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LPS injection reprograms the expression and the 3′ UTR of a CAP gene by alternative polyadenylation and the formation of a GAIT element in *Ciona intestinalis*

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ABSTRACT

The diversification of cellular functions is one of the major characteristics of multicellular organisms which allow cells to modulate their gene expression, leading to the formation of transcripts and proteins with different functions and concentrations in response to different stimuli. CAP genes represent a widespread family of proteins belonging to the cysteine-rich secretory protein, antigen 5 and pathogenesis-related 1 superfamily which, it has been proposed, play key roles in the infection process and the modulation of immune responses in host animals. The ascidian *Ciona intestinalis* represents a group of proto-chordates with an exclusively innate immune system that has been widely studied in the field of comparative and developmental immunology. Using this biological system, we describe the identification of a novel APA mechanism by which an intronic polyadenylation signal is activated by LPS injection, leading to the formation of a shorter CAP mRNA capable of expressing the first CAP exon plus 19 amino acid residues whose sequence is contained within the first intron of the annotated gene. Furthermore, such an APA event causes the expression of a translational controlling *cis*-acting GAIT element which is not present in the previously isolated CAP isoform and identified in the 3′-UTR of other immune-related genes, suggesting an intriguing scenario in which both transcriptional and post-transcriptional control mechanisms are involved in the activation of the CAP gene during inflammatory response in *C. intestinalis*.

1. Introduction

One of the hallmarks of eukaryotic genes is the presence of intervening sequences within protein coding information. The differential inclusion of exons and introns or portions of them in mature mRNAs results in the possibility of expressing multiple transcripts with different coding capacities from the same single gene (de Klerk and t Hoen, 2015). In this way, cells can enormously expand their flexibility, modulating their activities and displaying dynamic changes in response to independent stimuli (Anderson, 2010). Several studies have revealed how alternative splicing and alternative polyadenylation (APA) are exceedingly common events that occur throughout innate immunity and fine-tune almost all steps in the process. In this respect, immune genes are coordinately and temporally regulated in response to distinct stimuli. Transcriptional and post-transcriptional mechanisms that modify mRNA expression, stability and/or translation provide a rapid and flexible control of this process and are particularly important in coordinating the initiation and resolution of inflammation (Anderson, 2010).

The use of APA sites represents a regulatory level by which cells can generate different protein isoforms with different functions or with mRNAs differing in the length of their 3′ untranslated regions (UTR) (Batra et al., 2015). APA mechanisms can be divided into two major types: the simplest and more frequent are named UTR-APA (UnTranslated Region-Alternative PolyAdenylation) where the alternative poly(A) sites are located in the 3′ UTR of the mRNAs, with the majority of them residing closer to the stop codon (proximal) compared to canonical pA sites (distal). This kind of alternative mechanism results in the shortening of the 3′UTR without changing coding capacity. Through the alteration of the 3′ UTRs, APA

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potentially regulates the stability, cellular localization and translation efficacy of target RNAs as 3′ UTR serve as binding regions for factors that control these regulatory layers (i.e. micro RNAs and RNA binding proteins). Furthermore, 3′UTR of the mRNA of pro-inflammatory proteins can contain regulatory elements such as Interferon-γ-activated inhibitor of translation (GAIT) that direct their degradation and/or translational repression (Fox, 2015).

On the other hand, CR-APA (Coding Region-Alternative PolyAdenylation) is a mechanism by which the alternative pA sites reside in the upstream regions of genes. In particular, the less frequent intronic APA involves the recognition of a cryptic intronic poly(A) signal that involves premature polyadenylation within the coding region affecting the sequence of the protein (Elkon et al., 2013).

Ascidians (subphylum: Tunicata) occupy a key phylogenetic position in chordate evolution and are considered the sister group of vertebrates (Delsuc et al., 2006; Swalla et al., 2000; Tsagkogeorga et al., 2009). They are proto-chordates with an innate immune system, including inflammatory humoral and cellular responses. An inflammatory response induced by LPS injection in the body wall of *C. intestinalis* is a well-established model for analysing regulator and effector inducible host defence molecules of the innate immune system, as shown for a type IX collagen-like (Vizzini et al., 2008), CAP-like (Bonura et al., 2010), MBL-like (Bonura et al., 2009), TNFα-like (Parrinello et al., 2008; Parrinello et al., 2010), galectin-like (Vizzini et al., 2012), peroxinectin (Vizzini et al., 2013a), Interleukins 17 (Vizzini et al., 2015a), the proPO-system (Cammarata et al., 2008; Vizzini et al., 2015b) and TGF-β (Vizzini et al., 2016). In addition, our group described the first LPS-induced APA mechanism in this species with an intronic polyadenylation signal within the first intron leading to the upregulation of a short variant (Ci8 short) of an annotated mRNA and the modification of the tissue localization of this transcript (Vizzini et al., 2013a). Following this line of evidence, we decided to pursue our analysis investigating the existence of novel APA events induced by LPS injection in the body wall of *C. intestinalis*. For this reason, we decided to analyse the expression pattern of a previously isolated CAP protein (Bonura et al., 2010) in *C. intestinalis*. The CAP superfamily represents a large group of proteins in several species, ranging from the yeast *S. cerevisiae* to mammals, which are involved in several cellular processes including reproduction, development, immune functions and several pathologies including cancer, nerve damage, pancreatitis and heart failure [reviewed in (Gibbs et al., 2008)].

In this manuscript, we report the identification of a novel APA mechanism by which an intronic polyadenylation signal is activated by LPS injection, leading to a shorter CAP mRNA capable of expressing the first CAP exon plus 19 amino acid residues whose sequence is contained within the first intron of the annotated gene. Furthermore, by this mechanism, the short isoform shows a 3′ UTR translational regulatory element identified in immune related genes which is not present in the previously isolated CAP isoform, suggesting an intriguing scenario in which both transcriptional and post-transcriptional control mechanisms are involved in the activation of the *Ci*CAP gene during LPS mediated inflammatory response in *C. intestinalis*.

2. Materials and methods

2.1. Tunicates and LPS injection

Ascidians were collected within Sciacca Harbour (Sicily, Italy), maintained in tanks with aerated sea water at 15 °C, and fed every second day with a marine invertebrate diet of coraliquid (Sera Heinsberg, Germany). Lipopolysaccharide (LPS) (*Escherichia coli* 055:B5, Sigma-Aldrich, Germany) solution was prepared in sterile sea water (12 mM $CaCl_2$, 11 mM KCl, 26 mM $MgCl_2$, 43 mM Tris HCl, 0.4 M NaCl, pH 8.0). LPS solution (100 μg LPS in 100 μl sea water per animal) was injected into the tunic tissue at the median body region. Untreated ascidians were used as controls.

2.2. Total RNA extraction

Ascidian tissue fragments (200 mg) explanted at various times (from 1 to 48 h) were immediately soaked in RNAlater Tissue collection (Ambion, Austin, TX), and stored at −80 °C. Total RNA extraction was performed by using an RNAqueous™-Midi Kit purification system (Ambion, Austin, TX). RNA quality was assessed by agarose gel electrophoresis, and RNA concentration and purity were determined by measuring absorbance at 260 and 280 nm.

2.3. Gene RACE strategy and cDNA isolation

Total RNA (2 μg) from ascidians treated with LPS (4 h) was used in a PCR-based Gene RACE strategy, according to the manufacturer's instructions (GeneRacer™ Kit, Invitrogen, USA).

The strategy allows an RNA oligo to be ligated to 5′ decapped mRNA molecules followed by the reverse transcription of the ligated mRNAs using the GeneRacer™ oligo dT primer and the SuperScript™ III RT Module (Invitrogen, USA). To amplify alternative CiCAP cDNAs, the ATG forward primer (all the primers are reported in Table 1) was used in a 3′RACE (94 °C 1 min, 52 °C 30 s, 72 °C 1 min for 35 cycles) paired with the GeneRacer™ 3′primer. These reactions were diluted to 1:100, and nested PCRs were performed using the Exon 1 forward primer and the GeneRacer™ 3′Nested primer. This reaction yielded a fragment of 638 bp which differed from the expected *Ci*CAP molecular weights (1392 bp); the 638 bp amplification product was purified, cloned in pJET 1.2 blunt vector (CloneJET PCR Cloning Kit, ThermoFisher Scientific, USA) and sequenced. The 5′ RACE PCR (94 °C 1 min, 52 °C 30 s, 72 °C 1 min for 30 cycles) was performed with the GeneRacer™ 5′ primer and the Intron 1 Reverse primer; the reaction was diluted to 1:100 and a hemi-nested PCR was performed using the same Intron 1 Reverse primer and the GeneRacer™ 5′Nested primer. The product was purified, cloned in pJet 1.2 blunt and sequenced.

2.4. Sequence analysis

Sequence similarity searches were performed using the FASTA (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html) and BLAST (http://www.ebi.ac.uk/Tools/sss/ncbiblast/nucleotide.html) nucleotide programs. The deduced amino acid sequence was obtained using the Expasy translation tool (http://web.expasy.org/translate/) and a databank homology search was conducted using the BLAST protein tool (http://web.expasy.org/blast/). The presence of conserved domains was evaluated with the Delta Blast program. Alignments between the *Ci*CAP and the *Ci*CAP-2 proteins were accomplished with the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/). A signal peptide in the *Ci*CAP-2 protein was identified using the Signal IP algorithm (http://www.cbs.dtu.dk/services/SignalP). The Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php) was used to analyze the amino acid sequence of the mature *Ci*CAP-2. To characterize the 3′UTR region of *Ci*CAP-2, a computational analysis was performed using the Regulatory RNA Motifs and Elements Finder tool (http://regrna.mbc.nctu.edu.tw/html/prediction.html), and the mfold algorithm was employed to build up the two dimensional structure of the GAIT domain (http://unafold.rna.albany.edu/?q=mfold).