected to the stress imposed by global warming. In particular, members of the Symbiodiniaceae family in mutualistic symbioses with reef forming corals, suffer when the sea water temperature elevates and the symbiotic association is lost and, in turn, the coral polyps die leaving their calcareous skeletons in a condition known as coral bleaching (Hoegh-Guldberg et al., 2007). Numerous reports have documented the negative effects of heat stress on these cells, especially those causing perturbations of their photosynthetic machinery (Iglesias-Prieto et al., 1992; Warner et al., 1999; Takahashi et al., 2004). For example, it is known that in corals exposed to light, a moderate increase of temperature could induce coral bleaching through photobleaching of algal pigments and the primary target for damage was the photosystem II (PSII) (Takahashi et al., 2004). Heat-dependent photoinhibition was ascribed to inhibition of the repair of photodamaged PSII. Thus, the loss of efficiency of the photosynthesis repair machinery directly affected the bleaching susceptibility of coral species under high seawater temperature (Takahashi et al., 2004). In fact, earlier studies indicated that severe damage to PSII in remaining symbionts from bleaching corals showed a direct correlation between loss in PSII activity and a significant decline in the D1 reaction center protein of PSII (Warner et al., 1999). This was further demonstrated in laboratory experiments using temperature-sensitive and temperature-tolerant cultured symbiotic dinoflagellates. It was concluded that the perturbation of the PSII protein turnover rates during photoinhibition at elevated temperatures underlies the physiological collapse of symbionts in corals susceptible to heat-induced bleaching (Warner et al., 1999). In addition to elevated temperature stress, light exposure itself in the form of solar irradiance has been reported as a secondary factor associated with coral bleaching (Lesser & Farrell, 2004). Studies on the coral Montastraea (Orbicella) faveolata showed that exposure to high solar radiation and elevated temperatures resulted in a decreased efficiency of PSII and the concentration of D1 protein was significantly lower in high light when it was compared to low light treatments. The bleaching effect was also correlated with a possible increase in reactive oxygen spe-
cies (ROS), thus linking light irradiance with ROS generation (Lesser & Farrell, 2004).

We have previously reported the presence of a BiP-like 75 kDa protein of the heat shock protein 70 (HSP70) family in S. microadriaticum (initially named SmicHSP75), based on de novo partial peptide sequence identity, which matched Cryptophyceae chlorii and Oryza sativa BiP sequences. BiP’s are chaperones localized in the endoplasmic reticulum (RE), where they assist the folding and assembly of newly synthesized proteins. They also bind to misfolded, underglycosylated or unassembled proteins (Otero et al., 2010). On the other hand, BiP’s also participate in the unfolded protein response (UPR) process as a protective mechanism against stress events (Lewy et al., 2017). These proteins are regulated at the transcriptional level by transcription factors (Anckar & Sistonen, 2011), but they can also be regulated by several post-translational modifications that include AMPylation, ADP-ribosylation, methylation, ubiquitination, and acetylation. In addition, controlling BiP activity through phosphorylation appears to be a commonly used mechanism used by eukaryotic cells (Nitika et al., 2020). It was first reported that phosphorylated BiP was associated to oligomeric forms and that they probably represented an inactive state of BiP (Hendershot et al., 1988). Afterward, it was observed that phosphorylation decreased with thermal stress or when cells were exposed to cycloheximide or tunicamycin, inhibitors of protein synthesis or of N-glycosylation bonds of nascent proteins in the ER, respectively (Freiden et al., 1992; Satoh et al., 1993; Díaz-Troya et al., 2011). Overall, the most accepted model proposes that phosphorylation promotes an inactive form of BiP and that stress events or increases in protein synthesis in the ER regulate the activation of BiP through its dephosphorylation, allowing BiP-client complex formation (Crespo, 2012; Pérez-Pérez et al., 2017). We previously documented that in S. microadriaticum, SmicHSP75 was significantly phosphorylated in Thr under darkness, but the phosphorylation levels decreased after a 30 min light stimulus (Castillo-Medina et al., 2019). Since the protein is a member of the HSP70 family and its dephosphorylation is stimulated by light, we had particular interest in exploring the effect of low and high temperature stress on the modulation of the Thr phosphorylation of this protein. In this work, we obtained a specific antibody and determined that the changes in Thr phosphorylation were not due to changes in the SmicHSP75 protein expression. We also evaluated the effect of low and elevated temperature stress on the levels of Thr phosphorylation in order to explore if a correlation with activation/inactivation of the chaperone with stress/normal growth temperature existed. We found that the light induced Thr dephosphorylation of SmicHSP75 was not due to a decreased protein expression and that high, but not low temperature, prevented the phosphorylation even under dark conditions suggesting an activation of the chaperone activity and inactivation during normal non-stressed growth throughout the dark phase of the photoperiod.

MATERIAL AND METHODS

Antibodies and reagents

Polyclonal anti-phosphothreonine (anti-pThr) antibodies were purchased either from Cell Signaling Technology, Inc. (Danvers, MA, USA) (cat. 9381S) or from Abcam (Cambridge, UK) (cat. AB9337). Polyclonal anti-bodies to a specific fragment of SmicHSP75 (anti-SBiP1; see below) were raised in a female white New Zealand rabbit (see below) and a fraction purified on a Protein G-sepharose column (Sigma; St. Louis, MO, USA) was used for the experiments. Alkaline-phosphatase (AP) conjugated polyclonal anti-rabbit IgG antibody raised in goat was from Zymed-Life Technologies (Grand Island, NY, USA). Reagents 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) were from Promega (Madison, WI, USA).

Symbiodinium microadriaticum cell cultures

Dinoflagellate cultures of Symbiodinium microadriaticum Subsp. microadriaticum (clade A, also known as MAC-CassK88) originally isolated from the jellyfish Cassiopea xamachana, and Brevisidium pygmyphilum (clade B, formerly referred to as Symbiodinium sp. MH1.05b.01; Castillo-Medina et al., 2019) were a kind gift of Dr. Mary Alice Coffroth (State University of New York at Buffalo). Furagium kawagutii (clade F, previously referred to as Symbiodinium kawagutii, Castillo-Medina et al., 2019) were a kind gift of Dr. Robert K. Trench (University of California at Santa Barbara). Cultures were routinely maintained in our laboratory in ASP-8A medium under photoperiod cycles of 12 h light/dark at 26°C. Light intensity for routine culture was maintained at 80–120 μmol photons m⁻² sec⁻¹.

Multiple sequence alignment of BiP sequences

B. minutum, S. kawagutii and S. piloua ortholog sequences were identified in the Reefgenomics database (https://reefgenomics.org/), the corresponding sequences from Toxoplasma gondii chaperonin protein BiP (Tgon, MF49 XP_002364404.1), Cryptophyceae chlorii and heat shock protein 70 (Cmr, RN66 XP_00124079.1), Plasmidium malariae heat shock protein 70 (Pmal, SBT70952.1) and Arbolidopsis thaliana (Arth, CAD5333706.1) were also included. The multiple sequence alignment was generated by the MULTALIN multiple alignment tool (Corpet, 1988).

SDS-PAGE gel electrophoresis and western blot

The protein extracts were prepared with dark-adapted 6-d-old S. microadriaticum cells (5–9×10⁶ cells/ml) which were concentrated by centrifugation at 2600×g, 25°C for 5 min, the supernatant discarded, and the cell pellet suspended in 300 μl Laemmli sample buffer (Laemmli, 1970). This suspension was immediately added to a tube containing a matrix volume of 250 μl glass beads (425–600μm) and subjected to strong agitation at 48000 oscillations/minute for 3 min at 4°C in a MINI-BEAD BEATER-1® (BioSpec Products; Bartlesville, OK, USA). The lysate was then heated at 95°C for 5 min, centrifuged at 13000×g for 10 min, and the supernatant containing the proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in discontinuous denaturing gels (Laemmli, 1970) of 10% polycrylamide in the separation zone [375 mM Tris-HCl, pH 8.8; 10% acrylamide/bis-acrylamide; 0.1% SDS; 0.1% ammonium persulphate (APS); 0.106% N, N, N',tetramethylethlenediamine (TEMED)], and 4% polyacrylamide in the stacking zone [125 mM Tris-HCl, pH 6.8; 4% acrylamide; 0.137% bisacrylamide; 0.1% SDS; 0.1% APS; 0.06% TEMED] in a Mini-PROTEAN®3 System (BIO-RAD, Hercules, CA, USA). After electrophoresis, the proteins were transferred to PVDF membranes in “friendly buffer” (25 mM Tris-HCl, 192 mM...
glycine, 10% isopropanol; Villanueva, 2008) at a constant current of 300 mA for 1 h. The membranes were blocked with gentle agitation in a solution of: 3% bovine serum albumin (BSA) in PBS (2.79 mM NaH₂PO₄, 7.197 mM Na₂HPO₄, 136.9 mM NaCl, pH 7.5) for 1 h at 50°C when anti-pThr antibodies from Cell Signaling Technology were to be used; or 5% BSA in TBS (20 mM Tris-Base, 150 mM NaCl, pH 7.5) for 1 h at 4°C for the equivalent antibodies from Abcam. After blocking, the primary antibodies anti-pThr (1:2500; Cell Signaling Technology) or anti-SBiP1 (1:2500) were diluted in 0.01% Triton X-100 in PBS (PBS-T), added to the membranes, and incubated overnight with gentle rocking at room temperature. For all subsequent manipulations with anti-pThr from Abcam (1:500), dilutions were in 0.01% Triton X-100 in TBS (TBS-T), and incubations and washes at 4°C. Then, the membranes were washed five times, 5 min each, in PBS-T or TBS-T, and incubated with alkaline-phosphatase conjugated anti-rabbit IgG at 1:2500 dilution for 2 h at room temperature or 4°C. Subsequently, the membranes were washed again five times, 5 min with PBS-T or TBS-T. Finally, they were developed with a commercial solution of BCIP and NBT according to manufacturer’s instructions except that for anti-pThr from Cell Signaling Technology the development buffer used was PBS-T.

**Enrichment and separation by two-dimensional gel electrophoresis of SmicHSP75 isoforms and subsequent analysis with anti-SBiP1 and anti-pThr antibodies**

Protein extracts from dark-adapted *S. microadriaticum* cells were prepared as above and analyzed by two-dimensional (2D) gel electrophoresis. The extracts were run on two preparative 10% SDS-PAGE gels (7 cm×6 cm), and then cut widthwise between the 70 and 90 kDa markers. Subsequently, both strips of the gels were macerated in liquid nitrogen, and the powder suspended in 3 ml PBS followed by dialysis in an 8 kDa MWCO Spectra/Por® (Spectrum Chemical Mfg. Corp.; New Brunswick, NJ, USA) dialysis bag against PBS overnight at 4°C. The dialyzed proteins were then precipitated with the Cleanup ReadyPrep® 2D kit (BioRad), according to the manufacturer’s methodology. The proteins were suspended and separated by isoelectric focusing as previously described (Castillo-Medina et al., 2019). After isoelectrofocusing, the strips were briefly rinsed in running buffer and run in the second dimension on a 10% polyacrylamide SDS-PAGE gel (16 cm×16 cm). After the run, the gel was stained with coomassie blue and the spots corresponding to SmicHSP75 were analyzed separately with both anti-pThr and anti-SBiP1 antibodies by western blot. As previously described (Islas-Flores et al., 2019), using the oligonucleotides Forward (Fw1) 5’-gaggatgctgagatgagacagaagaag-3’ and Reverse (Rv1) 5’-gccgctgtagttctgagagg-3’ that flank the 285 bp stretch encompassing the c-term-SmicHSP75 fragment. The PCR product was purified from a band separated on a 1% agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer’s instructions. The purified fragment was inserted in a pGEOM-T easy vector with T4 ligase (Promega) after which, it was transformed into chemically competent DH5α *E. coli* cells according to the manufacturer’s instructions. Transformed bacteria were grown in 950 μl SOB (2% bacto-triptone, 0.5% yeast extract, 0.01 M NaCl, 0.0024 M KCl, pH 7) media for 2 h at 37°C, 200 RPM (Lab companion SI300R incubator; Billerica, MA, USA), followed by inoculation on solid LB (1% bacto-triptone, 0.5% yeast extract, 0.171 M NaCl, 1.5% agar, pH 7.4) media added with 100 μg/ml ampicillin for selection. Transformed colonies were further selected by PCR amplification with T7 and Rv1 primers. This amplicon consisted of a 363 bp fragment that contained the 285 bp c-term-SmicHSP75 sequence and flanking fragments of the plasmid. Positive colonies were picked and propagated overnight in liquid LB media with 100 μg/ml ampicillin at 37°C, 200 RPM. Plasmids were purified from grown bacteria using a Gene JET Plasmids Miniprep kit (Thermo Fisher Scientific; Waltham, MA, USA) according to the manufacturer’s instructions. The selected plasmids were sequenced at the sequencing facility of the Institute of Biotechnology-UNAM (Cuernavaca, Morelos, México) to confirm the correct sequence identity. The c-term-SmicHSP75 fragment was extracted from the pGEOM-T easy vector with EcoRI (Thermo Fisher Scientific), and the corresponding band excised from a 1% agarose gel was extracted with the QIAquick Gel extraction kit (QIAGEN; Hilden, Germany) according to the manufacturer’s instructions. This fragment was inserted in the pCR®TT7/NT-TOPO® expression plasmid previously linearized with EcoRI at the insertion site, followed by T4 enzyme ligation (Promega). This plasmid harboring the insert (c-term-SmicHSP75-pCR7/NT-TOPO) was transformed into chemically competent DH5α *E. coli* cells.

**Cloning and heterologous expression of a specific peptide from SmicHSP75**

A 95 amino acid sequence from the identified SmicHSP75 isoform near the C-terminus (Fig. 1B, bolded amino acids) was selected based on both, low identity (49% identity to the most similar *S. microadriaticum* BiP-like isoform), and antigenicity according to the EMBOSS explorer program (https://www.bioinformatics.nl/emboss-explorer/). The corresponding nucleotide sequence named c-term-SmicHSP75 was amplified by PCR as previously described (Islas-Flores et al., 2019), using the oligonucleotides Forward (Fw1) 5’-gaggatgctgagatgagacagaagaag-3’ and Reverse (Rv1) 5’-gccgctgtagttctgagagg-3’. Two PCR amplicons (lane 2) corresponding to c-term-SmicHSP75 in an agarose gel exposed to UV light. Molecular size markers (lane 1) and their size in base pairs (bp’s), are indicated by the arrows on the left. B) Nucleotide and corresponding translated amino acid sequences of the c-term-SmicHSP75-pCR7/NT-TOPO plasmid fragment that contained the 375 bp (lower case) encoding the hybrid fusion protein (named as c-term-SmicHSP75a) and containing c-term-SmicHSP75 (shaded in grey). The c-term-SmicHSP75 peptide sequence is shown (bolded amino acids). The six histidines that confer affinity to nickel are underlined. Bold italicized letters show an acidic region of four aspartic acids where enterokinase cleaves.
lowed by selection as described above. Finally, plasmids were purified and sent for sequencing as above to confirm the correct sequence and in-frame assembly of the insert (Fig. 1B). For the plasmid expression, the c-term-SmicHSP75-pCRT7/NT-TOPO vector was transformed into chemically competent BL21 D3 E. coli cells, as described above, except that the liquid LB media contained 100 μg/ml ampicillin. Cells from the same strain were transformed with the empty pCRT7/NT-TOPO vector in parallel as negative controls. Expression tests were carried out according to the manufacturer’s methodology (pCR®T7/NT-TOPO® TA Expression Kits, Version J). We named the expressed hybrid c-term-SmicHSP75 protein fusion as c-term-SmicHSP75a (Fig. 1B). The protein from the transformed bacteria was visualized after 1, 2 and 4 h of growth by SDS-PAGE and coomassie blue staining.

c-term-SmicHSP75a purification and enterokinase digestion

Two hundred ml of a bacteria culture grown for 7 h in LB media (with 100 μg/ml ampicillin) was centrifuged for 8 min at 2600×g, 25°C. The bacterial pellet was resuspended in 20 ml binding buffer (0.1 M sodium phosphate, 0.4 M NaCl, 0.1 M KCl, 30 mM imidazole, 6 M urea, 0.5% TX-100, pH 7.4, filter-sterilized through a 0.22 μm Millipore filter; Millipore; Burlington, MA, USA), and lysed by 3 passages through a French pressure cell press (SLM/Aminco Inc.; Urbana, IL, USA) at 20 000 psi. The lysed bacterial homogenate was centrifuged for 20 min at 20000×g, 4°C and the supernatant was filtered-sterilized through a 0.22 μm filter. All subsequent procedures were performed at 4°C. The sample was applied onto a column packed with 1.5 ml agarose-nickel matrix (HS-Select® Nickel Affinity Gel; Sigma), previously equilibrated with binding buffer and flow set at 10–15 ml/h with a peristaltic pump (P-1, Pharmacia Biotech; Erice, PA, USA). After binding, the column was washed with 100 ml binding buffer; finally, the bound proteins were eluted with 10 ml elution buffer (binding buffer adjusted to 200 mM imidazole). Aliquots from the loaded solution, flowthrough, wash, and elution were analyzed on 16% polyacrylamide gels by SDS-PAGE and coomassie blue staining. The fractions containing the purified c-term-SmicHSP75a peptide were dialyzed against PBS overnight at 4°C in an 8 kDa MWCO Spectra/Port® membrane (Spectrum Chemical Mfg. Corp.), and then run on a 16% polyacrylamide SDS-PAGE gels stained with coomassie blue. It was found that a 48 h digestion was optimal for removal of the extra histidines from the c-term-SmicHSP75a peptide that could interfere with the expected antigenic response; this digested peptide was termed c-term-SmicHSP75b.

Preparation of polyclonal antibodies against the c-term-SmicHSP75b peptide

The whole digestion mixture (300 μl) containing the c-term-SmicHSP75b peptide was mixed with one third volume of 4X Laemmli buffer (Laemmli, 1970), heated at 95°C and run on two preparative 16% polyacrylamide gels by SDS-PAGE. With the aid of the coomassie blue-stained reference lanes, the zones corresponding to c-term-SmicHSP75b were excised from the gels, homogenized in liquid nitrogen and resuspended in 2 ml PBS. This solution was dialyzed (3.5 kDa MWCO Spectra/Port® membrane) overnight against PBS at 4°C. The dialysate was clarified by centrifugation at 2600×g, 4°C and run on a 16% polyacrylamide gel by SDS-PAGE to confirm that it contained the pure c-term-SmicHSP75b peptide. One hundred and fifty μg of c-term-SmicHSP75b in 500 μL PBS were combined 1:1 with complete Freund’s adjuvant and emulsified. The suspension was injected subcutaneously to a female white New Zealand rabbit in three different areas. Two more booster immunizations in incomplete Freund’s adjuvant were applied after 5 and 31 d, respectively. Bleeds from the rabbit ear were collected: prior to immunization (preimmune serum), and 5, 31 and 38 d post-immunization. The sera from the bleeds were collected and stored at −70°C and some portions further purified on a Protein G-Sepharose column (Sigma), previously equilibrated with PB (PBS without NaCl). After loading, the column was washed with 10 vol PB and eluted with 10 m glycine-HCl, pH 3. The eluate was immediately neutralized with one tenth volume of 1 M Tris-HCl, pH 6.8 and adjusted to
0.05% final vol with NaN₃. The purified antibodies were named anti-SBiP1 (see below) and were stored at 4°C for future use.

**Light stimulation and preparation of protein extracts from Symbiodiniaceae**

All light stimulation treatments and subsequent analyses were carried out as three independent experiments and following the previously reported procedures (Castillo-Medina et al., 2019). Six-d-old cultures from *S. microadriaticum*, *F. kawagutii* or *B. pygmyphilum* collected 2 h before the dark phase of the photoperiod, were concentrated and suspended in 40 ml of fresh ASP-8A medium to reach 5–9×10⁶ cells/ml (2–3.6×10⁷ cells total). The cells were placed in four 10 ml aliquots (~5–9×10⁶ cells total) in Falcon tubes wrapped with aluminum foil. The cells in the tubes were allowed to adapt in the dark (12 h) during their night cycle. After this period, each tube was exposed to a light intensity of 100 μmol photon m⁻² s⁻¹ for 30, 60, and 240 min in the case of only light exposure at 26°C. For light stimulation along with previous low or elevated temperature treatments, the tubes with cells were exposed to 650 μmol photon m⁻² s⁻¹.

Then, the cells from each tube were sedimented by centrifugation as previously described (Castillo-Medina et al., 2019), suspended in Laemmli buffer (Laemmli, 1970), supplemented with 0.2 mM NaVO₃, 10 mM NaPPI and a cocktail of protease inhibitors (Complete®, Roche, Basel, Switzerland), mixed with glass beads (425–600 μm diameter) and lysed with a MINI-BEAD BEATER-1® (Bio-spec Products). The lysate was heated at 95°C for 5 min and the supernatant was used for western blot analysis. Equal loads of proteins were adjusted with equal aliquots of cells and also standardized by staining with coomassie blue on 10% polyacrylamide SDS-PAGE gels. Finally, the extracts were analyzed by western blot which included anti-SBiP1 antibodies as an internal loading control and to determine possible changes in protein expression.

The hands from the triplicate samples were centrifuged with a ChemiDoc-It®2 Imager (UVP-Analytik Jena, Upland, CA, USA) and analyzed by densitometry with the system’s VisionWorks LS software, normalized with the internal SmicHSP75 control to obtain an arbitrary level of intensity for each antibody reaction. The results were integrated into graphs displaying the average band intensity of the three biological replicates for each treatment.

**Treatments of *S. microadriaticum* cells adapted to darkness with low and elevated temperature**

For treatments of *S. microadriaticum* cells adapted to darkness with moderately low and elevated temperature, a similar approach to the light stimulation experiment was used. Samples from 6-d-old cultures were centrifuged and resuspended in fresh ASP-8A medium to reach 5–9×10⁶ cells/ml. Then, the suspension was divided in three sets of two 15 ml Falcon tubes, each containing 10 ml aliquots (~5–9×10⁶ cells total/sample) for each treatment, covered with foil and incubated 12 h in the dark at either 21, 26 or 32°C. After the incubation period, cells from one tube after each temperature treatment were placed in 3.5 cm diameter polystyrene wells and stimulated with 650 μmol photon m⁻² s⁻¹ for 30 min. The cells from the remaining tubes/treatments were kept in the dark during that time. The cells from all samples were then lysed as described above and the samples were analyzed by western blot with anti-pThr or anti-SBiP1 antibodies. An equal load of protein per well in the gels was assured at all times as described above. As previously, three independent experiments were carried out for the treatments and analyses. To evaluate short-term heat stress, two additional samples of dark-adapted cells were incubated, one at 26°C (control) and the other under darkness at 32°C (heat stressed) for 30–240 min only. Then, they were processed immediately for analysis by western blot with anti-pThr or anti-SBiP1 antibodies.

**RESULTS**

**Cloning, expression and purification of the c-term-SmicHSP75a peptide, a specific peptide absent in all other related sequences**

We previously identified several distinct sequences with identity to the SmicHSP75 homolog from the *S. microadriaticum* genome database. From these, at least three sequences, including SmicHSP75, corresponded to BiP-like proteins (Accession No’s. OLP91134, OLP86850 and OLP81269; Castillo-Medina et al., 2019). Further analysis by multiple alignment revealed a high identity to BiP-like proteins from the three Symbiodiniaceae species *F. kawagutii*, *B. minutum*, and *S. pilorum* (Supplementary Fig. 1 at https://ojs.ptbioch.edu.pl/index.php/abp/), and to BiP-like proteins from other apicomplexans such as *Toxoplasma gondii*, *Cryptosporidium muris*, and *Plasmodium malariae* (Supplementary Fig. 1 at https://ojs.ptbioch.edu.pl/index.php/abp/). Furthermore, a close alignment was also observed with a BiP-like protein from *Arabidopsis thaliana* (Supplementary Fig. 1 at https://ojs.ptbioch.edu.pl/index.php/abp/). These data further confirmed our previous report (Castillo-Medina et al., 2019) that SmicHSP75 is indeed a BIP-like protein from *S. microadriaticum*.

From all the sequences identified in the *S. microadriaticum* genome only one matched 100% with the peptide sequences obtained from 2-D gel spots (Castillo-Medina et al., 2019). We identified a 95 amino acid stretch near the C-terminus from such sequence (Fig. 1B, bolded amino acids). These data further confirmed our previous report (Castillo-Medina et al., 2019) that SmicHSP75 is indeed a BIP-like protein from *S. microadriaticum*.

**Figure 2. Expression and affinity purification of the c-term-SmicHSP75a peptide**

The c-term-SmicHSP75a peptide was expressed in BL21 D3 *Escherichia coli* cells, the cells lysed, and the clarified lysate purified on a nickel affinity column. The figure shows the coomassie blue stained proteins, separated on a 16% polyacrylamide SDS-PAGE gel of the lysate before loading (lane 2), the flowthrough after (lane 3), first wash (lane 4) and eluate from (lane 5) the column. The c-term-SmicHSP75a peptide can be observed as a major protein of the total bacterial lysate (lane 2, asterisk), absent in the column flowthrough (lane 3) and wash (lane 4), and eluted as the main protein (lane 5, right arrow). Lane 1 shows the molecular weight markers with their values indicated in kDa next to the arrows on the left.
Digestion of c-term-SmicHSP75a with enterokinase and raising of specific antibodies

The purified c-term-SmicHSP75a recombinant peptide contains an extra amino acid stretch with the six histidines (Fig. 1B, underlined amino acids) required for purification by nickel-affinity chromatography, but also four additional aspartates (Fig. 1B, bolded, italicized amino acids) that could elicit an important but undesirable immune response. Thus, the peptide was digested with the AEF, which targets the four aspartates and removes the immune response. Thus, the peptide was digested with the AEF, which targets the four aspartates and removes the immune response.

In contrast, the presence of a lower molecular weight peptide gradually appeared after 12, 24 and 36 h of digestion (Fig. 3, lanes 4–6, respectively). Interestingly, the presence of a lower molecular weight peptide gradually appeared after 12, 24 and 36 h of digestion (Fig. 3, lanes 4–6, respectively). Consequently, the antibody was named anti-SBiP1. Both the whole serum and IgG fraction of the anti-SBiP1 antibodies showed a specific reaction against a 75 kDa protein from a total homogenate of *S. microadriaticum* (Fig. 4A, lanes 3 and 5, respectively) and which co-migrated with the 75 kDa band detected with anti-pThr antibodies (lane 4). The anti-SBiP1 IgG fraction cross-reacted and specifically recognized a 75 kDa band (right arrow) in western blot analysis of total homogenates from *Brevisilum psammophilum* (lane 2) and *Fugacium kawaiuguti* (lane 3). Lanes 1 from both panels show the molecular weight markers with their values indicated in kDa next to the arrows on the left.

Anti-SBiP1 antibodies specifically detect the phosphorylated isoforms of SBiP1

To demonstrate that the antibodies against SBiP1 immunoreact with the same Thr phosphorylated 75 kDa protein, we separated the proteins by SDS-PAGE and the
proteins were analyzed by 2D-gel electrophoresis and visualized by coomassie blue (A). The various spots migrating at the SBiP1 position were localized (white frame and inbox), and excised from the gel, then ran individually on a second dimension and analyzed by western blot with anti-pThr (B) or anti-SBiP1 (C) antibodies. Immunostaining was observed with both anti-pThr (B, lanes 1–4) and anti-SBiP1 (C, lanes 1–4) antibodies, which correspond to spots 1–4, respectively. A random spot (circle in inbox) was also excised and ran in parallel as a negative control showing no signal for both immunoblots (B, C, lanes 5). The arrows on the left of (A) and (B) indicate the molecular weight markers in kDa; the top bar in (A) shows the pH values of the gradient after isoelectrofocusing.

75 kDa region was excised and processed for further separation by 2D-electrophoresis (Fig. 5A). This procedure allowed us to detect four distinct isoforms recognized by the anti-pThr antibody (Fig. 5A, inbox, arrows). We then excised the equivalent spots from a parallel gel and analyzed them separately by western blot with either anti-pThr or anti-SBiP1 antibodies. We observed the specific phosphorylated isoforms of the same 75 kDa protein. Furthermore, when the same spots were analyzed by anti-SBiP1 antibodies, they recognized the 75 kDa protein (Fig. 5C, lanes 1–4) indicating that they were specific against the 75 kDa Thr-phosphorylated protein. A random spot excised from the 2-D gel, which was also analyzed in parallel, showed no reaction with either antibody (Figs. 5B, C, lanes 5).

**Detected changes in the level of phosphorylation of SBiP1 (SmichSP75) do not arise from changes in protein expression**

Previous studies demonstrated that SBiP1 was highly phosphorylated on Thr during the dark phase of growth of *S. microadriaticum* and that, after 30 min of light stimulation, this phosphorylation level decreased ~50% (Castillo-Medina et al., 2019). With the availability of specific antibodies to SBiP1, we repeated the experiments to compare the variations in phosphorylation levels with possible variations in total SBiP1 protein levels. We observed the expected decrease in Thr phosphorylation after 30, 60 and 240 min of light stimulation on dark-adapted cells (Fig. 6A, upper panel, lanes 2, 3, 4, respectively) analyzed with anti-pThr antibodies. In contrast, analysis with anti-SBiP1 antibodies showed that the total SBiP1 levels remained unchanged, either with (Fig. 6A, lower panel, lanes 2–4) or without (Fig. 6A, lower panel, lane 1) the light exposure treatments. Furthermore, these results were reproduced when identical analyses were carried out on *B. psammosiphon* and *F. kawagutii* (Figs. 6B and C, respectively). This indicated that the detected changes in Thr phosphorylation levels of SBiP1 after light stimulation were not due to changes in expression of the protein.

**Stress at elevated but not at low temperature under dark conditions induces both SBiP1 Thr dephosphorylation and re-phosphorylation to basal levels after light exposure**

*S. microadriaticum* cells normally grow at 26°C and under light and dark photoperiod. Under dark-adapted conditions, SBiP1 displays a high level of Thr phosphorylation (Fig. 7A upper panel, lane 26°C-dark and Fig. 7B, grey bars above 26°C) that is markedly decreased after 30 min of light exposure at either 100 (Fig. 6A–C, lanes 2, 3, respectively) or 650 µmol photon m⁻² s⁻¹ light and dark photoperiod. Under dark-adapted conditions at the lower temperature of 21°C showed a high SBiP1 Thr phosphorylation level similar to that from cells grown under dark-adapted conditions at 26°C after light stimulation (Fig. 7A upper panel, lane 26°C-light and Fig. 7B, white bars above 26°C). In contrast, when *S. microadriaticum* cells were incubated at 32°C for 12 h in the dark, SBiP1 Thr phosphorylation levels were minimal (Fig. 7A upper panel, lane 32°C-dark; Fig. 7B, dark bar above 32°C). However, 30 min after the 32°C stressed cells were exposed to light, SBiP1 showed a re-phosphorylation (Fig. 7A upper panel, lane 32°C-light; Fig. 7B, white bar above 32°C) to levels comparable to those observed in the control of dark-adapted cells at 26°C after light stimulation (Fig. 7A upper panel, lane 26°C-light; Fig. 7B, white bar above 32°C). Interestingly, cells growing under dark conditions at the lower temperature of 21°C showed a high SBiP1 Thr phosphorylation level similar to that from cells incubated at the control 26°C temperature (Fig. 7A upper panel, lane 21°C-dark; Fig. 7B, black bar above 21°C). Consequently, a dephosphorylation behavior after exposure to light, similar (but not identical) to that from cells incubated at the control 26°C temperature was observed (Fig. 7A upper panel, lane 21°C-light; Fig. 7B, white bar above 21°C). The Thr dephosphorylation response of SBiP1 was also observed in short-term heat-shocked cells. The high levels of Thr phosphorylation observed at 26°C under dark conditions (Supplementary Fig. 2, lane 1 at https://ojs.pbioch.
BiP-like iso
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Ser, Thr, and Tyr in three different species of Symbio


depHosphorylation in all three species analyzed. The
present and responded to a light stimulus through Thr

Figure 7. High levels of Thr phosphorylation of SBiP1 under
darkness are disrupted by high but not low temperature long-
term treatments.

Western blot analysis of total extracts prepared from dark-adapted
(Dark) Symbiodinium microadriaticum cells incubated at 21, 26 or
32°C, followed by exposure to light for 30 min at 650 μmol photono
t m2 s−1 (Light). The SBiP1 band detected with anti-pThr anti-
odies (upper panel) was observed highly phosphorylated under
darkness at 21 (Dark, 21°C) and 26°C (Dark, 26°C) but not when
the darkness adaptation period was carried out at 32°C (Dark, 32°C). Light exposure caused a decrease in SBiP1 Thr phospho-
rylation at 21 (Light, 21°C) and 26°C (Light, 26°C) whereas at 32°C
(Light, 32°C), re-phosphorylation to basal levels occurred. Total
SBiP1 levels (lower panel) remained unchanged regardless of the
treatment applied. Densitometric analysis normalized with the
corresponding total SBiP1 bands confirmed that the differences in
the levels of phosphorylation were statistically significant (*).

DISCUSSION

Even though protein modifications by phosphoryla-
tion are fundamental for cellular regulation and signal-
transduction pathways, this field has been largely unex-
plored in Symbiodiniaceae. We have previously reported
the presence of numerous proteins phosphorylated on Ser, Thr, and Tyr in three different species of Symbio-
diniaceae (Castillo-Medina et al., 2019). We also demonstrated that one of these proteins of M~75 kDa was
present and responded to a light stimulus through Thr
dephosphorylation in all three species analyzed. The
protein was identified as a BiP-like protein from the
HSP70 protein family (Castillo-Medina et al., 2019). The
sequence analysis revealed at least 6 related sequences in
S. microadriaticum, and blast analysis against Symbio-
diniaceae also revealed homologs in other species. Fur-
thermore, although the BiP orthologs of B. minutum and
F. kawagutii have not yet been characterized, the corre-
sponding sequences were identified in the Reefgenomics
database and a multiple alignment confirmed their iden-
tity as BiPs (Supplementary Fig. 1 at https://ojs.ptbioch.
.edu.pl/index.php/abp/); therefore, we named this par-
cular isoform as SBiP1 (see above).

Multiple potential Thr phosphorylation sites in SBiP1 are
likely to be the targets of the kinase(s) responsible for the
differentially phosphorylated isoforms previously detected
(Castillo-Medina et al., 2019) and also reported here (Fig. 5).
In addition, several genes encoding at least 6 HSP70-related
proteins were found in the annotated S. microadriaticum
genome (Aranda et al., 2016). The genomic isoforms along
with those arising from post-translational phosphorylations
suggest a tightly controlled regulation of the pathways in
which these proteins are involved. Phosphorylation is also
the switch for regulation of the BiP chaperone function
whereby phosphorylated/dephosphorylated BiPs are the
inactive/active forms, respectively (Hendershot et al., 1988;
Gaut, 1997; Díaz-Troya et al., 2011). Therefore, external
factors capable of inducing changes in the SBiP1 phospho-
rylated status would also be the result of those factors caus-
ing changes in its chaperone activity. We have previously
shown that under dark conditions, SBiP1 is highly phos-
phorylated in Thr, thus reflecting an inactive chaperone sta-
tus; upon light exposure of the S. microadriaticum cells, SBiP1
is dephosphorylated in Thr, suggesting a light-activated
mechanism that ultimately results in the activation of the
chaperone function.

In order to rule out that the observed decrease in Thr
phosphorylation was due to changes in the expression of
the protein, we used a specific antibody against SBiP1.
Since there were several BiP-related sequences in S. mi-
croadriaticum, we required a specific antibody. Thus, we
raised polyclonal antibodies using a recombinant peptide
derived from a sequence of 95 amino acids near the C-
terminus of SBiP1 (Fig. 1B, bolded amino acids). This
peptide was chosen based on low identity (49% identity
to the most similar S. microadriaticum BiP-like iso-
form) and high antigenicity (according to the EMBOSS
explorer program). The resulting anti-SBiP1 antibodies
were highly specific towards all detected SBiP1 isoforms
from S. microadriaticum (Fig. 5), confirming their useful-
ness for assessing possible changes in protein expression.
Furthermore, the antibodies also recognized a similar
~75 kDa protein from B. psygmophilum and F. kawagutii,
indicating that they cross-reacted with the putative SBiP1
homologs in these other Symbiobiaceae species. We
then reproduced the experiment of light induced Thr
dephosphorylation using the antibodies in parallel with
anti-pThr antibodies. The results confirmed that the
detected light-induced BiP dephosphorylation was ind-
ed due to changes in the Thr phosphorylation levels
and not to changes in protein expression as the SBiP1
protein levels remained unchanged throughout the time-
course of the experiment. Even though transcriptomic
data of differential expression in S. microadriaticum upon
long-term exposure to high temperature (31°C) have re-
vealed up-regulated expression of proteins of the HSP70
family (Gierz et al., 2017), there are several important
differences that could account for our observations of
constant SBiP1 expression. First, our long-term exposure
at high temperature consisted of a maximum of 12 h
in the dark followed by analyses through a maximum of
4 h after light exposure, whereas Gierz and collabora-
tors (Gierz et al., 2017) observed up-regulated expression
of HSP70-like proteins in S. microadriaticum after 4 d
of the heat treatment; second, changes in gene expression
may be subtle and not detected in protein analyses by
western blot; and last, gene expression is not necessarily strictly correlated to protein expression (Liu et al., 2016). As mentioned above, both gene expression and post-translational modifications are important for highly regulated processes; post-translational modifications may be the initial early responses followed by changes in gene expression at later stages. A transcriptomic study using a short-term heat shock approach should reveal whether changes in expression of these proteins in *S. microadriaticum* also occur as early responses to heat stress. Finally, the observation of a homogeneous pattern in all the lanes analyzed with anti-SBiP1 antibodies also allowed for their use in the control of uniform protein load on the gels and, at the same time, normalization of band intensity in the densitometric analyses.

A phosphoamino acid analysis of mammalian BiP demonstrated that this protein is predominantly phosphorylated on threonine and to a lesser extent on serine residues (Hendershot et al., 1988). Consistent with this, we previously detected at least two isoforms of SBiP1 (SmicHSP75; Castillo-Medina et al., 2019); however, a more detailed analysis revealed at least four SBiP1 isoforms (Fig. 5). This suggests that several SBiP1 sites are phosphorylated on threonine, although we cannot rule out a single Thr phosphorylation and isoforms arising from other types of post-translational modifications. In fact, other post-translational modifications such as ADP ribosylation and AMPylation of mammalian BiPs have been reported (Hendershot et al., 1988; Ham et al., 2014). On the other hand, there are no reports to date of modifications of ADP ribosylation or AMPylation of BiP in plants or photosynthetic microalgae. In fact, AMPylation is presumed to be a modification restricted to animal cells (Preissler & Ron, 2019).

We observed a light induced Thr dephosphorylation response of SBiP1 in *S. microadriaticum* cells, which, based on the available evidence, we interpret as activation of its chaperone activity regulated by this post-translational modification. Indeed, regulated activation/inactivation by phosphorylation/dephosphorylation of BiP chaperones has led to their conceptualization as regulatory masters of the ER because they play crucial roles in the processes of synthesis, folding, assembly and translocation of proteins through the ER (Hendershot, 2004). Under basal conditions, light could be the stimulus that triggers these processes in the Symbiodiniaceae ER. Only a few studies describe a relationship between BiP and other ER chaperone complexes and light; much less, changes in their phosphorylation state in response to light. One of these reported that, in *Chlamydomonas reinhardtii*, light induced the expression of a chloroplast HSP70 (Kropat et al., 1995). Protein synthesis is another pathway in which regulation of BiP by phosphorylation has been reported to play a role. For example, cGrBiP (the *C. reinhardtii* homolog of BiP) is activated by dephosphorylation at the onset of de novo protein synthesis, which occurs downstream of the TOR (Target of Rapamycin) pathway (Díaz-Troya et al., 2011). More studies are necessary to determine whether SBiP1 is also involved in similar protein synthesis regulatory pathways in Symbiodiniaceae.

It has been clearly demonstrated that stresses that increase the need for active BiP, such as glucose starvation or tunicamycin treatment, promote its dephosphorylation and thus its association with client proteins (Hendershot et al., 1988; Satoh et al., 1993; Díaz-Troya et al., 2011). The light induced Thr dephosphorylation response, and thus activation of the BiP chaperone activity, may be part of a protective mechanism that allows the cells to contend with a number of new external conditions imposed by the irradiated light, and that are translated as stressful conditions to the cells. A number of these could be the light incidence itself and generation of ROS (Erickson et al., 2015), and UV irradiation (Rastogi et al., 2020) when the stimulus involved is daylight. In line with this hypothesis, stressful conditions such as heat stress would produce similar effects in the activation/inactivation of the chaperone that should be detected as changes in its level of Thr phosphorylation. In fact, in *C. reinhardtii*, stress induced by high temperature promoted CrBiP dephosphorylation (Díaz-Troya et al., 2011). Therefore, we treated the dark-adapted cells (highly phosphorylated BiP) with temperatures of 21 (cold stress) or 32°C (heat stress) and analyzed their response upon light exposure. Cold stress did not produce an observable effect on the Thr phosphorylation level under dark conditions as it remained high (inactive SBiP1), similar to the control 26°C condition. As expected, light exposure induced the SBiP1 Thr dephosphorylation response (active SBiP1) in these cells similar to those of the control 26°C condition. This suggests that the lower temperature did not have an effect in the normal chaperone function, or at least no detectable changes could be observed under our experimental conditions. We also observed that the lower 21°C temperature does not impose a significant stress on the cells under dark conditions, which suggests that the SBiP1 chaperone is maintained inactive. Upon light exposure under these conditions, the cells detect the need to contend with their new environment and SBiP1 Thr dephosphorylation occurs in order to activate the chaperone activity, similar to what was observed at the normal 26°C conditions. On the other hand, heat stress under dark conditions did induce Thr dephosphorylation at both long (12 h) and short (30 min) incubation times at 32°C, suggesting that the normally silenced SBiP1 chaperone activity during the dark phase of the growth cycle is required when a stressful high temperature condition is sensed by the cells. Upon light exposure, the heat-stressed cells displayed an altered phosphorylation response as the SBiP1 Thr phosphorylation level increased but only marginally; namely, to the basal levels normally found in light-exposed control cells. It is unclear why the combination of both heat stress and light would restore, at least in part, SBiP1 Thr phosphorylation. It is well known that in Symbiodiniaceae, heat stress primarily targets proteins that allow the proper function of a light-stimulated mechanism, the photosynthetic machinery (Iglesias-Prieto et al., 1992; Warner et al., 1999; Takahashi et al., 2004). The function of the photosynthetic apparatus eventually leads to a generalized failure of cell functions that could lead to cell death in both Symbiodiniaceae and their hosts (Venn et al., 2008). In addition, increased mortality and apoptosis as indicated by an increase in caspase 3-like activity in NO production in *S. microadriaticum* after 24 h incubation at 32°C, has been reported (Bouchard et al., 2009). One possibility is that in heat-stressed *S. microadriaticum* under the conditions tested, ROS generation and/or cell death programs, and apoptosis pathways that are induced (Venn et al., 2008), overwhelm the normal signal-transduction pathways involved in perception of stimuli under normal functioning. Under such conditions, the latter would become completely impaired and irreversibly damaged so that they could no longer respond properly to restore normal cellular function. Our data also suggest that regulation of chaperone activity of BiP-like proteins is one of the early responses to heat stress in *S. microadriaticum*.

In conclusion, our results indicate that: a) Thr phosphorylation changes in SBiP1 from *S. microadriaticum* are not due to changes in protein expression; b) post-translational modification by Thr phosphorylation could be the early response of the cells to activate the SBiP1 chaperone activity upon light stimulation; c) long-term
incubation at low temperature neither induced changes in the Thr phosphorylation levels of SBiP1, nor altered the dephosphorylation response after light exposure of the cells; d) both short- and long-term incubation at high temperature induced Thr dephosphorylation of SBiP1 suggesting activation of the chaperone activity under stress; and e) the Thr phosphorylation level of SBiP1 was restored to basal levels upon light exposure of the cells suggesting an irreversible alteration of the activation/inactivation responses of the chaperone activity.

Acknowledgements

We are grateful for the technical help of M.I. Miguel Ángel Gómez-Real, M.O. Edgar Escalante-Mancera, M.T.I.A. Gustavo Villarreal-Brito, and Dr. Edén Magaña-Gallegos. We also acknowledge the help of M.C. Laura Celis-Gutiérrez.

Author's contributions

Raúl Castillo-Medina: Experiments, data acquisition, formal analysis, and reviewing the manuscript. Tania Islas-Flores: Conceptualization and formal analysis, experimental help and reviewing the manuscript. Marco A. Villanueva: Conceptualization, writing, review, and editing. All authors have read and agreed to the published version of the manuscript.

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https://doi.org/10.1007/s004380000271


