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# Structural but not functional conservation of an immune molecule: a tachylectin-like gene in *Hydractinia*

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**Abstract**

Tachylectin-related proteins are a recently characterized group of pattern recognition molecules, functioning in the innate immunity of various animals, from the ancient sponges to vertebrates. Tachylectins are characterized by six internal tandem repeats forming β-propeller domains. We have identified and characterized a tachylectin-related gene in the colonial marine hydroid, *Hydractinia echinata*. The predicted gene product, termed CTRN, contained an N-terminal signal peptide and had a well-conserved tachylectin-like structure. RT-PCR analyses revealed only post-metamorphic expression while no mRNA was detected during embryonic development or in planula larvae. Exposure of colonies to LPS under conditions known to activate an immune response in *Hydractinia* did not result in upregulation of the gene. In situ hybridization analysis of metamorphosed animals detected *CTRN* transcripts only in a small subpopulation of neurons and their precursor cells, localized in a ring-like structure around the mouth of polyps. The same ring-like structure of *CTRN* expressing neurons was also observed in young polyp buds, predicting the position of the future mouth. This type of expression pattern can hardly be attributed to an immune-relevant gene. Thus, despite high structural similarity to tachylectins, this cnidarian member of this group seems to be an exception to all other tachylectins identified so far as it seems to have no function in cnidarian innate immunity.
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*Keywords:* *Hydractinia*; In situ hybridization; Lectin; Evolution; Neuron; Invertebrate immunity

## 1. Introduction

Tachylectin-related proteins are a recently identified group of lectins [1]. Initially found in horseshoe crabs [2], in slime molds [3], and later also in vertebrates [4,5], tachylectins are structurally characterized by having six tandem repeats of 31–37 amino acids in length, forming a beta-propeller structure [6], and are related structurally to mammalian fibrinogen [7]. In the horseshoe crab, *Tachypleus* (to which the name of these lectins refers) a variety of tachylectins were identified in haemocytes and plasma; they have been characterized biochemically [1,2,8,9] and found to bind various components of bacterial cell walls and, thus, to function in the crab's innate immunity.

*Abbreviations* EST, expressed sequence tags; LPS, lipopolysaccharid; RT-PCR, reverse transcription-polymerase chain reaction.

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In the slime mold *Physarum polycephalum*, tachylectin-related molecules, termed tectonins, were proposed to facilitate phagocytosis of bacteria, also by binding to bacterial cell wall components [3]. In vertebrates, tachylectin-like proteins were identified in a differential gene expression analysis aimed at finding immune-relevant genes [5] and as bacteria-binding proteins in fish eggs [4], indicating that they function in immunity also in vertebrates.

Interestingly, tachylectins' occurrence and function in innate immunity are conserved in metazoans. A single copy of a tachylectin-related gene was recently identified in the sponge *Suberites dumuncula* [10]. The product of this gene binds LPS and is upregulated upon exposure of the animals to LPS. Since sponges are the earliest diverging metazoans, tachylectins appear to be an ancient, highly conserved group of pathogen associated molecular pattern (PAMP)-binding proteins, functioning in innate immunity already in the metazoan ancestor, and perhaps earlier.

In an attempt to find immune-relevant molecules in the phylum Cnidaria, we have screened our recently established EST library, constructed of RNA from the colonial marine hydroid, *Hydractinia echinata*, and identified a tachylectin-like sequence. Surprisingly, molecular characterization of the full-length cDNA revealed that, in contrast to other tachylectin-related molecules, and despite highly conserved primary structure, this *Hydractinia* tachylectin-related gene does not appear to have any function in immunity.

## 2. Materials and methods

### 2.1. Animals

Wild type colonies of *H. echinata* were sampled at the Island of Sylt (North Sea) and cultured in the laboratory in artificial seawater as described [11]. Fertilized eggs were collected daily following light induced spawning and allowed to develop until the planula larva stage was reached within about three days. Larvae were induced to undergo metamorphosis by a pulse treatment of 100 mM CsCl in seawater [12]. They were then positioned on glass coverslips, where they completed metamorphosis within 1 day. Young colonies, about 7–10 days old, were exposed to 100 μg/ml *Escherichia coli* LPS (Sigma) for 1 h. This treatment was already shown to activate a TRAF/NF-κB-like pathway in the animal (Mali and Frank unpublished).

### 2.2. Tachylectin-related sequence from Hydractinia

A cDNA fragment of 504 bp, displaying significant sequence similarity to tachylectins of other organisms, was identified in our EST database. The cDNA library used for sequencing was constructed of pooled RNAs from animals in various developmental stages, and also animals challenged by LPS and allogeneic grafts. The library was arrayed in 384 well plates and following the identification of the clone we sequenced it from both ends using vector-specific primers to obtain the full-length cDNA of the gene. We have termed the gene *CTRN* for cnidarian tachylectin-related gene in neurons.

### 2.3. Sequence analysis

The sequences of eight tachylectin-related proteins were obtained from online databases and aligned with the predicted protein sequence of *CTRN* using Clustal W at the European Bioinformatics Institute's web site (http://www.ebi.ac.uk/clustalw/). The presence of a signal peptide was predicted using the SignalIP 3.0 software at http://www.cbs.dtu.dk/services/SignalP/.

### 2.4. RNA extraction and RT-PCR

Animals at the following developmental stages were used for RNA extraction: early embryos (1–5 h post-fertilization), post-gastrulation embryos (about 48 h post-fertilization), planula larvae (3 days post-fertilization), early metamorphosing animals (3–6 h post-CsCl treatment), primary polyps (24 h post-CsCl treatment), and mature colonies. In addition, we also extracted RNA from animals following a 1 h exposure to LPS, immediately after exposure, 1, 3, 6 and 12 h later, respectively. Total RNA was extracted using acid guanidinium thiocyanate [13] and its quality and quantity assessed on a 1.2% formaldehyde agarose gel. Two μg total RNA per developmental stage were reverse transcribed separately using PowerScript reverse transcriptase (Life Technologies) in a 20 μl reaction tube. Two microliter of 1:10 diluted cDNA

were used for RT-PCR using *CTRN* specific primers F 5′-attgttggggatctccttcgtga-3′ and R 5′-ctcacttgtttcaatcctcctga-3′ and *Hydractinia* actin primers F 5′-aaaccctttccaaccatcctt-3′ and R 5′-tggg ccagattcatcgtattct-3′ as control for equal amounts of cDNA load. Following calibration of the reaction to identify the log phase, 25 PCR cycles were performed. PCR products were resolved on a 1.2% agarose gel, stained with ethidium bromide and photographed.

### 2.5. In situ hybridization

The following oligonucleotides were synthesized to serve as PCR primers to amplify a 360 bp fragment from a cDNA template: F 5′-attgttggggatctccttcgtga-3′ and R 5′-ctcacttgtttcaatcctcctga-3′. PCR product was cloned into a pGEM-T vector (Promega) and sequenced. The vector was linearized by *Not*1 and *Nco*1 restriction enzymes to create templates for sense and antisense RNA probes synthesis, respectively. Digoxigenin labelled RNA probes were synthesized using the digoxigenin RNA labelling kit according to the manufacturer's recommendation (Roche). The probes were evaluated on a formaldehyde agarose gel and frozen at −70 °C until used. In situ hybridization was performed as described [14] on metamorphosed animals only (see below).

## 3. Results

The cDNA of *CTRN* contained an open reading frame (ORF) of 837 bp corresponding to 279 amino acid residues with a predicted molecular mass of 30 kDa. The nucleotide sequence has been deposited in EMBL Bank under accession number AJ920381. The ORF contained a signal peptide at its N-terminus with the most probable cleavage site after position 15 (predicted molecular mass of secreted protein 28.3 kDa). The primary structure of the protein was most similar to horseshoe crab tachylectin 1, to tectonin 1 from the slime mold *P. polycephalum*, and to the lectin from the sponge *S. dumuncula* (36, 35 and 33% amino acid identity, respectively: Fig. 1). CTRN included tachylectin-typical six tandem repeats of 31–33 amino acid residues (Fig. 2) with constant intervals of 11 amino acids between each repeat except of the interval between repeats 1 and 2 which was 17 amino acids long and the fragment between the sixth repeat and stop codon which was six amino acids long. All inter-repeat sequences, however, shared several amino acids at their N-terminus.

RT-PCR performed along a developmental gradient of *Hydractinia* revealed that the gene was only expressed in post-metamorphic life (Fig. 3). *CTRN* mRNA levels in early embryos and planula larvae were undetectable by RT-PCR. Based on the RT-PCR results, we performed in situ hybridization only with metamorphosed animals. The analysis visualized a ring-like structure around the mouth of polyps (Fig. 4). cRNA probes of *CTRN* did not find any hybridization target, except to this location. Moreover, in young polyp buds on stolons, in situ hybridization with *CTRN* cRNA probes also stained a ring-like structure of cells around the predicted position of the future mouth opening. This was already evident in very early buds when only a slight swelling of the stolon indicated the position of the future polyp. Higher magnification of the areas expressing *CTRN* showed that transcripts were only present in neurons and in their precursor interstitial cells. No other cell type was found to express the gene.

To study a possible modulation of *CTRN*'s transcriptional activity during an immune response we also performed RT-PCR on LPS challenged animals to mimic a gram-negative bacterial infection. The results show that *CTRN*'s expression is independent of LPS exposure (Fig. 5). The *CTRN* transcripts showed a constant level for all time points following LPS exposure and the expression was equal to the control animals.

## 4. Discussion

We have cloned and characterized the first cDNA of a tachylectin-related molecule in the phylum Cnidaria. The predicted 279 amino acids long protein had a N-terminal signal peptide and the putative secreted protein contained six tandem repeats, characteristic of tachylectins and related proteins. Alignment of CTRN with tachylectins, tectonins and some other related proteins showed that the core of the *Hydractinia* protein is well conserved (Fig. 1). Whereas cnidarian proteins often show higher similarity to

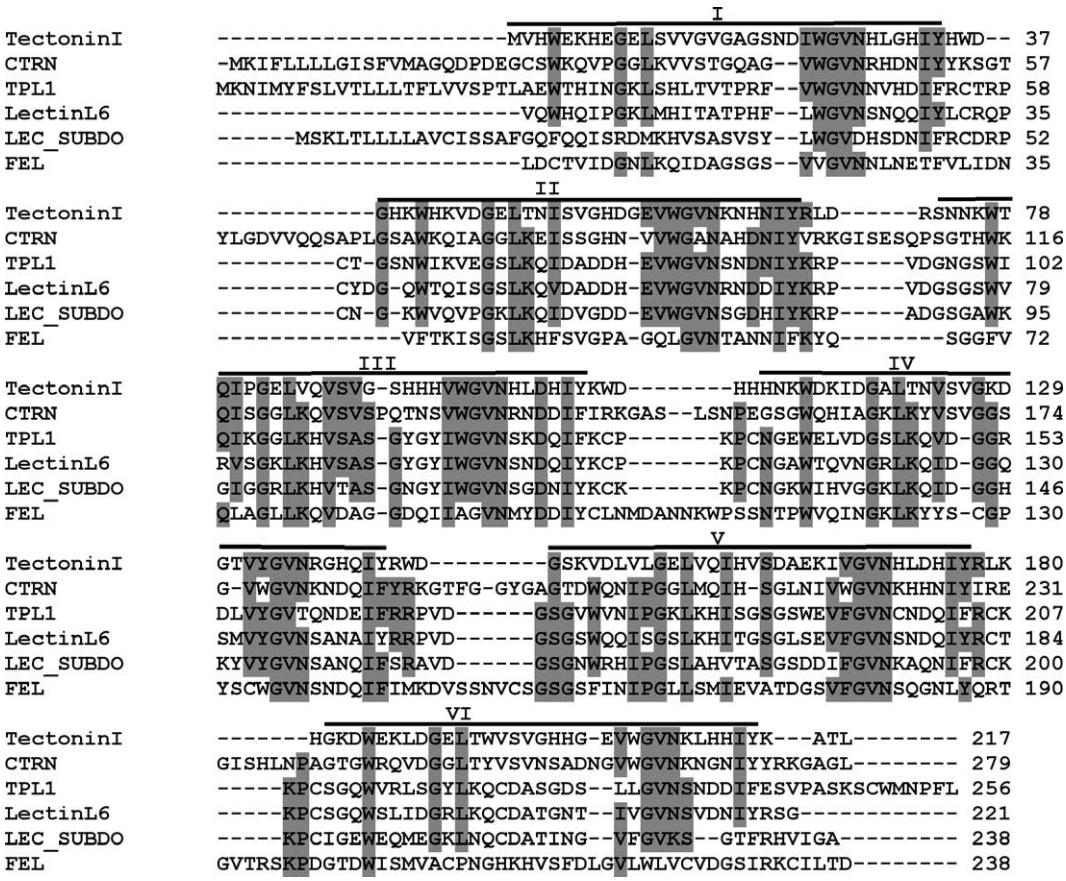


Fig. 1. Alignment of CTRN with related proteins from other organisms. Similar amino acids are highlighted. The six tandem repeats are marked by a line and numbered as I–VI. Abbreviations and accession numbers: tectonin1 from *Physarum polycephalum* (Genbank accession number: AF041455); CTRN, cnidarian tachylectin-related gene in neurons (Genbank accession number: AJ920381); TPL-1, *Tachypleus* plasma lectin 1 (Genbank accession number AAF74773); Lectin-L6 from *Limulus polyphemus* (Genbank accession number A56941); LEC_SUBDO, lectin of *Suberites dumuncula* (Genbank accession number CAD79378); FEL, fish egg lectin from *Cyprinus carpio* (Genbank accession number P68512).

their vertebrate orthologues than to other invertebrate counterparts [15–17], this was not the case here; the primary structure of the *Hydractinia* tachylectin homologue, CTRN, was most similar to horseshoe crab tachylectins, to tectonins from slime molds, and to a sponge lectin. The overall low number of characterized proteins in this family may be the cause and the picture may change after identification of more vertebrate tachylectin-related proteins in the future.

Internal alignment of the six tandem repeats of CTRN revealed a highly conserved structure (Fig. 2). A glycine residue was always the first in the repeat, and the domain contained several consensus sequences (e.g. VWGVN). Similar primary structures from other tachylectin-like proteins were proposed to form β-propeller domains. The inter-repeat stretches of CTRN also showed striking similarity among

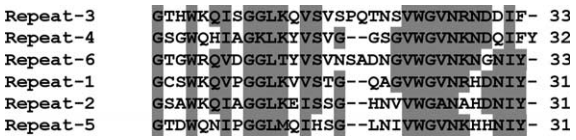


Fig. 2. Alignment of the six internal repeats of CTRN. Similar amino acids are highlighted.


Fig. 3. RT-PCR of *CTRN* in development. From the left: early development (1–5 h post-fertilization), pre-planula (48 h), planula larva, 3 h post-metamorphosis induction, primary polyp, and mature colony. *CTRN* is only expressed following metamorphosis.

themselves, being mostly 11 amino acids long (except interval #1).

Tachylectins in other organisms are immune molecules. In horseshoe crabs, where they were first identified, they bind to pathogen associated molecular patterns (PAMP) of gram-positive and gram-negative bacteria with different specificity spectra. Some horseshoe crab tachylectins were isolated from hemocytes that function as immune cells [2]. Others were isolated from the hemolymph [9], in which case it is unclear which cells synthesize the protein. Tectonins, the tachylectins orthologues from slime molds, are located on the exterior surface of the plasma membrane. They are thought to function in phagocytosis of bacteria. In sponges, a tachylectin-related protein was isolated and found to bind LPS and to be upregulated following exposure to LPS [10]. Vertebrate tachylectin-related proteins were isolated from, e.g. fish eggs [4], and from liver, head kidney, and spleen of salmon following a bacterial challenge [5]. Thus, it appears that tachylectins also play a role in vertebrate immunity.

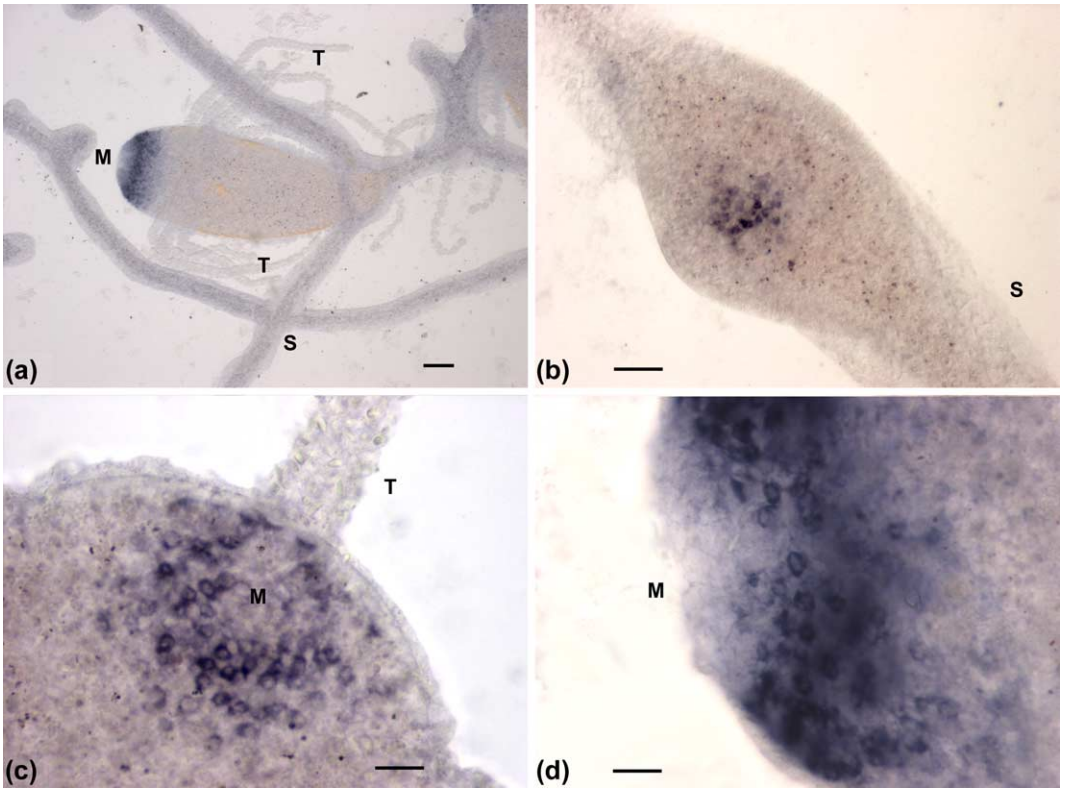


Fig. 4. In situ hybridization of *CTRN* cRNA in *Hydractinia*. (a) An overview of a young colony. *CTRN* positive cells form a ring around the mouth. (b) An early polyp bud developing from a stolon. *CTRN* expressing neurons form a ring-like structure around the area of the future mouth. (c) A higher magnification of the mouth region of a polyp showing *CTRN* expressing neurons. (d) The same like the previous picture, view from the side. M, mouth; S, stolon; T, tentacle. Scale bars approximately 50 μm.

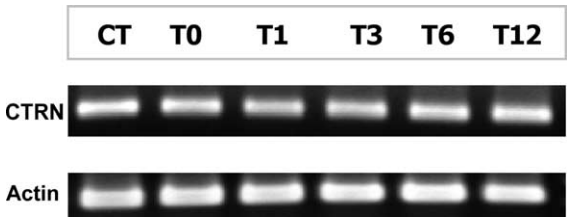


Fig. 5. RT-PCR of a colony treated with 100 μg/ml *E. coli* LPS for 1 h. CT, control; T, time in hours following treatment. *CTRN*'s expression is not altered by LPS treatment.

Given the apparent conserved structure and function of tachylectins in the animal kingdom, one would expect a homologous gene in cnidarians to have a similar function too. We have addressed this point using three approaches: first, we performed RT-PCR along a developmental gradient of the animal. This experiment showed hat *CTRN* was only expressed following metamorphosis. Its mRNA levels during embryogenesis and in the larval stage were undetectable. Second, we exposed animals to LPS under conditions that, according to our experience, activate an immune response in *Hydractinia*. We then extracted RNA from colonies and subjected it to RT-PCR, revealing that *CTRN* expression levels were not modulated by LPS. This does not role rule out induction by an unknown elicitor. Finally, we performed in situ hybridization to localize tissues and cells transcribing the gene. These results showed that *CTRN* had a defined, restricted spatial expression; its mRNA was found solely in ectodermal tissues, limited to particular neurons and their precursor cells around the mouth.

The cnidarian immune system is poorly studied, especially at the cellular and molecular levels. Based on cnidarian morphologies and life histories (see below), however, it is clear that these animals must have very efficient defense mechanisms to cope with seawater-borne pathogens. Cnidarians have a rather simple morphology with only few differentiated organs and (morphologically characterized) cell types. Their body wall is composed of an epithelial bilayer, ectoderm and endoderm, separated by a basal lamina, the mesoglea. *Hydractinia* colonies have also a simple circulatory system. A colony is composed of many, genetically identical polyps that share one gastrovascular space via a network of tubes, the stolons. The lumen of the stolons is filled with seawater and secretory products of the endoderm and is connected to the outside via the numerous polyps' mouths, which also serve as an anus. The ectoderm of the polyps is directly exposed to the surrounding seawater, while a chitineus cuticle covers the outside of the stolonal compartment. Keeping the colony's morphology and life history in mind, a putative immune gene in this animal may be expressed by any tissue (there are no known specialized immune cells in cnidarians). However, expressing cells must be distributed in a way ensuring efficient accessibility of their product to potential pathogens. An immune molecule in *Hydractinia* is likely to be upregulated following metamorphosis. Earlier life stages are also exposed to the natural seawater environment; however, the potential risk pathogens represent would increase upon completion of metamorphosis and the associated loss of mobility. Also, all pre-metamorphic stages do not feed and, thus, are less likely to become infected. A post-metamorphic expression was observed for a chitinase, a putative immune molecule [18]. Finally, an antimicrobial lectin could be expressed 'upon demand', i.e. following exposure to a potential pathogen or PAMPs, like other tachylectins.

Taken together, although immune-relevant molecules in *Hydractinia* could theoretically display many types of expression pattern, *CTRN*'s behaviour in this regard was clearly not among them. The gene was expressed by only few neurons around the mouth. A protein secreted by these neurons would be 'trapped' in the interstitial space without access to potential pathogens. *CTRN*'s post-metamorphic expression is not indicative and could also be attributed to many developmental genes. Hence, despite the lack of a direct functional study we can conclude that this cnidarian homologue of tachylectins, proteins that appear to function in immunity in all other studied animals, does not fulfil any direct role in the innate immune response of *Hydractinia*. The gene may function during neuronal development in signalling to migratory neuronal precursors, or in neurite path finding. A developmental role has also been assigned to several immune molecules, with *Drosophila* Toll as a prominent example [19]. CTRN may fit into this paradigm.

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