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SHORT COMMUNICATION

H_V1 Proton Channels in Dinoflagellates: Not Just for Bioluminescence?

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ABSTRACT

Bioluminescence in dinoflagellates is controlled by H_V1 proton channels. Database searches of dinoflagellate transcriptomes and genomes yielded hits with sequence features diagnostic of all confirmed H_V1, and show that H_V1 is widely distributed in the dinoflagellate phylogeny including the basal species *Oxyrrhis marina*. Multiple sequence alignments followed by phylogenetic analysis revealed three major subfamilies of H_V1 that do not correlate with presence of theca, autotrophy, geographic location, or bioluminescence. These data suggest that most dinoflagellates express a H_V1 which has a function separate from bioluminescence. Sequence evidence also suggests that dinoflagellates can contain more than one H_V1 gene.

BIOLUMINESCENCE in dinoflagellates takes place in subcellular structures known as scintillons (Hastings and Dunlap 1986; Johnson et al. 1985; Nicolas et al. 1987a,b; Schmitter et al. 1976). Scintillons contain the biochemical machinery (luciferase enzyme, luciferin, and luciferin-binding protein) for bioluminescence (Desjardins and Morse 1993; Fogel and Hastings 1972; Hastings and Dunlap 1986; Lecuyer et al. 1979; Morse et al. 1989), while nonbioluminescent dinoflagellates lack these molecules (Valiadi and Iglesias-Rodriguez 2013). In response to mechanical stimulation, the vacuolar membrane of bioluminescent dinoflagellates experiences an action potential (Eckert and Reynolds 1967), most likely propagated by protons (Nawata and Sibaoka 1979; Rodriguez et al. 2017). In 1972, J. Woodland Hastings and colleagues proposed that a voltage-gated proton channel in the tonoplast of the bioluminescent species *Lingulodinium polyedrum* responds to the action potential and conducts protons from the acidic vacuole into the scintillon lumen, triggering the light reaction at pH < 7 (Fogel and Hastings 1972). This hypothesis was confirmed recently when LpH_V1, a bona fide H_V1 proton channel identified in *L. polyedrum*, was shown to localize to scintillons (Rodriguez et al. 2017).

H_V1 is a family of voltage-gated proton-conducting channels with four transmembrane helices, S1–S4. The exquisite proton selectivity of H_V1 arises from a critical Asp in S1 (Chaves et al. 2016; Musset et al. 2011; Smith et al. 2011) that interacts with one or more arginines in S4 (Dudev et al. 2015; Kulleperuma et al. 2013). H_V1 opens with depolarization but both internal and external pH strongly influences its voltage dependence such that a 1 unit change in pH gradient ($\Delta pH = pH_o - pH_i$, where pH_o is external pH and pH_i is internal pH) shifts the conductance–voltage (g_H–V) relationship by 40 mV (Cherny et al. 1995). The three-dimensional structure of the H_V1 protein (Takeshita et al. 2014) shows cytoplasmic and extracytoplasmic aqueous vestibules separated by a narrow hydrophobic region identified as the gating charge transfer center (Tao et al. 2010).

Previously, a H_V1 was identified in *Karlodinium veneficum* (Smith et al. 2011), raising the question of whether H_V1's appearance in this nonbioluminescent dinoflagellate is anomalous. We interrogated recently acquired dinoflagellate transcriptomes and genomes for the presence of H_V1, and found that H_V1 is widespread in the dinoflagellate phylogeny, suggesting that it has another function separate from bioluminescence.

MATERIALS AND METHODS

Sequence searches of public transcriptome and genome data

Transcriptome data from dinoflagellates at the National Center for Biotechnology Information (NCBI) and from iMicrobe (http://imicrobe.us/), genome data from *Symbiodinium*, and sequence data from protists at NCBI, were searched using BLAST, PSI-BLAST, and/or tBLASTx with protist H_V1 (Rodriguez et al. 2017; Smith et al. 2011; Taylor et al. 2011) as probes. Hits were aligned using MAFFT (Katoh and Standley 2013) to previously identified H_V1s (Musset et al. 2011; Smith et al. 2011); sequences lacking any transmembrane helix, the S4 signature sequence RxWxxRxxR (where x is any amino acid character) or the critical D in S1 (Musset et al. 2011) were discarded. This alignment was submitted to HMMer (Alva et al. 2016; Finn et al. 2011) to search the UniProt database for protist Hv1, which were aligned and filtered as for dinoflagellate sequences.

Sequence alignment and clustering

All nonidentical sequences were retained in the MAFFT multiple sequence alignment (MSA). We used the SIAS program (http://imed.med.ucm.es/Tools/sias.html, Secretaria General De Ciencia, Tecnologia E Innovacion Of Spain) with default parameters to compute pairwise similarity scores of sequences based on the MSA. Two sequences identified in the *S. minutum* genome (Shoguchi et al. 2013) as two different genes had SIAS score 0.64. We clustered sequences from the same dinoflagellate species with SIAS scores greater than 0.64 (i.e. more similar than the two separate genes in *S. minutum*) and arbitrarily chose a representative from each cluster to retain in a dinoflagellate-only MSA.

Phylogenetic analysis

The dinoflagellate-only MSA was trimmed to the voltage sensor domain (VSD) (Smith et al. 2011; Takeshita et al. 2014) and realigned with MAFFT. Chromerid and diatom sequences were added, aligned, and trimmed to the VSD. This MSA was trimmed for phylogenetic analysis using Trimal (Capella-Gutiérrez et al. 2009) with the gappyout option, and identical sequences were removed. MSAs prepared for phylogenetic analysis were submitted to the Seaview program (Gouy et al. 2010), with default settings and 1,000 bootstraps and replicates for neighbor-joining and parsimony analyses; and to MrBayes (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003) to perform 8 Markov Chain Monte Carlo Bayesian analyses with sampling every 100 generations (10,000,000 generations total). A maximum likelihood phylogenetic tree was generated by PhyML (Guindon and Gascuel 2003) using Prottest predictions (LG amino acid substitution model (Le and Gascuel 2008) with I [proportion of invariant sites], F [observed amino acid frequency], and G [gamma shape]) along with nearest neighbor plus subtree pruning and regrafting tree improvements, and Shimodaira–Hasegawa-approximate likelihood ratio test (aIRT-SH-like) to estimate branch support. PhyML analyses were also performed on three different Trimal-trimmed VSD-only H_V1 MSAs: one with randomized order, one with different Trimal settings, and one including all dinoflagellate Hv1s found. Trees were visualized at iTOL (Letunic and Bork 2007). Sequence logos were produced using WebLogo 3 (Crooks et al. 2004; Schneider and Stephens 1990).

Identification of specificity determining sites

Dinoflagellate-only alignments were submitted to the SPEER server (Chakraborty et al. 2012) for prediction of specificity determining sites, using Sci-Phy with default settings and RELATIVE ENTROPY term, PC Property Distance term, and TYPE options, with or without the "include unclustered/singleton sequences" option. We identified sites with p-value < 0.05 in all analyses. A two-dimensional representation of kH_V1 (Smith et al. 2011) was prepared using TOPO2 (Johns, S.J., TOPO2, Transmembrane protein display software, http://www.sacs.ucsf.edu/TOPO2/).

RESULTS AND DISCUSSION

H_V1 is not just for bioluminescence

We probed 63 publicly available dinoflagellate transcriptomes for H_V1 sequences. Thirty dinoflagellate species out of 38 examined contained high-confidence H_V1 transcripts. We found two Hv1 sequences in the *S. minutum* genome (Shoguchi et al. 2013) and one from the *S. kawagutii* genome (Lin et al. 2015). H_V1 sequences from the *S. microadriaticum* genome (Aranda et al. 2016) were excluded because their gene models were unusual. H_V1 appears in armored and unarmored species, and in autotrophic, heterotrophic, mixotrophic, and symbiotic species sampled from disparate geographic origins (Table S1).

Eight species had transcriptomes without detectable H_V1 sequence. Two of these had transcriptomes under 50 MB, explaining the lack of H_V1. The six species with transcriptomes over 100 MB that lacked H_V1 are distributed over a wide range of the dinoflagellate phylogeny and do not correlate with autotrophy, theca, or geographical location. The absence of H_V1 in these six species could be explained by low H_V1 abundance, by loss of the H_V1 gene in the species, or by a bona fide H_V1 that does not fit our current profile. We are unable to distinguish among these possibilities. As expected, none of the species lacking H_V1 are bioluminescent, while all assemblies from bioluminescent species sampled contained H_V1.

Importantly, H_V1 appears in 23 nonbioluminescent dinoflagellate species including *Oxyrrhis* which is basal to the dinoflagellate lineage (Bachvaroff et al. 2014; Janouškovec et al. 2017; Orr et al. 2012). All of these data taken together provide good evidence that H_V1 has an ancestral function in dinoflagellate biology.

Dinoflagellates can have more than one H_V1 gene

We observed that the *S. minutum* genome contains at least two H_V1 genes, as evidenced by two hits appearing on different scaffolds with different surrounding genes. Although the significance is not understood, to our knowledge this is the first report of multiple H_V1 genes in the same organism.

Fifteen species contained two or more transcriptomic H_V1 sequence clusters (Fig. 1A) that were more different from each other than the two *S. minutum* gene sequences. We did not include *Symbiodinium* because samples may contain multiple species. In *Kryptoperidium foliaceum* and *Glenodinium foliaceum*, the number of H_V1 sequence clusters (i.e. the number of sequences that differ more than the *S. minutum* H_V1 genes) per transcriptome was highly variable, reducing confidence that clusters represent different genes in these species. In the other 13 species with multiple transcriptomes, however, the number of H_V1 sequence clusters per transcriptome was similar, giving confidence that different clusters represent different H_V1 genes.

Phylogenetic analysis revealed three major groups (subfamilies) (Fig. 1). In eight species, H_V1 sequences from two or three of the different subfamilies appear in the same organism, strong evidence that these sequences represent different genes, although we cannot rule out alternative splicing or inaccurate transcript assembly. In humans, H_V1 appears in three alternatively spliced forms that result in two different protein sequences, designated short and long (Hondares et al. 2014). The short protein results from splicing out the first intron (i.e. the first 20 residues of the protein) leaving the rest of the sequence identical to the long form. In contrast, alternative forms of H_V1 in dinoflagellates differ in several sequence locations (Fig. 1), indicating that any alternative splicing would be more complex than for human H_V1.

Characteristics of H_V1 sequence subfamilies

The three branches of the tree (Fig. 1A) are supported with 96% confidence by aIRT-SH-like branch support in maximum likelihood analysis; this division was observed in all trees we produced (Fig. 1, S1). Preliminary phylogenetic trees (data not shown) placed H_V1 sequences from chromerids (considered to be dinoflagellates' closest relatives) and coccolith (H_V1 from which have been experimentally verified (Taylor et al. 2011)) basal to the Group 1 subfamily; for simplicity we retained just the chromerid H_V1 sequences as the outgroup. The H_V1 from *Oxyrrhis marina*, the species considered basal to the dinoflagellate phylogeny (Bachvaroff et al. 2014; Janouškovec et al. 2017; Orr et al. 2012), was always basal to the Group 1 subfamily. Many Group 2 species are "dinotoms"; diatom H_V1s cluster basal to Group 2, suggesting that H_V1 from nominally dinoflagellate transcriptomes in this subfamily could instead be symbiont or prey sequences. In the other subfamilies, sequences from autotrophs cluster with nonautotrophic sequences, providing confidence that these are bona fide dinoflagellate H_V1s. As shown in Fig. 1A and in Table S1, the major division of H_V1 sequences does not correlate with a species' bioluminescence, theca, trophic type, or geographical origin (Fig. 1).

Sequence logos (Fig. 1C) from the trimmed alignments of the three subfamilies show several regions in which the subfamilies differ. Consensus results of SPEER (Chakraborty et al. 2012) analysis identified 11 sites (Fig. 1B, C) predicted to distinguish subfamilies. These 11 sites appear in both external and internal aqueous vestibules, and in the hydrophobic gasket region of the protein (Fig. 1C). For six of these (represented by *K. veneficum* H_V1 [kH_V1] sequence numbers H41, G58, A105, E109, L126, H140), the side chain characteristics change dramatically, e.g. from positive to negative or from charged to hydrophobic (Fig. 1C). Despite these differences, no position is completely diagnostic for separating sequences into the three main subfamilies.

In human H_V1 (hH_V1), the position corresponding to E109 of kH_V1 was shown to be an important determinant of divalent metal binding in hH_V1 (DeCoursey et al. 2016). In hH_V1, the position corresponding to L126 of kH_V1 is probably involved in an internal salt bridge stabilizing the closed state, while the position corresponding to E154 of kH_V1 is probably involved in an external salt bridge stabilizing the open state (DeCoursey et al. 2016). The significance of these positions in dinoflagellate H_V1 has not been tested. No functional significance has been ascribed to the other sequence positions that differ among the sequence subfamilies.

KH_V1's threshold voltage has a unique set point negative to the reversal potential (Smith et al. 2011). In contrast, like all other experimentally verified H_V1, the threshold voltage of *L. polyedrum* H_V1 (LpH_V1) is positive to the reversal potential (Rodriguez et al. 2017). A simple explanation for this difference might be that kHv1 represents an isoform with a unique function. However, the experimentally verified LpH_V1 is in Group 3, as is kH_V1. This observation makes the simple explanation unlikely and the molecular basis of kH_V1's unique characteristics remains mysterious.

Cellular and physiological characteristics of dinoflagellate H_V1

LpHv1 has been localized to the vacuole and also likely to the plasma membrane (Rodriguez et al. 2017). Thus, both tonoplast and plasma membranes are probable sites for H_V1 in all dinoflagellates. Interestingly, the Group 1 *L. polyedrum* H_V1 sequence lacks the epitope used in the localization study and thus would probably not have been detected (Rodriguez et al. 2017).

Because kHv1 was found in a cDNA library from a dark-maintained culture and not in a cDNA library from a light-maintained culture (Smith et al. 2011), we speculate that H_V1 is important to dinoflagellate nutrition. In coccolithophores, H_V1 at the plasma membrane mediates H^+ conductance regulating cytoplasmic pH and promoting

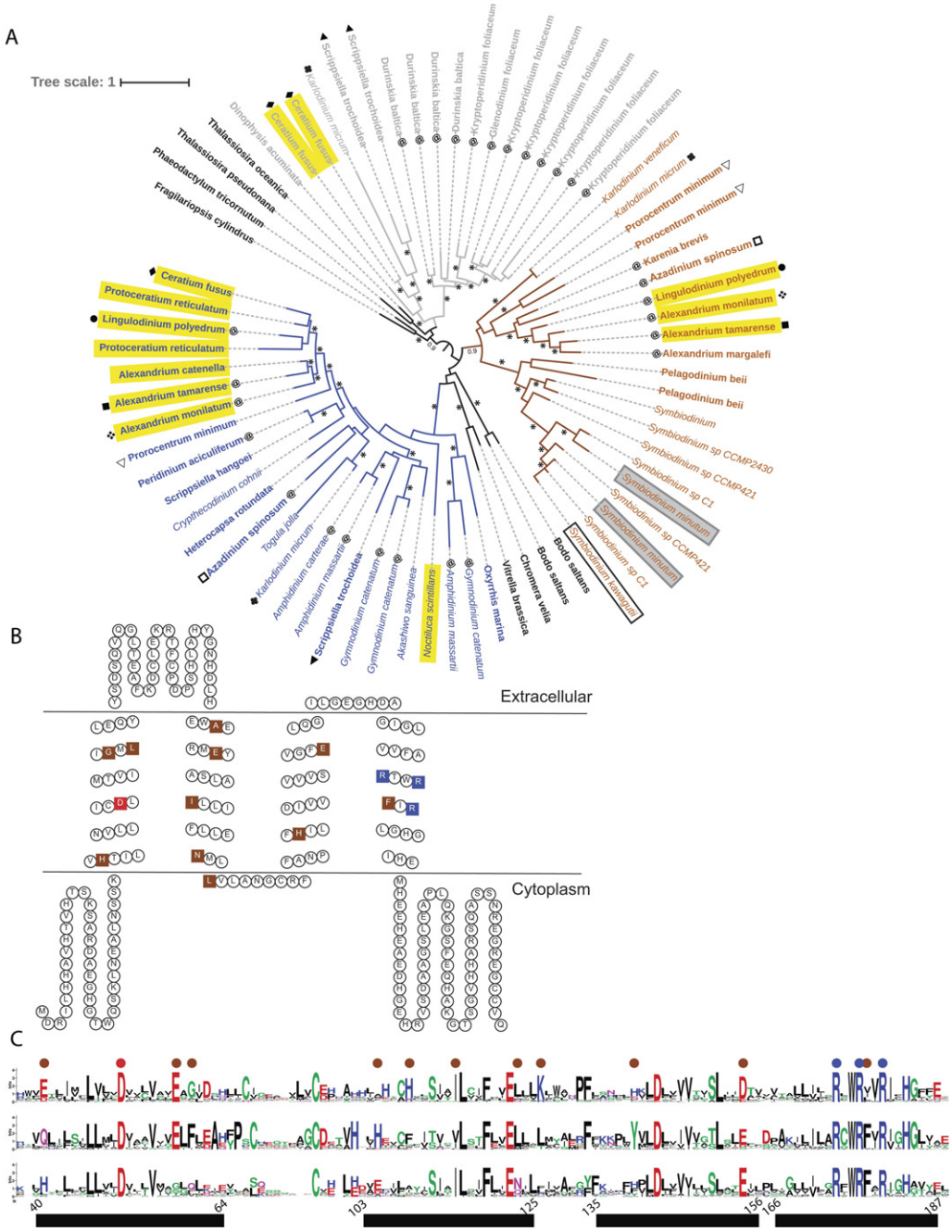

Figure 1 Characteristics of H_V1 sequences found in dinoflagellate transcriptomes and genomes. (A) Maximum likelihood tree of high-confidence dinoflagellate H_V1 sequences shows three major sequence subfamilies (Group 1 in blue, Group 2 in gray, Group 3 in brown). Chromerid and diatom sequences are indicated in black. Yellow shading indicates bioluminescent species; @ indicates autotrophic species; italics indicates nonthecated species; boxes indicate sequences found in genomic data; gray highlighting indicates two distinct sequences found in the same genome. Matching symbols indicate sequences in different subfamilies found in the same species. Asterisks indicate aIRT-SH branch support > 0.8. (B) Two-dimensional depiction of kH_V1 sequence produced by TOPO2. Extents of transmembrane helices were defined as in DeCoursey et al. (2016). Red indicates proton selectivity filter on S1, blue indicates arginines involved in voltage sensing on S4. Brown indicates specificity determining sites as identified by SPEER analysis. (C) Sequence logos from the three main subfamilies. The trimmed multiple sequence alignment that was submitted to maximum likelihood analysis (tree shown in A) was separated into three alignments corresponding to Group 1 (top), Group 2 (middle), and Group 3 (bottom) and a sequence logo was obtained for each subfamily. Black bars indicate the extent of transmembrane helices S1–S4 as defined in (B); numbers indicate beginnings and ends of transmembrane helices in kH_V1. Note that the trimmed alignment contains only well-conserved sequence positions and does not reflect the full length of the VSD. Brown circles indicate specificity determining sites as identified by SPEER analysis. Red circle indicates proton selectivity filter on S1, and blue circles indicate arginines involved in voltage sensing on S4.

sustained calcification (Taylor et al. 2011). In several cell types from multicellular organisms, H_V1 is important for controlling cytoplasmic pH (DeCoursey, 2013). It is thus likely that H_V1 also contributes to pH homeostasis in dinoflagellates. This function could be critical to the survival of these ecologically important producers in an acidifying ocean (Bopp et al. 2013).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Multiple phylogenetic analysis methods confirm three distinct subfamilies of dinoflagellate H_V1s.

Table S1. H_V1 sequences and associated characteristics of source dinoflagellates.