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Evolution of Developmental Control Mechanisms

NF-κB is required for cnidocyte development in the sea anemone *Nematostella vectensis*

Francis S. Wolenski [a], Cynthia A. Bradham [a,b], John R. Finnerty [a,b], Thomas D. Gilmore [a,*]

[a] Boston University, Department of Biology, 5 Cummington Mall, Boston, MA 02215, United States
[b] Program in Bioinformatics, Boston University, Boston, MA 02215, United States

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ABSTRACT

The sea anemone *Nematostella vectensis* (Nv) is a leading model organism for the phylum Cnidaria, which includes anemones, corals, jellyfishes and hydras. A defining trait across this phylum is the cnidocyte, an ectodermal cell type with a variety of functions including defense, prey capture and environmental sensing. Herein, we show that the Nv-NF-κB transcription factor and its inhibitor Nv-IκB are expressed in a subset of cnidocytes in the body column of juvenile and adult anemones. The size and distribution of the Nv-NF-κB-positive cnidocytes suggest that they are in a subtype known as basitrichous haplonema cnidocytes. Nv-NF-κB is primarily cytoplasmic in cnidocytes in juvenile and adult animals, but is nuclear when first detected in the 30-h post-fertilization embryo. Morpholino-mediated knockdown of Nv-NF-κB expression results in greatly reduced cnidocyte formation in the 5 day-old animal. Taken together, these results indicate that NF-κB plays a key role in the development of the phylum-specific cnidocyte cell type in *Nematostella*, likely by nuclear Nv-NF-κB-dependent activation of genes required for cnidocyte development.

Introduction

The phylum Cnidaria is an ancient lineage of primarily marine animals comprising over 11,000 species, including sea anemones, corals, hydras and jellyfishes (www.cnidtol.com, Cnidarian Tree of Life; Steele et al., 2011). Cnidarians populate a wide range of marine habitats including coral reefs, estuaries, and even deep water thermal vents. The survival of corals and many sea creatures that live in coral reefs is in peril as environmental changes contribute to the demise of coral reefs around the world (Weis, 2008). Thus, an understanding of the molecular basis of cnidarian physiology and development will be important for assessing environmental effects on cnidarian health.

One phylum-specific cell type that distinguishes Cnidaria from other lineages is the cnidocyte (also known as the nematocyte). There are at least 28 morphologically distinct types of cnidoctye, and they contribute to a range of biological functions including defense, prey capture, environmental sensing and anchoring the animal to its substrate (Kass-Simon and Scappaticci, 2002; Mariscal et al., 1977). The mature cnidocyte contains a collagenous capsule (the cnida or cnidocyst) that is connected to an eversible thread. Many cnidarians have a stinging cell subtype of cnidocyte that discharges venom through a harpoon-like thread in order to capture prey or deter predators (Watson and Mariscal, 1983). Other cnidocytes are thought to be involved in sensing of environmental substances, and may behave somewhat like neuronal sensory cells (Kass-Simon and Scappaticci, 2002). Moreover, treatment of anemones with NANA, an *N*-acetylated sugar derived from mucin, enhances the ability of stinging cnidocytes to fire, supporting the idea that they are regulated by a chemosensory response (Kass-Simon and Scappaticci, 2002; Krayesky et al., 2010; Marlow et al., 2009; Watson et al., 2009).

Nematostella vectensis (Nv) is a small burrowing sea anemone native to estuaries on the Atlantic coast of North America (Reitzel et al., 2008) and is one of the leading model organisms among cnidarians (Darling et al., 2005). In part, this prominence is due to the ease of culturing this small (1–2 cm) anemone in laboratory conditions (Darling et al., 2005), as well as the availability of a complete genome sequence and substantial EST data (Putnam et al., 2007; Sullivan et al., 2006). In *N. vectensis*, cnidocytes are abundant in the tentacles and are also scattered among the ectodermal cells of the external body column and within a limited number of internal sites (Stephenson, 1935). Morphology, size, distribution, and the expression of particular minicollagens (a structural protein in the capsule) have been used to classify *N. vectensis* cnidocytes into three distinct subtypes: basitrichous haplonema, microbasic mastigophores, and spirocysts (Zenkert et al., 2011). While the basitrichous haplonema and spirocyst subtypes are both abundant in the head region, the basitrichous

Abbreviations: Nv, *Nematostella vectensis*; ASW, artificial sea water; BH, basitrichous haplonema; MM, microbasic mastigophore; SC, spirocyst

* Corresponding author. Fax: +1 617 353 6340.
E-mail address: gilmore@bu.edu (T.D. Gilmore).

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haplonema subtype is the dominant cnidocyte in the body column and foot (Zenkert et al., 2011).

NF-κB is an inducible eukaryotic transcription factor that is held inactive in the cytoplasm bound to an inhibitory IκB protein (Hayden and Ghosh, 2004). Activation of cytoplasmic NF-κB generally requires signal-induced degradation of IκB, which enables NF-κB to enter the nucleus where it alters the expression of target genes to bring about cellular and organismal responses. NF-κB can be activated to enter the nucleus in response to a wide range of physiochemical (chemicals, ultraviolet light) and biological (pathogens, cytokines, growth factors, etc.) factors (Gilmore and Wolenski, 2012; Oeckinghaus et al., 2011). NF-κB also plays critical roles in immune system and organ development in a variety of organisms ranging from protostomes to deuterosomes (Hayden and Ghosh, 2004). For example, nuclear localization of the *Drosophila* NF-κB protein Dorsal is necessary to establish dorsal-ventral patterning in the developing fly embryo (Lynch and Roth, 2011). In mammals, NF-κB signaling is also critical for the development of many immune cell types (Gerondakis et al., 2006), the liver (Beg et al., 1995), and the limb bud (Bushdid et al., 1998; Kanegae et al., 1998).

Recently, NF-κB homologs have been identified in a diverse range of simple marine animals (sponges, anemones, and corals) (Gauthier and Degnan, 2008; Shinzato et al., 2011; Sullivan et al., 2007) as well as a unicellular holozoan (Sebé-Pedrós et al., 2011). While several researchers have proposed a role for NF-κB in innate immunity in these basal animals (Augustin et al., 2010; Lange et al., 2011; Meyer et al., 2009; Miller et al., 2007; Shinzato et al., 2011; Sullivan et al., 2007), there is no direct evidence supporting this hypothesis, and the biological function(s) of the NF-κB signaling pathway in these simple marine organisms is unknown.

We previously showed that transcription factor NF-κB is expressed in a subset of ectodermal cells in the body column of *N. vectensis* (Wolenski et al., 2011b). In this report, we show that the majority of the Nv-NF-κB-positive ectodermal cells in juvenile and adult animals are cnidocytes. Moreover, by morpholino-mediated knockdown, we demonstrate that NF-κB is required for cnidocyte development in *N. vectensis*. These results provide the first description of a biological function of NF-κB in an organism basal to arthropods.

Materials and methods

Antisera

Custom antisera were prepared by OpenBiosystems (Fisher Scientific). An affinity-purified rabbit polyclonal antiserum against a C-terminal peptide (PADFLQQGVFSTQNPSNM) of the Nv-NF-κB protein has been described previously (Wolenski et al., 2011b). An affinity-purified guinea pig polyclonal antiserum was prepared against a C-terminal peptide (DNVRPMLQLPESPF) of Nv-IκB. A complete list of antisera, dilutions, and blocking buffers used is provided in Supplemental Table 1.

Plasmid construction, cell culture and transfection

The cDNA encoding Nv-IκB (EU092641.1) was described previously (Wolenski et al., 2011b). Gene-specific primers were used to amplify the Nv-IκB cDNA which was then subcloned into the pcDNA3.1 (+) (Invitrogen) expression vector. Details about the plasmid construction are included in supplemental material.

DF-1 chicken fibroblasts and human A293 cells were grown in Dulbecco's modified Eagle's Medium (Invitrogen) supplemented with 10% fetal bovine serum (Biologos), 50 U/ml penicillin, and 50 μg/ml streptomycin as described previously (Sullivan et al., 2009). Transfection of cells with expression plasmids was performed using polyethylenimine (Polysciences, Inc.) essentially as described previously (Sullivan et al., 2009). Briefly, on the day of transfection, cells were incubated with plasmid DNA and polyethylenimine at a ratio of 1:6. Media was changed 24 h post-transfection, and whole-cell lysates were prepared 24 h later. Alternatively, if cells were used for immunofluorescence, they were passaged onto glass coverslips on the day prior to fixation.

Collection of N. vectensis embryos

The routine maintenance and mating of *N. vectensis* adults were performed as previously described (Fritzenwanker and Technau, 2002; Genikhovich and Technau, 2009; Hand and Uhlinger, 1994). Anemones were raised and spawned in bowls of 1/3 strength artificial seawater (ASW; ~12 parts per thousand). Spawned eggs that were part of an egg mass were de-jellied and collected essentially as described previously (Fritzenwanker and Technau, 2002).

Fixation of anemones

Anemones were prepared for fixation by first placing whole animals in a solution of 7% (w/v) $MgCl_2$ freshly prepared in ASW, and then gently shaking them for 10 min on a rocker as previously described (Wolenski et al., 2011b). After the incubation, anemones were washed 3X with ASW. Anemones were then transferred to 1.5-ml microcentrifuge tubes and fixed in ice cold 4% (v/v) formaldehyde in ASW, with rocking overnight at 4 °C.

Indirect immunofluorescence

Whole-mount indirect immunofluorescence was performed using unfertilized eggs, embryos (0–2 day post-fertilization), juvenile polyps (3–14 days post-fertilization), and small adults (less than 1 cm in size) to determine the expression patterns of Nv-NF-κB and Nv-IκB proteins. Anemones were transferred from the fixative to 12-well plastic dishes and washed 3X with PBT (1X PBS [pH 7.4], 0.2% [v/v] Triton X-100].

Except when anemones were stained with DAPI to detect cnidocytes (see below), antigen retrieval was performed to enhance staining by heating samples in urea (technique modified from Shi et al. (1993)). Briefly, anemones were transferred to glass tubes containing 10 ml of 5% (w/v) urea that had been heated to 80 °C. The tubes were placed in a 1450-watt microwave, heated at the lowest setting for 5 min, and the final temperature of the solution was not allowed to exceed 95 °C. The urea was allowed to cool for 30 min at room temperature, anemones were transferred to a 12-well dish, and washed 4X with PBT. Anemones were incubated overnight in blocking buffer (1X PBT, 5% [v/v] normal goat serum [Gibco], 1% [w/v] bovine serum albumin [Sigma]) at 4 °C.

The next day, the blocking buffer was removed and replaced with primary antiserum (anti-Nv-NF-κB, 1:100; anti-Nv-IκB antiserum, 1:200) diluted in blocking buffer, and samples were incubated with gentle shaking (~80 rpm) for 1.5 h at 37 °C. The primary antiserum was removed and samples were washed 4X with PBT for 10 min each. Secondary antiserum was added and samples were incubated with gentle shaking (~80 rpm) for 1.5 h at 37 °C. In a darkened environment, the samples were washed 4X with PBT for 10 min each. Anemones were then transferred onto a microscope slide and mounted in Vectashield HardSet mounting medium containing DAPI (Vector Labs). Slides were imaged with an Olympus FluoView FV10i confocal microscope. Information on