

Cloning and expression of a novel component of the CAP superfamily enhanced in the inflammatory response to LPS of the ascidian *Ciona intestinalis*

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Abstract The CAP superfamily is a group of proteins that have been linked to several biological functions such as reproduction, cancer, and immune defense. A differential screening between lipopolysaccharide (LPS)-challenged and naive *Ciona intestinalis* has been performed to identify LPS-induced genes. This strategy has allowed the isolation of a full-length 1471-bp cDNA encoding for a 413-amino-acid protein (CiCAP). In silico analysis has shown that this polypeptide displays a modular structure with similarities to vertebrate CAP-superfamily proteins and to a collagen-binding adhesin of *Streptococcus mutans*. Domain organization analysis and alignment of CiCAP to other vertebrate CAP proteins have revealed a novel structure suggesting that this protein originated from a common ancestor gene that gave rise to many subfamilies of mosaic proteins with novel functions. Quantitative mRNA expression performed by real-time polymerase chain reaction analysis has demonstrated that this gene is rapidly activated in the pharynx of *C. intestinalis* a few hours after LPS injection. Moreover, *in situ* hybridization has shown that CiCAP mRNA is highly expressed by hemocytes with large granules contained inside the pharynx vessels. Thus, CiCAP represents a protein with novel structural domains involved in ascidian immune responses.

Keywords Innate immune system · Differential display · CAP protein · Molecular biology · *Ciona intestinalis* (Tunicata)

Introduction

The CAP superfamily represents a group of diverse proteins that have been isolated in several species ranging from *Saccharomyces cerevisiae* to mammals. Within mammals, CAP proteins have been identified in several tissues in which they perform endocrine or paracrine functions linked to several cellular processes including reproduction, development, immune functions, and several pathologies including cancer, nerve damage, pancreatitis, and heart failure (for a review, see Gibbs et al. 2008). The CAP nomenclature is derived from the three major groups of proteins originally identified, viz., the cysteine-rich secretory proteins (CRISPs), antigen 5 (Ag5) and pathogenesis-related 1 protein families (Pr-1), suggesting a functional link between the plant and the animal immune system (Szyperski et al. 1998). The CAP superfamily is characterized by several signature sequences that are usually present in a small disulfide-bond stabilized and structurally conserved 17- to 21-kDa domain that has been referred to as the CAP domain. Although a large number of proteins within the superfamily contain a CAP domain in isolation (e.g., the Ag5 and Pr-1 proteins found in insects and plants), many others contain additional C-terminal extensions that can impart diversity of biological functions. Most of them have a short C-terminal extension containing four cysteines, which has been referred to as a Hinge region (Guo et al. 2005). Additional domains are sometimes entirely unique and are observed only in association with the CAP domain, such as the ion channel

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regulator at the C terminus of the CRISPs (Gibbs et al. 2006); some others display homology to domains of diverse protein families, such as the C-type lectin domain (Zelensky and Gready 2005) and the LCCL domain (Trexler et al. 2000). Among non-mammalian proteins, various CAP proteins have been found in *Drosophila* (for a review, see Kovalick and Griffin 2005), *Xenopus*, *Heloderma horridum*, and *Conus textile* (for a review, see Yamazaki and Morita 2004). CAP proteins have been identified in several species of both free-living (*Caenorhabditis elegans*) and parasitic nematodes. These proteins are produced at the crucial early stage of parasitic invasion to evade the host immune system and facilitate feeding (Del Valle et al. 2003; Hawdon and Hotez 1996; Moyle et al. 1994).

Ascidians (subphylum Tunicata) are hermaphroditic invertebrates belonging to the same phylum of vertebrates (Chordata) (Delsuc et al. 2006; Swalla et al. 2000) and occupying an intermediary phylogenetic position between invertebrates and vertebrates (Azumi et al. 2003). For this reason, they represent an intriguing model for studying the evolution of the innate immune system. In particular, the inflammatory reaction in the body wall of *C. intestinalis* (Parrinello et al. 1984, 1990) is a well-established model for the analysis of inducible host defence molecules of the innate immune system (Bonura et al. 2009; Cammarata et al. 2007, 2008; Parrinello et al. 2008; Vizzini et al. 2008). The pharynx is the main hemopoietic organ in which circulating hemocyte populations are renewed, and it can be challenged by inoculating inflammatory agents. CAP proteins have previously been isolated from ascidians. *Halocynthia roretzi* (sea squirt) presents two CAP proteins (HrUrabin and HrUrabin-long) (Urayama et al. 2008) with a high percentage of homology to glioma pathogenesis-related proteins (GLIPR1L1 and GLIPR1L2) respectively, which are expressed within testes (Ren et al. 2006) and on sperms (Gibbs et al. 2010), representing the first CAP proteins identified within invertebrate sperm. These proteins seem to play a role as adhesion molecules involved in the negative selection against “self” oocytes. However, little is known about other ascidian CAP proteins and their involvement in biological functions apart from the control of self-fertilization.

In the present paper, we report a *C. intestinalis* CAP (CiCAP) cDNA that exhibits a novel modular structure. CiCAP shows a NH₂ terminal domain with a high level of homology to the vertebrate CAP superfamily, and a COOH terminal domain highly homologous to the collagen-binding adhesin of *Streptococcus mutans*. Real-time polymerase chain reaction (PCR) analyses and in situ hybridization (ISH) has revealed that inoculation of lipopolysaccharide (LPS) into the ascidian pharynx up-regulates the CiCAP gene suggesting that the CiCAP gene is involved in the innate immune response of *C. intestinalis*.

Materials and methods

Tunicates

Ascidians were collected from Sciacca Harbor (Sicily, Italy), maintained in tanks with aerated sea water at 15°C, and fed every second day with a marine invertebrate diet (coraliquid) (Sera Heinsberg, Germany). *C. intestinalis* were kept in facilities compatible with their normal requirements and under conditions that minimize stress, by following guidelines for marine invertebrate maintenance.

LPS injection

LPS (*Escherichia coli* 055:B5, LPS; Sigma-Aldrich, Germany) solution was prepared in sterile marine solution (MS: 12 mM CaCl₂ × 6H₂O, 11 mM KCl, 26 mM MgCl₂ × 6H₂O, 43 mM TRIS HCl, 0.4 M NaCl, pH 8.0). LPS solution (100 µg LPS in 100 µl MS per animal) was injected through the tunic into the body wall (pharyngeal region) at the mid-portion referred to the longitudinal axis. Ascidians, both untreated and injected with MS only (100 µl), were used as negative controls.

Total RNA extraction and poly(A)⁺ purification

Ascidian tissue fragments (200 mg) explanted at various times (from 1 to 48 h) were immediately soaked in RNAlater Tissue collection (Ambion, Austin, Tex., USA) and stored at -80°C. Total RNA extraction was performed by using an RNAqueous-Midi Kit purification system (Ambion).

Poly(A)⁺ RNA was prepared from control and injected animals (1 h) by using an Illustra mRNA Purification Kit (GE Healthcare, UK) according to the manufacturer's instructions. The RNA quality was assessed by agarose gel electrophoresis, and the RNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm (data not shown).

Subtractive hybridization

Subtractive hybridization was performed with the PCR-Select cDNA Subtraction Kit (Clontech Laboratories, USA) according to the manufacturer's instructions. This strategy is based on a PCR-based method for the selective amplification of differentially expressed sequences, thereby allowing the isolation of transcript from activated tissues (pharynx). Briefly, 2 µg poly(A)⁺ RNA from non-injected (driver) and injected (tester) animals were retro-transcribed. The tester and driver cDNAs were digested with the restriction enzyme *RsaI* to yield blunt ends. The tester cDNA was then subdivided into two parts, and each was ligated with a different cDNA adaptor (ADAPTOR 1: 5'-CTAATAC GACTCACTATAGGGCTCGAGCGGCCGCCCCGGG

CAGGT-3'; ADAPTOR 2: 5'-CTAATACGACTCACTA TAGGGCAGCGTGGTCGCGGCCGAGGT-3'). The ends of the adaptor did not contain a phosphate group, and so only one strand of each adaptor attached to the 5' ends of the cDNA. Two hybridizations were then performed. In the first run, an excess of driver was added to each sample of tester. The samples were then heat-denatured and allowed to anneal. In the second run of hybridization, the two primary hybridization samples were mixed together without denaturing to allow the subtracted single-strand tester cDNAs to re-associate. These new hybrids were molecules that had different ends but that corresponded to the sequences of the two adaptors. After filling in the ends by DNA polymerase, the differentially expressed sequences displayed different annealing sites for the nested primers on their 5' and 3' ends. The entire population of molecules was then subjected to PCR in order to amplify the desired differentially expressed sequences by using the following primers (Nested PCR Primer 1: 5'-TCGAGCGGCCGCCC GGGCAGGT-3'; Nested PCR Primer 2: 5'-AGCGTGG TCGCGGCCGAGGT-3') and PCR conditions (94°C for 30 s, 68°C for 30 s, 72°C for 90 s; 12 cycles).

Cloning and sequence analysis

Differentially expressed cDNAs were cloned in the pCR4-TOPO vector (Invitrogen, USA) and sequenced. Sequence analysis showed a cDNA fragment of 94 nucleotides. Similarity searches performed by using the FASTA algorithm (<http://www.ebi.ac.uk/Tools/fasta/>) showed relevant homology to some expressed sequence tag clones from mature adult *C. intestinalis* (data not shown). The full-length sequence of the cDNA clone was obtained by using the GeneRacer kit (Invitrogen, USA). The kit ensures the amplification of only full-length transcript via elimination of truncated messages from the amplification process. 5' Rapid amplification of cDNA ends (RACE) was performed by PCR (94°C for 60 s, 52°C for 60 s, 72°C for 60 s; 30 cycles) with the CiCAP 3'RACE-specific oligonucleotide 5'-AAGTTCTGCCAGTTCCTC-3' (see Fig. 1 for details) and the GeneRacer 5'-oligonucleotide, viz., 5'-CGACTGG AGCAGGAGGACTGA-3'. This reaction was diluted 1:100, and a nested PCR was performed with the GeneRacer 5' nested-oligonucleotide (5'-GGACTGA CATGGACTGAAGGAGTA-3') and the CiCAP 3' nested specific oligonucleotide (5'-CTGTGGACGACATTGT-3').

3' RACE was performed with the *Ciona* CiCAP 5'-specific oligonucleotide (5'-ACAATGTCGTCCACAG-3') and the GeneRacer 3' oligonucleotide (3'-GCAATGCA TCGCATAGCAACTGTGCG-5'). PCR products were diluted 1:100 and re-amplified with the CiCAP 5' specific oligonucleotide and the GeneRacer 3' nested oligonucleotide (3'-GTGACAGTACGGCAATGCATCGC-5'; see Fig. 1 for

details). Fragments were purified, ligated in the pCR4-TOPO vector (Invitrogen, USA), and sequenced.

Sequence and phylogenetic analysis

Similarity searches were performed by using the FASTA program (<http://www.ebi.ac.uk/Tools/fasta/>). They showed a high degree of homology of CiCAP to several CAP-superfamily proteins from vertebrates and invertebrates. Multiple alignments were accomplished with the Clustal W program (Thompson et al. 1994). A phylogenetic tree was constructed by the neighbor-joining method, and 1000 bootstrap replicates were performed. The respective accession numbers are as follows: human cysteine-rich secretory protein LCCL domain-containing 2 (CRLD2), Q9H0B8; *Mus musculus* cysteine-rich secretory protein LCCL domain-containing 2 (CRLD2), Q8BZQ2; *Xenopus laevis* cysteine-rich secretory protein LCCL domain-containing 2 (CRLD2), Q4V9Y5; human GLIPR1, P48060; *Bos taurus* GLIPR1, Q32LB5; *Mus musculus* GLIPR1, Q9CWG1; *Osmerus mordax* GLIPR1, C1BLT0; human CRISP2, P16562; human GLIPR2, NP071738; *Mus musculus* GLIPR2, NP081726; *Salmo salar* GLIPR1, ACI33913; *Xenopus laevis* CRISP, NP001082594; *Schistosoma mansoni* CRISP, XP002578074; *Mus musculus* peptidase inhibitor 15 (PI15), NP444421; human PI15, AAI26293; *Danio rerio* PI15, NP_001153449; human PI16, AAH35634; *Mus musculus* PI16, CH466606.1; *Bos taurus* PI16, Q58D34; *Polistes fuscatus* Ag5, P35780; *Vespa vulgaris* Ag5, CAB42887.

Real-time PCR analysis

Tissue expression of the CiCAP gene was studied in four distinct LPS-challenged ascidians by real-time PCR with the Sybr-Green method. Primers were designed by using Custom Primers OligoPerfect Designers software (<https://tools.invitrogen.com/>) and synthesized commercially (Eurofins MWG Operon, Ebersberg, Germany). Real-time PCR analysis was performed on the Applied Biosystems 7500 real-time PCR system. Tissue expression was performed in a 25- μ l PCR containing 2 μ l cDNA converted from 250 ng total RNA, 300 nM CiCAP 1F forward (5'-GTATCTCCGCGAAGAGTTGG-3') and CiCAP 1R reverse primers (5'-TCGGTATAACGTGCGCTCT-3'), 300 nM actin forward (5'-TGATGTTGCCGCACTCGTA-3') and actin reverse (5'-TCGACAATGGATCCGGT-3') primers, and 12.5 μ l Power Sybr-Green PCR Master Mix (Applied Biosystems). The 50 cycles of the two-step PCR program consisted of initial polymerase activation for 3 min at 95°C followed by a denaturing step at 95°C for 15 s, and then annealing/extension was carried out at 60°C for 45 s when the fluorescent signal was detected. Each set of samples was run

1 gaaaattctatttgaataaggcgaatTTTgctaaaaggctttctattcaatttaatttaa 60
 61 taagactgaacattgctca^{CiCAP ATG}**ATGGATTCTAAACTGCATGTTACTTTTCGCTGTGTTGCTTTT** 120
 121 ACTGCTCGCTTCTTTTACAAATGCTATAGAATGGTCCGAGTCCATAAATGCTGAAGAAGC 180
 181 ACACCAGCGGTGGAAGCGCAGCCCTGACCAGCTGCTAGAGACAGTCGTTTTAACCAGGC 240
 241 GGAAAAGGATCAAATCGTGGCCTTGCACAATAAGTTCCGTTCTGATGAAATGGCGTCAAA 300
 301 TATGCTAGCAATGTCTGGGATAACCAAATCGCGAATTTTCGCATCATCCTATATATCAAA 360
 361 TTGTGAGTTTTTCGCACAGTAGTTCGGCGGAGCGGAGCCGGCATCCCATATTTAGTTATTT 420
 421 AGGAGAAAACCTTGGAGTGACCGTTTTCTTCGTCTCAACCAGATCAAGGGTTTTATTAT 480
 481 CCGTGTCAATCAATTGTGGTACGATGAAATAAATGACTATACGTACAGTATTTTACCGTG 540
 541 TGCTGCTGGCAAAGCATGCGGCCATTACTCAACAAGTTTGGGCTAGTACCTACAAGAT 600
 601 AGGTTGCGGAGCTGCATACTGCGCTCGTGGGAACGGTGGTTCCGGATATCAACTCATGGT 660
 661 AGCGTGTCAATACGGACCTGGTGGCAATCTCCTTAATACGAAT^{CiCAP 1F}**GTATCTCCGCGAAGAGT** 720
 721 **TGGCCAACCTTACAATAAAAATGGTGCACCGTGTCTGAATGCTCATCGGCGGATACATG** 780
 781 **TGTTAACAATCTGTGCATGAATCTTGCAGAGGGCAGCTTATAACCGACCAACAGTACACC** 840
 841 AACAACCACAAGTACACCAACAACCTACAAGTACACCAACAACCTACAAGTACACCAACAAC 900
 901 TACAAGTACACCAACAACCTACAAGTACACCAACAACCTACAAGTACACCAACAACCTAGCAG 960
 961 TACACCAACAACCTACAAGTACACCAACAACCTACAAGTACACCAACAACCTACAAGTACACC 1020
 1021 AACAACCTAGCAGTACACCAACAACCTAGCAGTGCACCAACAACCTAGCAGTACACCAACAAC 1080
 1081 CAGCAGTACCTCTCCACCTACAACGAGCTCAGGGCCACCTTCCACAACCTGAAAGACCAAG 1140
 1141 CAGTACACCCCGCCTACAATGTCTCCACAGCACCCACCAGCACCTCAGCACCGACCAC 1200
 1201 TGACCCTACGACCGGTCAATGCCAGGCGATTCAAGAGTACAAAGAAGCTTTTAGCAGCATA 1260
 1261 CCAGAC**CGGA**ACTGAGGA**ACTGGCAGAACCTTTTCAATGCTTGGTTGGCTGCACAGCCGTG** 1320
 1321 **A**aaacatacaaaaaccgcacttatacaattcgaaagatttgcacacatttactgcagtcgt 1380
 1381 catttttttgcttaataataacatctgtatacccgatatagcttatcaaataatagttg 1440
 1441 tactgcacagaaaaaaaaaaaaaaaaaaaaaaaaa 1471
 CAP 3'UTR reverse

Fig. 1 Nucleotide sequence of the full-length CiCAP. 5'- and 3'-Untranslated regions (UTR) are given in lower case letters. Upper case letters indicate the coding region with the first ATG and the stop codon highlighted in bold letters (arrows oligonucleotide used for cloning

procedures). The region from nucleotide 1320 to nucleotide 1450 is the probe used for the in situ hybridization (ISH) assay (CAP 3'UTR forward oligonucleotide vs CAP 3'UTR reverse oligonucleotide)

three times, and each plate contained quadruplicate cDNA samples and negative controls. The specificity of amplification was tested by real-time PCR melting analysis. To obtain sample quantification, the $2^{-\Delta\Delta C_t}$ method was used, and the relative changes in gene expression were analyzed as described in the Applied Biosystems Use Bulletin N.2 (P/N 4303859). The amount of CiCAP transcript from the various tissues was normalized to actin in order to compensate for variations in input RNA amounts. Relative CiCAP expression was determined by dividing the normalized value of the target gene in each tissue by the normalized value obtained from the untreated tissue.

Body wall explant preparation and histology

The ascidian body wall consists of the tunic, connective tissue with lacunae of the open circulatory system, muscles, and the large pharynx sac provided with vessels containing hemocytes. The tunic surface was cleaned with ethyl alcohol, and body wall fragments (200 mg), containing both tunic and pharynx tissue, were excised from the injection site at various times after injection (1–48 h).

For ISH studies, body wall fragments were fixed in Bouin's fluid (picric acid solution:formaldehyde:acetic acid 15:5:1) for 24 h, paraffin embedded, and serially cut at 6 μm (Leica RM2035 microtome, Solms, Germany).

ISH assay

To examine tissue excised from the inflamed body wall, ISH was carried out with digoxigenin-11-UTP-labeled riboprobes (1 $\mu\text{g}/\text{ml}$ final concentration). The CiCAP probe was generated by PCR-amplifying a cDNA fragment of 148 bp covering the 3' untranslated region from nt 1302 to nt 1450 of the isolated cDNA with the CAP 3'UTR forward (5'-GGCTGCACAGCCGTGA-3') and the CAP 3'UTR reverse (5'-CTGTGCAGTACAAC-3') oligonucleotides (see Fig. 1). The DNA fragment was cloned in the pCR4-TOPO vector (Invitrogen, USA). The CiCAP digoxigenin-11-UTP-labeled riboprobes were produced according to the manufacturer's instructions (Roche Diagnostics). The re-hydrated histological sections were digested with proteinase K (10 $\mu\text{g}/\text{ml}$) in phosphate-buffered saline (PBS) for 5 min, washed with PBS plus Triton X-100 (PBS-T), and treated for hybridization with 50% formamide, 5 \times SSC (1 \times SSC: 0.15M NaCl/0.015M sodium citrate, pH 7), 50 $\mu\text{g}/\text{ml}$ heparin, 500 $\mu\text{g}/\text{ml}$ yeast tRNA, and 0.1% Tween 20, at 37°C overnight. After exhaustive washes in PBS-T and 4 \times SSC (twice for 10 min), the sections were incubated for 1 h with anti-digoxigenin-Fab-AP conjugate (Roche Diagnostics) diluted 1:500 and washed in PBS-T. Finally, the sections were incubated in the 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium liquid

substrate system (Sigma-Aldrich, Germany). Color development was stopped after 30 min at room temperature. Four independent ascidians (untreated or inoculated with MS) were used as control animals.

Statistical methods

Student's *t*-test was used to estimate statistical significance. Multiple comparisons were performed with a one-way analysis of variance, and the different groups were compared by using Tukey's *t*-test. Standard deviations were calculated from four experiments. $P < 0.01$ was considered as being statistically significant.

Results

CiCAP is a member of the CAP-superfamily protein

A PCR-based subtractive hybridization strategy from the naive and inflamed pharynx of *C. intestinalis* allowed the isolation of a new LPS-induced form of CAP protein (CiCAP). 5' and 3' RACE was used to identify full-length mRNAs, and sequence analysis showed a 1471-nt cDNA with 5' (79 nucleotides) and 3' (150 nucleotides) untranslated regions (see Fig. 1 for details). A BLAST search in the Ensembl genome browser identified the predicted gene ENSCING00000004464 localized on chromosome 8q: 2,547,479–2,550,637. The cDNA encodes a 413-amino-acid protein with a deduced molecular size of 43895 Dalton (Fig. 2). In particular, *in silico* analysis showed that the CiCAP contained a 21-amino-acid-long signal peptide and displayed several CAP-superfamily domains identified in the 61–240 tract: the CiCAP3 motif [HNKFR] (63–67), the CiCAP4 motif [GENL] (115–118), the CiCAP1 motif [GHYTQQVWASTYKIGC] (161–176), and the CiCAP2 motif [MVACQYGGPNL] (193–204). In addition, a GLIPR1 motif [CX₂CX₅CX₄CX] was present in the 225–240 region. Sequence analysis showed a highly conserved "PY" dipeptide (amino acids 117–118) characteristic of the CAP-superfamily protein. Finally, the CiCAP C-terminal domain contained several threonine residues considered to be putative glycosylation sites in the T-N-TTT-N-TP repeat sequences (Fig. 2).

Molecular phylogenetic analysis

A similarity search with the FASTA algorithm showed a significant homology of CiCAP with vertebrate CAP-superfamily proteins. In particular, the amino-acid sequence 1–240 showed the highest similarity (61.4%) and identity (39.1%) to human GLIPR1 (P48060; Fig. 3a), and the amino-acid sequence 240–413 showed a significant similarity

Fig. 2 cDNA-deduced amino-acid sequence of the CiCAP protein. Locations of putative structural domains are based on the Predict Protein algorithm. The putative transmembrane domain is indicated in *italics*. Asterisks show the ten characteristic CAP-family cysteine residues, *double-underlined amino acids* the conserved “PY” CAP dipeptide, *boxed amino acids* the CAP domains and the GLIPR1 domain [CX₂CX₅CX₄CX]. The conserved amino acids within the domains are in *bold*. The *underlined threonine residues* are the putative glycosylation sites in the T-N-TTT-N-TP repeat sequences

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1      Transmembrane domain                                50
MDSKLVHTFA VLLLLLASFT NAI EWSE SIN AEEAHQR SKR SPDQLLETVV

51
LTQAEKDQIV AL CAP3HNKFRSDE MASNMLAMSW DNQIANFASS YISNCF*SHS

101
SSAERSRHPI FSYI CAP4GENLGV TVSSSSTRSR GFII RVNQLW YDEINDYTYS

151
IFTCAAGKAC CAP1GHYTQQVWAS TYKIGCGAAY *CARGNGGSGY QI CAP2MVACQYGP 200
* * * * * * * *

201
GGNLLNTNVS PRRVGQPYNK NGAP GLI-PR1 domainCSECSS ADTCVNNLCM NLARGDVIPT 250
* * * * * * * *

251
NSTPTTTSTP TTTSTPTTTS TPTTTSTPTT TSTPTTTSTP TTSSTPTTTS 300
* * * * * * * *

301
TPTTTSTPTT TSTPTTSSTP TTSSAPTSS TPTTSSTSP TTSSTGPPSTT 350
* * * * * * * *

351
ERPSSTPPPT MSSTAPTSTS APTTDPTTGQ CQAIQEYKEA LAAYQTEL RN 400

401      413
WQNLFNAWLA AQP

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(74.5%) and identity (53.8%) to the collagen-binding adhesin of *S. mutans* (B9A889; Fig. 3b). The CiCAP amino-acid sequence was aligned with the vertebrate and invertebrate members of CAP-superfamily proteins (CRISP, CRLD, CAPLD, GLIPR1, GLIPR2, PI15 and PI16). Figure 4 shows the phylogenetic tree constructed by the neighbor-joining method. Four main clusters were found. The first includes PI15, CAPLD2, and CRLD2 vertebrate proteins, the second contains invertebrate Ag5 and vertebrate PI16 proteins, the third includes CiCAP and the GLIPR1 human protein, and the last includes the CRISP vertebrate proteins. CiCAP appears to be most closely related to the human GLIPR1 protein.

Quantitative mRNA expression analysis by real-time PCR

Quantitative mRNA expression was examined, by real-time PCR analysis, in the pharynx excised from ascidians after LPS inoculation. In the inflamed pharynx, the expression pattern, compared with that of a housekeeping gene, showed an enhanced level in CiCAP mRNA expression after stimulation. At each time point (0, 1, 2, 4, 8, 12, 24, 48 h), the gene expression was evaluated in the pharynx from four ascidians. The LPS challenge up-regulated the gene activity, and the CiCAP mRNA level appeared to be increased at 4 and 8 h post-injection, reaching the highest peak at 8 h; expression decreased at 12 h and further increased at 24 h (Fig. 5). As a control for the inducing effect of LPS, the same number of MS-injected ascidians

were analyzed and compared with the LPS-injected ascidians. At 8 h and 24 h post-injection of MS, when the gene expression attributable to LPS treatment reached a peak, a significantly lower activation was detected (see Fig. 5).

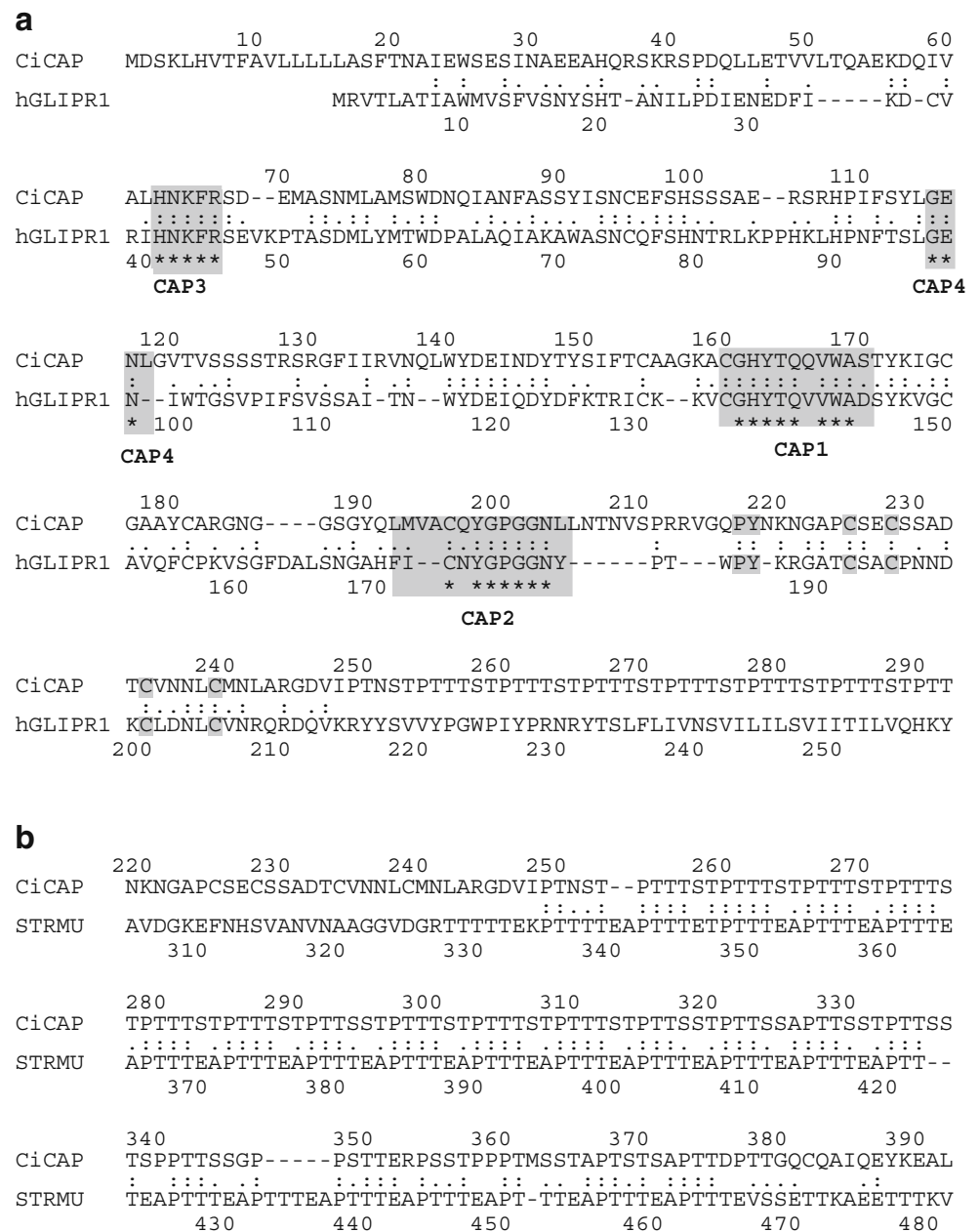
ISH assay

At 4–8 h after LPS inoculation, ISH analysis of transverse histological sections of the body wall showed numerous CiCAP-expressing hemocytes in the pharynx bars (Fig. 6a, c). In the corresponding sections from naive ascidians, a few positive haemocytes could be seen (Fig. 6b, d). Stigmata epithelium and associated hemocytes expressed CiCAP mRNA only after LPS activation (Fig. 6e); no expression was observed in untreated animals (Fig. 6f). Under the epidermis lining the tunic, the connective tissue lining the hemolymph lacunae frequently contained CiCAP-expressing cells as an effect of the LPS inoculation, whereas positive cells were rare in naive ascidians (Fig. 6g, h, respectively). In inoculated tissue, positive hemocytes appeared to be amoebocytes (Fig. 6g, inset). Controls with the sense-strand probe were negative, both in tissues and in circulating hemocytes (Fig. 6a–c, g, insets).

Discussion

The innate immune system is the first line of inducible host defense against bacterial, fungal, and viral pathogens.

Fig. 3 a Alignment of the amino-acid sequence of the CiCAP of *C. intestinalis* with human glioma pathogenesis-related 1 (*GLIP1*, P48060). **b** Alignment of the amino-acid sequence of the CiCAP of *C. intestinalis* with the collagen-binding adhesin of *Streptococcus mutans* (*STRMU*, B9A889). Gray-shaded boxes show CAP motifs, asterisks conserved amino acids within the domains, colons conserved residues, dots chemically similar residues, dashes gaps introduced for maximal alignment



Because both invertebrates and vertebrates respond to substances derived from pathogens, the identification of common patterns of activation of immune genes may shed some light on the action and evolution of the immune system.

CAP proteins represent a widely distributed family of proteins involved in both the innate and adaptive immune responses (Gibbs et al. 2008). Several components of this superfamily have been identified mainly in vertebrates, with few data existing on their role in invertebrates. We have used a subtractive hybridization strategy for selective amplification of differentially expressed sequences allowing the isolation of a CAP transcript from the *C. intestinalis* pharynx after LPS inoculation. This strategy has enabled us

to isolate a novel form of a CAP protein (CiCAP) that displays a modular structure with a NH₂ terminal region having features in common with other members of the CAP superfamily. In this region, CiCAP displays highly conserved motifs in the CAP domain (CAP1-4), which has been shown to be buried within the core of the tertiary structure of other CAP proteins, and which is probably involved in maintaining the overall structure of the protein (Shikamoto et al. 2005). Furthermore, an additional C-terminal extension of about 160 amino acids, with a significant similarity to the collagen-binding adhesin of *S. mutans* has been identified (Sato et al. 2004). This kind of architecture has not been found in any other protein belonging to the CAP superfamily so far. Domain organization analysis and

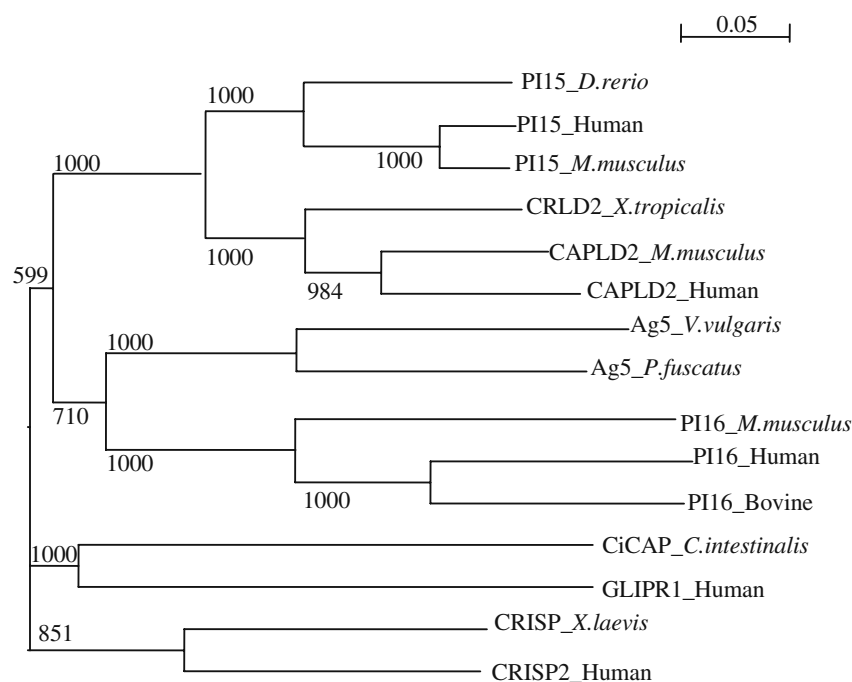


Fig. 4 Phylogenetic tree of deduced amino-acid sequences of CiCAP of *C. intestinalis* and of vertebrate and invertebrate members of CAP superfamily. The tree was constructed by the neighbour-joining

method and bootstrap analysis (numbers percentages of 1000 bootstrap replicates in which the same internal branch was recovered). Bar 0.05 (number of amino-acid residues substitutions for site)

alignment of the NH₂ terminal region of the CiCAP to CAP vertebrate members has shown that this protein originated from a common ancestor gene, and it appears to be related to the vertebrate GLIPR1 proteins. In this respect, the CiCAP protein might represent a case of opposing evolutionary

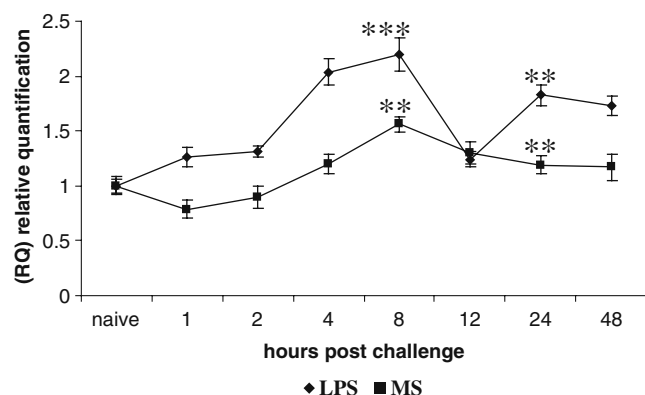
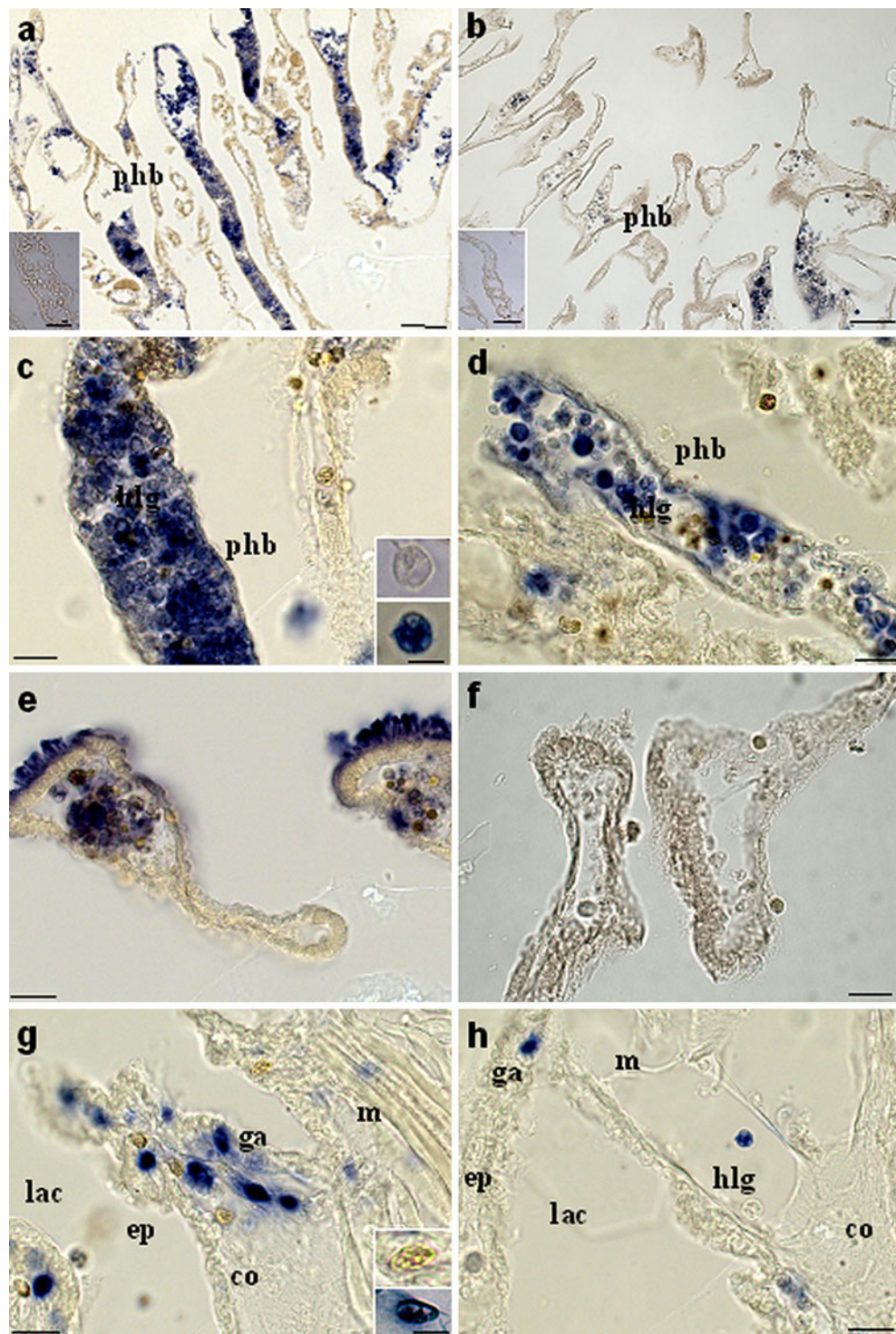


Fig. 5 Quantitative real-time PCR. Relative quantification (RQ) of the induction of the CiCAP gene compared with an internal control. Numbers on the x-axis indicate the time after injection of the animal with LPS or marine solution (MS) and RNA sampling. The data on the y-axis show the relative increase of the transcriptional activity. Significance was evaluated by comparing the values with the expression level of untreated pharynx from four ascidians. *** $P < 0.01$, **** $P < 0.001$. Multiple comparisons of the time-course profiles were performed by a one-way analysis of variance, and the different groups were compared by the Tukey *t*-test

patterns: the first leading to the strong conservation of several sequence motifs probably responsible for the three-dimensional structure of the protein, and the second leading to different C-terminal extensions that might justify independent biological functions. Indeed, genome sequencing of many organisms has revealed that species differ in the number and types of possessed genes. New genes arise at various points in evolutionary time, as a result of the juxtaposition of various pre-existing exons in new combinations (Gilbert 1978), by mutational modifications of duplicate genes and retroposition (Long et al. 2003), thereby conferring novel functions enabling organisms to tackle environmental challenges.

The involvement of CiCAP in *C. intestinalis* innate immunity responses has been detected by real-time PCR analysis of the inflamed pharynx. As previously reported, *C. intestinalis* tumor necrosis factor α (CiTNF α), the type IX-like collagen α -chain, collectins, and complement pathway components can be up-regulated following LPS challenge of the pharynx tissues (Parrinello et al. 2008, 2010; Bonura et al. 2009; Vizzini et al. 2008). In this study, we have found that LPS inoculation enhances the expression of CiCAP mRNA. In the pharynx, the CiCAP mRNA level increases in a statistically significant fashion at 4 h and 8 h, then decreases at 12 h, followed by a further increase at 24 h. A similar time course response characterizes the type IX-like collagen α -chain and *C. intestinalis* mannose-binding lectin

Fig. 6 ISH assay of transverse sections of the body wall of *C. intestinalis*. Histological sections from ascidians at 4–8 h after LPS inoculation. **a** Pharynx bars (*phb*). Bar 100 μm . *Inset* Section treated with sense probe. Bar 50 μm . **c** Pharynx bars (*phb*) and hemocyte with large granules (*hlg*). Bar 40 μm . *Inset* Hemocyte with large granules treated with sense probe (*top*) and with anti-sense probe (*bottom*). Bar 10 μm . **e** Stigmata. Bar 40 μm . **g** Connective tissue (*co*) under the epidermis (*ep*) with lacunae (*lac*) of the open circulatory system, granular amebocytes (*ga*), and muscles (*m*). Bar 40 μm . *Inset* Granular amebocyte treated with sense probe (*top*) and with anti-sense probe (*bottom*). Bar 10 μm . Histological sections from naive ascidians. **b** Pharynx bars (*phb*). Bar 100 μm . *Inset* Section treated with sense probe. Bar 50 μm . **d** Pharynx bars (*phb*) and hemocyte with large granules (*hlg*). Bar 40 μm . **f** Stigmata. Bar 40 μm . **h** Connective tissue (*co*) under the epidermis (*ep*) with lacunae (*lac*) of the open circulatory system, granular amebocytes (*ga*), hemocyte with large granules (*hlg*), and muscles (*m*). Bar 40 μm



(Bonura et al. 2009; Vizzini et al. 2008). The meaning of the second increase in the expression profiles of inflammatory molecules remains to be clarified. One possible explanation is a delayed inflammatory response by distinct tissues of the pharynx; this might include hemocytes inside the vessels and epithelial tissues. On the other hand, we do not know the regulatory effect of other inflammatory molecules in re-

sponse to external signals that might explain the second wave of CiCAP upregulation at 24 h post-injection. Moreover, we cannot exclude the possibility that the decrease in expression is attributable to cell death after LPS injection, whereas the subsequent increase might be attributable to hemocyte proliferation and the appearance of new CiCAP-expressing circulating cells.

Based on previously published papers (Bonura et al. 2009; Vizzini et al. 2008), ISH has been performed in order to identify challenged hemocytes inside the pharynx vessels at the time course corresponding to the main expression peak. At 4 h after inoculation, LPS induces a prompt *CiCAP* upregulation in hemocytes with large granules contained both in vessels and in associated with connective tissue lining the hemolymph lacunae of the open circulatory system. Although a cell count has not been performed, the *CiCAP*-expressing hemocytes seem to be more abundant than those found in naive ascidians. Interestingly, the epithelium of pharynx stigmata expresses *CiCAP* only after LPS challenge. This result is in agreement with a recent paper on *CiTNF α* expression (Parrinello et al. 2010) disclosing that several regions of the pharynx epithelial tissues are probably involved in the inflammatory response.

In conclusion, we have described the isolation of a novel *CAP* gene that is up-regulated after LPS injection in the body wall of *C.intestinalis*. The *CiCAP* expression profile together with studies on tissue localization suggest a possible role for this protein in the ascidian innate immune responses.

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