A putative helical cytokine functioning in innate immune signalling in

*Drosophila melanogaster*

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Abstract

In invertebrates and vertebrates, innate immunity is considered the first line of defense mechanism against non-self material. In vertebrates, cytokines play a critical role in innate immune signalling. To date, however, the existence of genes encoding for invertebrate helical cytokines has been anticipated, but never demonstrated. Here, we report the first structural and functional evidence of a gene encoding for a putative helical cytokine in *Drosophila melanogaster*. Functional experiments demonstrate that its expression, as well as that of the antimicrobial factors defensin and cecropin A1, is significantly increased after immune stimulation. These observations suggest the involvement of helical cytokines in the innate immune response of invertebrates.

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1. Introduction

Numerous reports documenting the presence of cytokine-like molecules in invertebrates have been recently reviewed [1]. Immunocytochemical approaches have demonstrated in different invertebrate species belonging to Mollusca, Insecta, Nematoda, Annelida and Tunicata the presence of cytokine-like molecules such as interleukin (IL)-1, IL-2, IL-6, IL-8, tumor necrosis factor-α, platelet-derived growth factor (PDGF)-AB, and transforming growth factor (TGF)-β.

Mammalian cytokines affect invertebrate immune functions, e.g. cell motility, chemotaxis, phagocytosis and cytotoxicity [1]. The cytokines PDGF-AB, TGF-β1 and IL-8 induce cell shape changes in molluscan circulating immunocytes throughout the phosphatidylinositol and cAMP pathways [2,3]. PDGF-AB and TGF-β1 also provoke a partial inhibition of cell death in the IPLB-LdFB insect cell line, activate the phosphatidylinositol 3-kinase, protein kinase A and protein kinase C pathways, promote the wound healing process, and intervene in stress response [2,4]. Furthermore, research on IL-2 and corticotrophin-releasing hormone has suggested the presence on molluscan immunocyte membrane of a receptor able to bind both cytokines and neuropeptides [5]. Altogether morphological and functional studies show a close overlap between the humoral factor effects in invertebrates and vertebrates.

Notwithstanding these findings, opposing views persist regarding the presence of cytokines in invertebrates. Mainly on the basis of the limited molecular biological data, it has been hypothesized that invertebrates lack genes orthologous to mammalian cytokines [6]. Though several proteins functioning as cytokines have been found in invertebrates [7–11], conventional bioinformatics approaches cannot detect invertebrate genes orthologous to mammalian helical cytokines, possibly due to evolutionary divergence.

In vertebrates, helical cytokines are a family of structurally related genes, including well-characterized proteins such as IL-2, IL-6, interferon α-1 and granulocyte-macrophage colony-stimulating factor, and they are distinguished by their unique four helical bundle topology [12]. In this investigation, we utilized a specialized bioinformatics method specifically devel-
oped to recognize the helical cytokine fold from sequence [12]. This led to the identification and isolation in the *Drosophila melanogaster* transcriptome of a sequence encoding for a protein that displays structural features indicative of a helical cytokine. This protein will be referred to here as DHF (*Drosophila Helical Factor*). In addition to the bioinformatics evidence, we have determined that the expression of DHF following immune stimulation implicates the molecule in insect innate immune response.

2. Materials and methods

2.1. Isolation and analysis of helical cytokines in *D. melanogaster*

The peptide section of the *D. melanogaster* genome BDGP4 from the Berkeley Drosophila Genome Project was downloaded from the Ensembl site ([www.ensembl.org](http://www.ensembl.org)) October 14, 2005. This section contained 7570 known *D. melanogaster* protein sequences derived from verified CDNA and EST evidence. An implementation of the QT helical cytokine fold recognition method described by Conklin [12] was applied to these data, looking for sequences which might be indicative of a helical cytokine fold. Secretory signal peptides were predicted by the QT method [12] which employs the von Heijne profiles [13], and signal peptides were also verified by the SignalP method [14] (Version 3.0 at [www.cbs.dtu.dk](http://www.cbs.dtu.dk)). Considering that many helical cytokines are glycosylated proteins, potential N-linked glycosylation sites were predicted by the NetGlyce method (Version 1.0 at [www.cbs.dtu.dk](http://www.cbs.dtu.dk)). The presence of transmembrane domains was explored with the TMHMM method (Version 2.0 at [www.cbs.dtu.dk](http://www.cbs.dtu.dk)). Coiled-coil motif prediction was performed with the Paircoil server ([paircoil.lcs.mit.edu](http://paircoil.lcs.mit.edu)).

2.2. Immune stimulation of *D. melanogaster* larvae

The flies (FBst0006971, y[1] w[+]; P [y[Scer] Scel RS crA] = y[donor] 1B Sb [1] TM6) used in this investigation were grown on *Formula4-24® Blue Drosophila* medium (Carolina Biological Supply Company, Burlington, NC 27215, USA) in an incubator at 23 ± 1 °C. It has been observed that both microinjection and pricking induce a small increase of antimicrobial peptides [15]. Therefore, in consideration of the role that mammalian cytokines play also in wound repair and in the consideration of the role that mammalian cytokines play also in wound repair and in the regulation of mammalian immune responses, we considered the possibility of using *D. melanogaster* larvae for this purpose. In order to demonstrate that the applied experimental conditions were able to elicit an immune response in *D. melanogaster* larvae and embryonic hemocytes, the expression of defensin [17] (F 5′-GCT TCT GCT TTT GCT CGT CT-3′ and R 5′-CCA TTT GGA GAG TAG GTC GC-3′) and cecropin A1 [18] (F 5′-ACA TCT CGG TTT TCG TCG CT-3′ and R 5′-CTT GTT GAG CGA TGA CCA GC-3′) was confirmed through sequencing performed by “Servizio Sequenziamento Automatico-Progetto Camilla” (Pomezia, Italy). Sequencing reactions were performed with primers DHF...F and DHF...R. The obtained sequences were confirmed by sequence alignment to correspond to the expected *dhf* coding sequence. In order to exclude any false positive results due to the presence of contaminating genomic DNA, negative control reactions were realized either by substituting for cDNA an equal amount of purified RNA or by replacing template with molecular biology grade water (Sigma).

2.5. Sequence amplification, sequencing and analysis of *dhf*

In order to demonstrate that the applied experimental conditions were able to elicit an immune response in *D. melanogaster* larvae and embryonic hemocytes, the expression of defensin [17] (F 5′-GCT TCT GCT TTT GCT CGT CT-3′ and R 5′-CCA TTT GGA GAG TAG GTC GC-3′) and cecropin A1 [18] (F 5′-ACA TCT CGG TTT TCG TCG CT-3′ and R 5′-CTT GTT GAG CGA TGA CCA GC-3′) was confirmed through sequencing performed by “Servizio Sequenziamento Automatico-Progetto Camilla” (Pomezia, Italy). Sequencing reactions were performed with primers DHF...F and DHF...R. The obtained sequences were confirmed by sequence alignment to correspond to the expected *dhf* coding sequence. In order to exclude any false positive results due to the presence of contaminating genomic DNA, negative control reactions were realized either by substituting for cDNA an equal amount of purified RNA or by replacing template with molecular biology grade water (Sigma).

2.3. Immune stimulation of SL2 cell line

*Drosophila* embryonic hemocytes (SL2 cell line) were maintained at 25 °C in Schneider medium (Sigma), supplemented with heat-inactivated 10% FCS. In LPS and MMS exposure experiments, 10⁶ cells were suspended in 5 ml of medium containing either 10 U/ml gel filtration purified *E. coli* LPS (Sigma) or 250 μl Lantigen B (Bruschettini). SL2 cells were subjected to a 24-h treatment, before RNA extraction. Each experiment was repeated in duplicate for three times.

2.4. Sequence amplification, sequencing and analysis of *dhf*

A coding sequence for *D. melanogaster* DHF was used to design the following pair of primers for use in RT-PCR reactions for amplification with either larval or hemocyte DNA as a template: DHF...F 5′-AGT GAA AGA AGA AGC CAG CCT TA-3′ and DHF...R 5′-AAC TGG GTG TGT AGG TGA AA-3′. Cytoplasmic actin expression was evaluated as loading control (see below). After LPS or MMS exposure, the larvae were collected, washed in sterile phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 10 mM NaH₂PO₄, pH 7.3 ±0.1), centrifuged for 3 min at 100 × g and total RNA was extracted using TRI REAGENT™ (Sigma), following the method described by the supplier. SL2 cells were centrifuged for 10 min at 800 × g in order to remove the culture medium then total RNA was extracted as indicated above for larvae.

cDNA of both larvae and SL2 cells was obtained using “RevertAid H Minus First Strand cDNA Synthesis Kit” (Fermentas AB, Vilnius, Lithuania) using 2 μg of total RNA as template. Semi-quantitative PCR reactions for *dhf* were performed with an annealing temperature of 54 °C; time of annealing was 60 s and the time of elongation was set at 60 s. The number of PCR cycles was set in order to maintain the reaction within the exponential phase, in this case between 25 and 35 cycles. Amplified fragment was extracted from agarose gel and purified with “QiAquick™ Gel Extraction Kit” (QIAGEN GmBH, Hilden, Germany). The correspondence between the expected and the amplified fragment was confirmed through sequencing performed by “Servizio Sequenziamento Automatico-Progetto Camilla” (Pomezia, Italy). Sequencing reactions were performed with primers DHF...F and DHF...R. The obtained sequences were confirmed by sequence alignment to correspond to the expected *dhf* coding sequence. In order to exclude any false positive results due to the presence of contaminating genomic DNA, negative control reactions were realized either by substituting for cDNA an equal amount of purified RNA or by replacing template with molecular biology grade water (Sigma).

3. Results and discussion

An implementation of a fold recognition method for helical cytokines [12], referred to in this study as the QT method, was performed on a database of 7570 known *D. melanogaster* protein sequences. The QT method indicated that the highest-scoring peptide in the helical cytokine search is the sequence referenced as GenPept accession no. AAF53861. This sequence was originally deposited by Jagadeeshan and Singh [19], and is annotated with the definition “olfactory-specific protein Os9”. However, subsequent searches of Entrez with
the term OS9 revealed additionally an entirely different
D. melanogaster gene named OS9, cloned by Raha and Carlson
[20] and deposited as GenPept accession no. AAB31188. The
ambiguity of the OS9 designation led us to refer to the GenPept
accession no. AAF5 3861 with the new name DHF (Drosophila
Helical Factor). DHF is a peptide of 214 amino acids, and the
QT method predicts that this sequence has a helical cytokine
fold with four core amphipathic helices placed as indicated in
Fig. 1. The overall secondary structure has in addition been
verified by several other prediction methods to be predomi-
nately helical (Fig. 1). The sequence is not predicted to contain
transmembrane domains and this is consistent with the QT
prediction of a secreted globular helical protein. The putative
signal peptide (Fig. 1) comprising Met1 through Ser32 was
predicted by the QT method, which employs the von Heijne
profiles [13], and was also verified by the SignalP method.
The DHF protein without the signal peptide has a predicted pl/
Mw of 6.72/21000. The dhf gene resides on D. melanogaster
chromosome arm 2 L and its coding sequence resides on 2
exons separated by a short intron of 63 bp. The exon/intron
junction is within the predicted signal peptide region and is in
phase 0. In terms of homology, BLAST searches reveal no
similar sequences other than DHF orthologues from other
species of the genus Drosophila; particularly, D. simulans
(95% identity), D. yakuba (87%) and D. pseudoobscura (61%).
The DHF protein is predicted by the NetNGlyc method to have
2 N-linked glycosylation sites at Asn76 and Asn160 (Fig. 1).
The glycosylation motif NX[S/T] is conserved at corresponding
positions within D. simulans and D. yakuba; in D. pseudo-
obscura the Asn76 site is not conserved, though an alternative
nearby glycosylation site is predicted. The DHF peptide is not
predicted by Paircoil to contain extended coiled-coil amphi-
pathic helical motifs incompatible with the loop topology of the
helical cytokines.

All fold recognition approaches, when calibrated for a zero
false negative rate, will produce false positive results: for the
QT method these will be proteins that are predominately helical
in structure though without a helical cytokine topology. Within
the 7570 known D. melanogaster proteins, we find two secreted
proteins with high QT scores (though below the score of DHF):
the products of genes Acp26Aa (accessory gland-specific
peptide 26Aa), and Obp19a (odorant-binding protein 19a).
Containing several pairs of basic amino acids, Acp26Aa is a
precursor to multiple peptide hormones [21] and is unlikely to
also function without posttranslational processing as a helical
cytokine. A helical fold for Obp19a can be inferred by homo-
logy to Obp76a (Protein Data Bank 1OOH) though it does not
have the helical cytokine topology.

Fig. 1. (a) Signal peptide of DHF predicted with the QT method. The predicted signal peptide is Met1 through Ser32. (b) Structure predictions of DHF performed with the QT helical cytokine fold recognition method and various other secondary structure prediction methods. The following secondary structure prediction approaches were applied to the predicted mature peptide (Ser33 through Ala214): 1 psipred; 2 sam-t02-dssp; 3 sam-t02-stride; 4 profsec; 5 QT (H: helix, T: turn, E: strand). The two predicted NX[S/T] glycosylation motifs at Asn76 and Asn160 are indicated in boxes.
As far as functional evidence is concerned, RT-PCR experiments performed on larvae exposed to 10 U/ml of LPS revealed a significant increase in dhf expression and antimicrobial peptides (Figs. 2, 3), but no increase in expression was observed after feeding with MMS (data not shown). Conversely, MMS stimulated dhf and antimicrobial peptide expression in SL2 cells (Fig. 4), while no induction was seen after treating the cells with LPS (data not shown). Experiments performed by other groups have demonstrated that microinjections of LPS in D. melanogaster larvae promote the synthesis of both defensin [22] and cecropin [23]. Regarding defensin synthesis, Lemaître et al. [15] observed that the Gram-negative and Drosophila pathogen Serratia marcescens does not induce antimicrobial peptides in adult flies when introduced into the food. However, the insects die within days as a consequence of the treatment. In larvae of D. melanogaster, it has been established that the synthesis of several peptides is increased after feeding with MMS, but neither defensins nor cecropins are reported to be induced by the treatment [16]. Accordingly, we observe here that feeding larvae with MMS does not affect defensin and cecropin expression, while LPS-contaminating medium provokes an immune stimulation as indicated by the induction of defensin and cecropin A1 (Fig. 3). In this context, it is possible that the induction we have observed as a consequence to LPS exposure is due to peptidoglycan impurities contained in our commercial preparation [24]. This should be taken into account for studies devoted to the unravelling of the pathway involved in dhf expression.

The concomitant induction of dhf and antimicrobial peptides after LPS feeding suggests a relationship between the predicted fly helical cytokine and innate immune response. This conclusion relies also on the observation that SL2 cells increase dhf expression together with that of defensin and cecropin A1 after exposure to MMS. Furthermore, the mRNA for the DHF peptide has also been isolated by an EST sequence from fat body of third instar larvae challenged with Gram positive and negative bacteria (see GenBank accession no. CO193348). The expression in an immune-related organ, and after Gram-positive and Gram-negative challenge, strengthen our hypothesis of an involvement of dhf in immunity.

Genes orthologous to mammalian helical cytokines have been identified in teleost fish but so far have not been reported in invertebrates [25]. Though the D. melanogaster genome encodes a protein belonging to the class I cytokine receptor family, with this receptor activated by the Unpaired (UPD) protein [7], from the results obtained with the QT method there is no evidence to indicate that UPD has a helical cytokine structure.

In the present investigation, we utilized the specialized QT fold recognition method for the identification of a conserved structure rather than a conserved sequence. The bioinformatics results presented here indicate that DHF has features consistent with that of helical cytokines. Our functional experiments indicate that dhf gene expression is significantly increased as a consequence of LPS ingestion by third instar larvae and after MMS exposure by SL2 embryonic hemocytes. In both cases, the increase in dhf expression is concomitant with the induction of defensin and cecropin A1, and this is consistent with a possible role for this molecule in immune functions. Obviously,
a crystal structure and a full characterization of the functions played by DHF is needed at this stage before definitely concluding that it has roles comparable to those of a mammalian cytokine.

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