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Caspase-like activity during aging and cell death in the toxic dinoflagellate *Karenia brevis*

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ABSTRACT

The observation of caspase-like activity during cell death has provided a new framework for understanding the evolutionary and ecological contexts of programmed cell death in phytoplankton. However, additional roles for this caspase-like activity, the enzymes responsible, and the targets of this enzyme activity in phytoplankton remain largely undefined. In the present study, the role of caspase-like activity in aging and ROS-mediated cell death were investigated and death programs both dependent on and independent of caspase-like activity were observed in the toxic dinoflagellate, *Karenia brevis*. The dual use of *in situ* caspase 3/7 and TUNEL staining identified previously undescribed death-associated morphotypes in *K. brevis*. *In silico* motif analysis identified several enzymes with predicted caspase-like activity in the *K. brevis* transcriptome, although bona fide caspases are absent. Lastly, computational prediction of downstream caspase substrates, using sequence context and predicted secondary structure, identified proteins involved in a wide range of biological processes including regulation of protein turnover, cell cycle progression, lipid metabolism, coenzyme metabolism, apoptotic and autophagic death. To confirm the computational predictions, a short peptide was designed around the predicated caspase cleavage site in a predicted novel *K. brevis* caspase 3/7-like target, S-adenosylmethionine synthetase (KbAdoMetS). Cleavage of the peptide substrate with recombinant caspase 3 enzyme was determined by MALDI-TOF MS, confirming that KbAdoMetS is indeed a bona fide caspase substrate. These data identify the involvement of caspase-like activity in both aging and cell death in *K. brevis* and identify novel executioner enzymes and downstream targets that may be important for bloom termination.

1. Introduction

Over the last decade, significant advances in understanding the molecular biology of the harmful algal bloom (HAB) forming dinoflagellate, *Karenia brevis*, have provided a foundation to explore cellular mechanisms underlying bloom progression and termination. *K. brevis* is responsible for the near annual HAB events in the Gulf of Mexico. Through the production of a suite of potent neurotoxins, the brevetoxins, blooms of *K. brevis* cause marine animal mortalities, human illness, and economic loss in the fisheries and tourism sectors (Larkin and Adams, 2007). Understanding the molecular mechanisms that dictate the ability to rapidly form blooms, persist for long periods at high concentration, and then rapidly terminate, is important for improving our ability to forecast its impacts in coastal waters.

Mechanisms hypothesized to regulate bloom termination include cell lysis by lytic bacteria and viruses, nutrient limitation, grazing by zooplankton and benthic filter feeders, dilution, or oceanographic processes disrupting advection and bloom concentration; however, each of these mechanisms alone appears insufficient to cause termination in *K. brevis* (Vargo, 2009). The potential role for intrinsic mechanisms to regulate the fitness and aging in *K. brevis* populations, until recently, has been overlooked. Programmed cell death (PCD) has now been identified in a wide range of phytoplankton species (reviewed below), but the extent to which *K. brevis* exhibits signs of this pathway has been largely unexplored.

Caspases, cysteine dependent aspartic acid proteases, are key metazoan enzymes involved in potentiating signals through the PCD pathway. Caspase enzymes are divided into two functional

Abbreviations: EST, expressed sequence tag; HAB, harmful algal bloom; PCD, programmed cell death; ROS, reactive oxygen species; MT, morphotype.

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categories that function in a cascade whereby the initiator enzymes (casp. 2, 8, 9, 10) work to regulate and activate the executioner caspases (casp. 3, 6, 7). The executioner caspase enzymes cleave a diverse set of targets, such as cytoskeletal elements, transcription, translation, DNA repair, and other metabolic processes (reviewed in Degterev and Yuan, 2008; Taylor et al., 2008). Until a decade ago, the PCD process was deemed unique to metazoans; however, molecularly regulated cell death has now been observed in plants, fungi, protozoa, bacteria, and archaea (Deponte, 2008; Lord and Gunawardena, 2012; Ludovico et al., 2005; Taylor-Brown and Hurd, 2013).

Caspase homologs, termed metacaspases, which contain a structurally homologous caspase/hemoglobinase fold, have been identified in plants, fungi, and protists, including phytoplankton (Uren et al., 2000). In addition to containing the well-conserved caspase catalytic domain (p20), metacaspases contain a C-terminal domain reminiscent of a p10 caspase domain. The presence of a conserved histidine/cysteine catalytic dyad at the predicted active site within the p20 domain initially suggested that observed caspase activities measured by fluorogenic probes in these organisms could be attributed to metacaspases. Further *in silico* and biochemical analyses of metacaspase enzymes have since distinguished metacaspases as coordinating strict cleavage after basic residues, namely arginine and lysine, rather than aspartic acid as do metazoan caspases (Vercammen et al., 2004; Watanabe and Lam, 2005). This distinct biochemical difference has led to contradictory reports on the connection between caspase-like activities and the functional role of metacaspases in unicellular organisms (reviewed in Tsiatsiani et al., 2011). The enzymes responsible for caspase-like activities observed in phytoplankton remain unresolved. A number of enzymes in plants are now known to possess caspase-like activity, including vacuolar processing enzyme (Hatsugai et al., 2004), proteasome subunit B1 (Hatsugai et al., 2009), and subtilisins (Chichkova et al., 2010). These provide clues for candidate enzymes responsible for the caspase-like activity observed in phytoplankton. While little is still known about the suite of enzymes responsible for caspase-like activities in phytoplankton, even less is known about the downstream targets of this activity.

Studies on caspase-like activity have provided key information for understanding the evolutionary lineage for the PCD pathway and have shifted the paradigm for understanding phytoplankton population regulation, fitness, and selection, and as such its ecological contexts and consequences (Franklin et al., 2006). While no bona fide caspase enzymes with aspartic acid specificity have been identified in any phytoplankton species, biochemical cleavage of canonical caspase substrates has been measured (reviewed in Tsiatsiani et al., 2011). Such activity is observed in death processes induced by various stressors such as nutrient starvation (Bidle and Bender, 2008), light deprivation (Segovia and Berges, 2009; Segovia et al., 2003), high irradiance (Berman-Frank et al., 2004), CO_2 limitation (Vardi et al., 1999), viral infection (Bidle et al., 2007), and protein synthesis inhibition (Segovia and Berges, 2005). Caspase-like activities associated with cellular aging have also been documented in the chlorophyte *Dunaliella viridis* (Jiménez et al., 2009), and during iron limitation in the diatom *Thalassiosira pseudonana* (Bidle and Bender, 2008), while more recent studies have further defined a larger context for caspase-like activity in regulating stress acclimation (Bidle et al., 2010; Thamatrakoln et al., 2011). The accumulation of reactive oxygen species (ROS) has been proposed as both an initiator as well as a by-product of the PCD process in phytoplankton. In one of the seminal studies in this field, the accumulation of ROS in response to environmental CO_2 limitation triggers bloom termination in the dinoflagellate *Peridinium gatunense*, which was shown to be mediated by cysteine protease activity (Vardi et al., 1999). The direct inhibition of caspase-like activity has also been shown to block ROS accumulation and subsequent cell death in the chlorophyte *Dunaliella teriolecta* (Segovia and Berges, 2009). Caspase 3-like activity has been previously documented in *K. brevis* (Bouchard and Purdie, 2011); however, the proteins responsible for this activity, the downstream targets, and their roles in aging and death remain unclear. Together, these findings have directed our interest in investigating the role of caspase-like enzymes in aging and cell death in *K. brevis*.

In the current study, a combination of biochemical analyses and *in silico* EST sequence data mining was employed to identify the role caspase-like activity may play in the aging and death processes in *K. brevis*. Quantification of caspase-like activities was carried out to gain an understanding of their presence, timing, and magnitude during aging. Next, ROS challenge was employed to assess a role for caspase-like enzyme activity in death processes. DNA fragmentation and caspase-like activity were used to define PCD-like morphological changes present in *K. brevis*. Targeted *in silico* bioinformatic mining was then used to identify enzymes potentially responsible for the activities observed, as well as their potential substrates. To validate the bioinformatics screen, one novel predicted substrate, a synthetic peptide resembling *K. brevis* S-adenosylmethionine synthetase (KbAdoMetS), was then examined to confirm that it is capable of being cleaved at the proposed caspase DEVD motif.

2. Methods

2.1. Culture maintenance and sampling regime

K. brevis (Wilson isolate) was maintained in batch cultures in 1-L glass bottles with autoclaved, 20 μm filtered seawater at 36 psu obtained from the Vero Beach Field Station seawater system at the Florida Institute of Technology. Seawater was enriched with modified *f*/2 medium with 0.01 mM selenous acid (final concentration) and ferric sequestrene used in place of $EDTA{\cdot}Na_2$ and $FeCl_3{\cdot}6H_2O$. Cultures were maintained on a 16:8 light–dark cycle, with illumination from cool white lights at a photon flux density of 50–65 $\mu E\ m^{-2}\ s^{-1}$ measured with a LiCor 2pi meter at 25 °C. Twenty-one 900 mL batch cultures were inoculated at a starting concentration of approximately 1000 cells/mL, from a mid-logarithmic stage starter culture on day 0. Independent triplicate cultures were sampled every other day from day 6 to 18. Cell abundance and growth rates were determined for each sample using a Beckman Coulter Multisizer 3. The specific growth rate and divisions per day were calculated as: Specific growth rate; K' = Ln $(N2/N1)/(t2 - t1)$, where $N1$ and $N2$ = biomass at time1 ($t1$) and time2 ($t2$), respectively, and divisions per day as; Div.day^{-1} = K'/Ln2 (Levasseur et al., 1993).

2.2. Photosynthetic efficiency (F_V/F_M)

The optimum photochemical quantum yield of photosystem II (PSII) fluorescence (F_V/F_M) was determined using the method of Vincent et al. (Vincent et al., 1984) with modifications. Briefly, 3 mL of *K. brevis* culture from each experimental sample were dark adapted for 30 min. The initial fluorescence emitted when all reaction centers are open (F_O) was measured on a Turner Designs Model 10-AU Fluorometer. Cultures were then exposed to 0.03 μM DCMU (3-3,4-dichlorophenyl)-1,1-dimethylurea (Sigma) to reduce Q_A, the first electron acceptor of photosystem II, and maximal fluorescence (F_M) was read. F_V/F_M, defined as $(F_M - F_O)/F_M$ was determined for each sample.

2.3. Cell viability

Cell viability was quantified using the mortal stain, SYTOX green (Invitrogen). 5 mL of culture sample was incubated in the