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Characterization of a novel EF-hand homologue, CnidEF, in the sea anemone *Anthopleura elegantissima*

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Abstract

The superfamily of EF-hand proteins is comprised of a large and diverse group of proteins that contain one or more characteristic EF-hand calcium-binding domains. This study describes and characterizes a novel EF-hand cDNA, CnidEF, from the sea anemone *Anthopleura elegantissima* (Phylum Cnidaria, Class Anthozoa). CnidEF was found to contain two EF-hand motifs near the C-terminus of the deduced amino acid sequence and two regions near the N-terminus that could represent degenerate EF-hand motifs. CnidEF homologues were also identified from two other sea anemone species. A combination of bioinformatic and molecular phylogenetic analyses was used to compare CnidEF to EF-hand proteins in other organisms. The closest homologues identified from these analyses were a luciferin binding protein (LBP) involved in the bioluminescence of the anthozoan *Renilla reniformis*, and a sarcoplasmic calcium-binding protein (SARC) involved in fluorescence of the annelid worm *Nereis diversicolor*. Predicted structure and folding analysis revealed a close association with bioluminescent aequorin (AEQ) proteins from the hydrozoan cnidarian *Aequorea aequorea*. Neighbor-joining analyses grouped CnidEF within the SARC lineage along with AEQ and other cnidarian bioluminescent proteins rather than in the lineage containing calmodulin (CAM) and troponin-C (TNC).

Keywords: Cnidarian; EF-hand; *Anthopleura elegantissima*; Calcium-binding protein; Molecular evolution

1. Introduction

Calcium is used to mediate a large variety of biological functions in animals, including bioluminescence, muscle contraction, neurotransmitter release, cell growth and development, and signal transduction. The EF-hand motif is often present in calcium-binding proteins. Familiar examples of these proteins include calmodulin (CAM), troponin-C (TNC), sarcoplasmic calcium-binding protein (SARC), and aequorin (AEQ), which functions in bioluminescence in hydrozoan cnidarians. The EF-hand domain consists of a helix-loop-helix structure formed by a highly conserved 12 residue calcium-binding loop, flanked on both sides by α-helices (Nelson and Chazin, 1998). This loop binds calcium with the coordination of seven ligands (Fig. 1). Five are from side-chain carboxylate oxygens donated by residues 1, 3, 5, and 12. One is a backbone carbonyl oxygen from residue 7. And finally an indirect association with residue 9, which is mediated by the seventh ligand of a water-molecule hydrogen bound to its side chain and also often associated with residue 3 (Malmendal et al., 1998; Nelson and Chazin, 1998). A variety of EF-hand subfamilies show conservation of the first position D (D_1) which provides a ligand binding group, a G_6 allowing for a 90° turn in the loop, and an E_{12} which provides two ligand binding groups (Jamieson et al., 1980; Malmendal et al., 1998; Yuasa et al., 2001).

There are at least 45 distinct subfamilies of EF-hand proteins which contain up to eight EF-hand domains that usually occur in pairs (Nakayama and Kretsinger, 1994; Kawasaki et al., 1998). The function of only 13 of these 31 subgroups is known. For the well-studied EF-hand proteins, in most cases, adjacent EF-hand domains cross-associate to form a calcium-binding pocket. The most significant differences between EF-hand proteins occur in the regions outside of the EF-hand domains. These can be catalytic, such as an oxygenase region encoded in AEQ, or structural, such as CAM which

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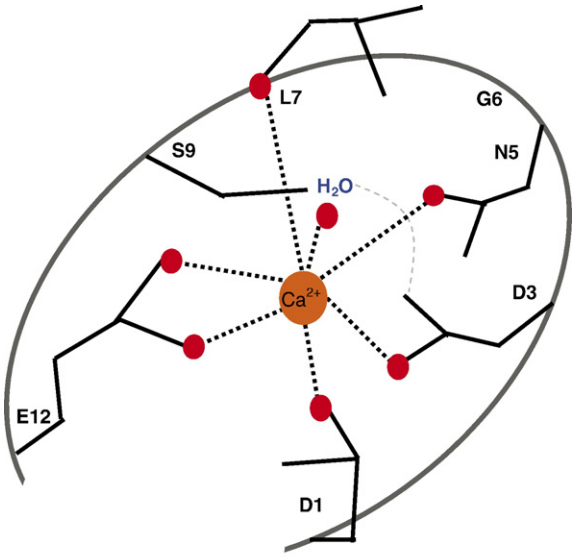

Fig. 1. Schematic representation of an EF-hand calcium-binding loop domain depicting the positions of the seven ligands that contribute to coordinated calcium binding.

transduces intracellular calcium signals through a conformational change (Nakayama and Kretsinger, 1994). The conformational change in CAM causes activation of the enzymatic or structural portion of the protein. Given the variety and importance of the many calcium-related biological functions, it is not surprising that this family of proteins has been well-studied, including its molecular evolution and phylogenetics (Nakayama and Kretsinger, 1994).

Several EF-hand proteins have been described in cnidarians, and are reviewed by Tsuji et al. (1995). These include AEQ, obelin (OBL), mitrocomin, and cyclin, all isolated from different hydrozoans, and all proteins that function in bioluminescence. Luciferin-binding protein (LBP), another bioluminescence protein, as well as a CAM have also been described in the anthozoan *Renilla reniformis*.

In this study, we describe a novel EF-hand protein, CnidEF, from another anthozoan, the North American Pacific coast temperate anemone *Anthopleura elegantissima*. CnidEF was first identified from *A. elegantissima* during screening of a subtracted library that was generated to characterize anemone genes expressed specifically as a function of its symbiosis with the dinoflagellate alga *Symbiodinium muscatinei* (Muscatine, 1971; LaJeunesse and Trench, 2000). Symbiosis between *A. elegantissima* and *S. muscatinei* is facultative; in high light environments anemones harbor symbionts in their tissues but in low light environments, such as caves and rock crevices, anemones are symbiont-free (Weis and Levine, 1996). This study describes comprehensive expression studies that revealed no correlation of CnidEF levels to symbiotic state. In addition, we have identified CnidEF homologues from two other anemone species and performed structural and phylogenetic analyses of CnidEF, both of which place CnidEF within the SARC lineage as a novel addition to the EF-hand family of calcium-binding proteins.

2. Methods

2.1. Isolation of CnidEF from A. elegantissima

CnidEF was originally isolated from a subtracted cDNA library made from *A. elegantissima* RNA and designed to identify sequences enhanced in the symbiotic state. See supplementary online information for details of the subtracted library.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from animals as described in Weis and Reynolds (1999). RNA was reverse transcribed using 0.5 μg oligo $(dT)_{12–18}$ and the Superscript Preamplification System (Life Technologies) to synthesize cDNA.

2.3. Northern hybridizations

To determine the relative level of CnidEF expression in field collected samples, Northern hybridizations were conducted. Total RNA (6.75 μg) from 56 animals was resolved on a 1% formaldehyde denaturing gel in 1× MOPS buffer (0.1 M MOPS, 40 mM sodium acetate, 5 mM EDTA). RNA was then transferred to a nylon membrane and hybridized overnight at 68 °C in DIG Easy Hyb (Roche) containing 100 μg/mL salmon sperm DNA and a 307 nucleotide digoxigenin-labeled RNA probe transcribed from a CnidEF clone with specific primers (see Fig. 2 for primer locations). The membrane was washed twice at room temperature with 2× SSC, 0.1% SDS. The hybridized RNA was then immunodetected using anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche). To quantify the relative expression levels, a pixilation analysis of the blot film was conducted using the ImageQuaNT phosphoimager (Molecular Dynamics, Inc.) according to the manufacturer's instructions. Optical density values were subjected to a 2-way ANOVA to test for differences in CnidEF expression between months and among groups (symbiotic versus aposymbiotic). They were also subjected to a post hoc Tukey HSD multiple comparison test to identify the difference in expression by month. All statistical analyses were performed using SPSS V. 9.0.0 (1989).

2.4. Motif recognition

Motif searches of homologues from the closest EF-hand families, and all CnidEF sequences, were conducted at the ExPASy Website hosted by The Swiss Institute of Bioinformatics (Bucher and Bairoch, 1994; Hofmann et al., 1999). Many EF-hand proteins, including those used in the phylogenetic analysis described below, contain more than two EF-hands. Motif searches were conducted on all sequence groups of the final alignment to determine the number and location of EF-hands. This information was used to verify the alignment parameters, and to search for evidence of evolutionarily lost EF-hands within the CnidEF sequence. General EF-hand conservation patterns, such as D_1, G_6, and E_{12} (Jamieson et al., 1980; Malmendal et al., 1998; Yuasa et al., 2001) were used to identify the possible locations of degenerate EF-hands.