ACCELERATED PUBLICATION

Transcriptomic analysis in the leech *Theromyzon tessulatum*: involvement of cystatin B in innate immunity

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At the present time, there is little information on mechanisms of innate immunity in invertebrate groups other than insects, especially annelids. In the present study, we have performed a transcriptomic study of the immune response in the leech *Theromyzon tessulatum* after bacterial challenge, by a combination of differential display RT (reverse transcriptase)–PCR and cDNA microarrays. The results show relevant modulations concerning several known and unknown genes. Indeed, threonine deaminase, malate dehydrogenase, cystatin B, polyadenylate-binding protein and α-tubulin-like genes are up-regulated after immunostimulation. We focused on cystatin B (stefin B), which is an inhibitor of cysteine proteinases involved in the vertebrate immune response. We have cloned the full-length cDNA and named the *T. tessulatum* gene as *Tt-cysb*. Main structural features of cystatins were identified in the derived amino acid sequence of *Tt-cysb* cDNA; namely, a glycine residue in the N-terminus and a consensus sequence of Gln-Xaa-Val-Xaa-Gly (QXVXG) corresponding to the catalytic site. Moreover, *Tt-cysb* is the first cystatin B gene characterized in invertebrates. We have determined by *in situ* hybridization and immunocytochemistry that *Tt-cysb* is only expressed in large coelomic cells. In addition, this analysis confirmed that *Tt-cysb* is up-regulated after bacterial challenge, and that increased expression occurs only in coelomic cells. These data demonstrate that the innate immune response in the leech involves a cysteine proteinase inhibitor that is not found in ecdysozoan models, such as *Drosophila melanogaster* or *Caenorhabditis elegans*, and so underlines the great need for information about innate immunity mechanisms in different invertebrate groups.

Key words: cDNA microarray, cystatin B, differential display reverse transcriptase (RT)–PCR, innate immunity, leech, *Theromyzon tessulatum*.

INTRODUCTION

The major characteristic of innate immunity is a fast and efficient response. Its study has generated further interest due to data obtained from invertebrate models [1–5] showing that mammalian innate immunity could be considered as a mosaic of invertebrate immune responses [5]. The main events following septic injury in both vertebrates and invertebrates are based on many mechanisms, such as the recognition of a pathogen's patterns, activation of specific receptors depending on the nature of the pathogens (Toll-like receptors), phagocytosis, antimicrobial peptide production, systemic response and cellular recruitment. Some mechanisms have been well studied in vertebrates and in a few invertebrates, e.g. the ecdysozoan model *Drosophila melanogaster* [6–10]. However, experiments performed on various invertebrate models have shown that this response is based on both basic events and specific signalling molecules. These are dependent on the environment, the life cycle and the external anatomy of each animal model.

In the light of these findings, it is important to study other invertebrate models that are different from ecdysozoans, such as annelids, which belong to the lophotrochozoan branch. This will allow a better understanding of the different defence strategies that adaptation has taken during the course of evolution [4].

In this context, the purpose of our study was to identify genes that are regulated after a bacterial challenge in the leech *Theromyzon tessulatum*. Using a combination of molecular techniques, including differential display (DD) RT (reverse transcriptase)–PCR and DNA microarrays, several immune-system-induced genes have been identified. Among them, we focused our studies on the leech homologue of the mammalian cystatin B gene (named *Tt-cysb*).

MATERIALS AND METHODS

Animals

T. tessulatum leeches were maintained in our laboratory as described elsewhere [11]. In each experimental set, total RNA of 40 leeches was pooled.

Bacterial challenge

Experimental leeches were injected with a mixture containing *Escherichia coli* and *Micrococcus luteus*. After 12 h of culture, centrifuged bacteria were resuspended in PBS, before mixing with PBS (1:1, v/v) to yield a final concentration of 10^9 bacteria/ml. The leeches were inoculated subepidermally by injection of 2×10^6 bacteria (2 μl). Control animals were injected with a sterile PBS solution (2 μl). All leeches were killed 24 h after injection.

RNA preparation and DD RT–PCR

Total RNA was extracted from complete animals with TRIzol® (Invitrogen) according to the manufacturer's instructions, before

Abbreviations used: AU, arbitrary units; (DD) RT–PCR, (differential display) reverse transcriptase–PCR; DE, differential expression; LPS, lipopolysaccharide; PABP1, polyadenylate-binding protein 1; TBS, Tris-buffered saline.

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DNase I treatment. Differential analysis was performed with the RNAimage kit, mRNA Differential Display system (GenHunter, Nashville, TN, U.S.A.), according to the manufacturer's instructions and as described by Liang and Pardee [12]. PCR amplifications were achieved using 24 5′-arbitrary oligonucleotides (HAP 1–24), each one combined with three 3′-oligo(dT) oligonucleotides (HT11M). Duplicate samples of each set were analysed in order to limit the number of false positives. PCR products were electrophoresed on a denaturing 4.5 % (w/v) polyacrylamide gel (100 W, 2500 V, 50 °C for 3.5 h) using the Genomyx LR electrophoresis system (Beckman Coulter). After autoradiography (Biomax; Kodak), detected differential products were excised from the gel and eluted in 50 μl of sterile water at room temperature overnight. They were amplified again by PCR with the same set of initial primers, before cloning into pGEM T-easy vector (Promega) before analysis in the DNA microarray control.

Cystatin B cDNA isolation

Control DNA-free total RNA (2 μg) was reverse-transcribed with 200 units of Superscript™ II RNase H$^-$ reverse transcriptase (Life Technologies) using 500 ng of an oligo(dT)$_{12-18}$ under the following conditions: 50 min at 42 °C in 50 mM Tris/HCl, pH 8.3, containing 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol and 0.5 mM dNTPs, followed by enzyme inactivation for 15 min at 70 °C.

The Marathon™ cDNA amplification kit (ClonTech) was used according to the manufacturer's instructions in order to obtain the complete 5′-end sequence of the cystatin gene [13]. A specific primer was designed from the partial cDNA sequence to clone full-length cDNA: 5′-GAGTTCAGGTTGGCCCCCAC-3′ (a specific reverse primer was used with AP1 Marathon Primer to amplify the 5′-end). All PCR products were cloned into the pGEM T-easy vector.

Sequencing

All DNA products were sequenced using the BigDye Terminator v3.0 polymerization kit before detection on the ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequence analysis used BLAST programs with E = 1000 and no filter [14,15].

Northern blot analysis

Total RNA (15 μg) was diluted in loading buffer [3.7 % formaldehyde/50 % formamide/20 mM phosphate buffer (pH 7.2)/0.004 % ethidium bromide/0.2 μM EDTA/0.025 % Bromophenol Blue/0.025 % Xylene Cyanol/5 % glycerol] and loaded on to a denaturing 1 % (w/v) agarose gel [20 mM phosphate buffer (pH 7.2)/7.2 % formaldehyde]. Total RNA was electrophoresed according to its size in electrophoresis buffer [20 mM phosphate buffer (pH 7.2)/3.7 % formaldehyde] for 3 h at 130 V. Total RNA was then transferred on to an uncharged nylon membrane (Hybond; Amersham Biosciences) by upward capillary transfer overnight. After an incubation for 2 h at 80 °C, the membrane was ready to use.

The full-length *Tt-cysb* cDNA clone and the partial 18 S rRNA clone were cultured in Luria–Bertani medium (with 100 μg/ml ampicillin). The extracted plasmid DNA was digested with 1 unit of *Eco*RI and then electrophoresed on a 1 % (w/v) agarose gel. DNA fragments were finally purified after excision from the gel (Qiaquick gel extraction; Qiagen). Samples (50 μg) of the fragments were used for the [α-^{32}P]dCTP-labelled probe synthesis, according to the manufacturer's instructions (DNA labelling kit, Ready To Go system; Amersham Biosciences). Probes were purified by gel filtration on a G-25 Sephadex column, according to the manufacturer's instructions (Quick Spin Column; Boehringer Mannheim).

The membrane was pre-incubated for 4 h at 42 °C in 50 ml of hybridization buffer [5 × SSPE (where 1 × SSPE is 0.15 M NaCl/10 mM sodium phosphate, pH 7.4)/1 mM EDTA containing 50 % formamide, 5 × Denhardt's solution (where 1 × Denhardt's solution is 0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.02 % BSA), 1 % SDS and 40 μg/ml of salmon-sperm DNA]. Quantified, labelled DNA was then denatured for 5 min at 95 °C and used at 2×10^6 c.p.m./ml in hybridization buffer (incubated at 42 °C overnight).

Membranes were washed sequentially in 2 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1 % SDS, 0.5 × SSC/0.1 % SDS and 0.1 × SSC/0.1 % SDS for 20, 5 and 5 min respectively, and then exposed for 24–48 h to X-ray film (XAR-5, X-Ommat; Kodak) at − 80 °C with an intensifying screen. Finally, autoradiograms were analysed on a densitometer (GS-710 calibrated imaging densitometer; Bio-Rad).

DNA microarrays

Slide preparation

Differential candidates from 300–600 bp were amplified with aminated oligonucleotides complementary to pGEM vector sequences flanking the DNA insert (candidates of a more extensive size are too long to be spotted on the chip). They were also amplified with internal oligonucleotides to generate expected size products up to 600 bp, and were cloned into the pGEM T-easy vector. They were then amplified with aminated oligonucleotides, as described above. Purified PCR products (50 pl of each) were spotted on to microarray slides (Genetix) by a pin-and-ring method (GMS417 Arrayer). The slides were then treated as described by Schena et al. [16].

Fluorescent labelling and hybridization reactions

Total RNA was reverse-transcribed in the presence of Cy3-dCTP or Cy5-dCTP (Amersham Biosciences) as follows: 15 μg of total RNA from naïve or challenged animals was incubated at 42 °C for 110 min in 30 μl of SuperScript™ buffer containing 1 μg of oligo(dT)$_{15}$ (Hoffmann-La Roche Ltd, Basel, Switzerland), 500 μM each of dATP, dGTP and dTTP, 200 μM dCTP, 100 μM Cy3-dCTP or Cy5-dCTP, 16 units of RNAsine, 10 mM dithiothreitol and 400 units of SuperScript™ II reverse transcriptase (Invitrogen). The samples were then treated with 1.5 μl of 1 M NaOH for 10 min at 65 °C to degrade the RNA and purified on a YM 30 Centricon centrifugal filter unit (Millipore) in a final volume of 5 μl. Hybridization buffer (10 μl) was then added to a final concentration of 40 % formamide, 2.5 × Denhardt's solution, 0.5 % SDS and 4 × SSC [16]. After incubation at 95 °C for 5 min, the probe was applied on to the microarray slides under a coverslip. They were hybridized with the probe and 30 μl of hybridization buffer for 14–16 h at 42 °C, washed sequentially in 0.1 × SSC/0.5 % SDS and 0.1 × SSC, and then dried.

The hybridization intensities for the different channels were normalized using four hop gene controls (see below). Before each labelling reaction, the samples received the four different *in vitro*-synthesized control RNAs (hop genes), for which four corresponding cDNAs were spotted on to the slide among the bacterial genes.

Microarray analyses

The slides were scanned using the Affymetrix 418 Array Scanner (Affymetrix). Separate images were acquired for each fluorophore

at a resolution of 10 μm/pixel. To normalize the two channels with respect to signal intensity, the photomultiplier and laser-power settings were adjusted such that the signal ratios of the hop control genes were as close to 1.0 as possible. The average fluorescence intensity was determined by using the ScanAlyze program (http://rana.lbl.gov/EisenSoftware.htm). Background fluorescence was calculated as the median fluorescence signal of non-target pixels around each gene spot. Spots showing a signal value lower than three times the local background in both Cy3 and Cy5 channels were not considered. For every experiment, the DE (differential expression), which represents the degree of induction or repression between the control and the experimental animals, was defined for each of the genes and expressed as the natural logarithm value of the ratio. Statistically significant differences in gene expression between the different sets of animals were assessed by a Student's *t* test implemented on the Cyber-T interface (http://www.genomics.uci.edu). In addition, a Bonferroni correction value of 0.25 was applied to all the data to reduce the number of false positives. This statistical analysis allowed us to distinguish between significant and non-significant DE. The mean DE is considered as significant when a P value > 0.005 is obtained.

In situ hybridization

Probe synthesis

A plasmid containing the original DD RT–PCR partial cystatin sequence (512 bp) was used as a template for the preparation of the probes. [^{35}S]UTP-labelled antisense and sense probes were generated from linearized cDNA plasmids by *in vitro* transcription using [^{35}S]UTP (Amersham Biosciences) and an RNA labelling kit (Hoffmann-La Roche Ltd.), according to the manufacturer's instructions.

Tissue preparation

Control and challenged stage 2 animals were fixed in a solution containing 4 % (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 16 h. After dehydration, tissues were embedded in Paraplast and 8 μm sections were cut, mounted on to poly(L-lysine)-coated slides and stored at 4 °C until use.

Hybridization

[^{35}S]UTP-labelled riboprobes (100 ng or 1×10^6 c.p.m./slide) were hybridized to tissue sections, as described previously [17,18]. The [^{35}S]UTP hybridization signal was visualized using autoradiography. Samples were coated by dipping in LM1 liquid emulsion (Amersham Biosciences), immediately dried and exposed for a 28-day period. At the end of the exposure period, the autoradiograms were developed in D19b (Kodak), fixed in 30 % sodium thiosulphate (10 min at room temperature), stained with Toluidine Blue and mounted with XAM (Merck). Slides were observed under a Zeiss Axioskop microscope, and images were captured with a system monitored with the Leica IM1000 software. Quantification of the radiolabelling at the cellular level was performed using a Zeiss Axiophot microscope and a BIOCOM quantification system, as described previously [18].

Controls

Controls for *in situ* hybridization consisted of replacing antisense riboprobe with sense riboprobe. RNase control sections were obtained by adding a pre-incubation step with 10 μg/ml RNase A before hybridization.

Immunocytochemical procedures

Antibodies

The potentially immunogenic region of the Tt-CYSB protein (Tyr^7–Val^{21}; derived from the *Tt-cysb* gene) was chemically synthesized coupled with ovalbumin, and then used for the immunization of five Balb/c mice, according to the protocol of Agrobio (La Chavannerie, La Ferté St Aubin, France).

Immunofluorescence

Tissue sections obtained as described above were pre-incubated for 1 h in TBS [Tris-buffered saline; 0.1 M Tris/HCl (pH 7.4)/0.9 % NaCl] containing 1 % normal goat serum, 1 % ovalbumin and 0.05 % Triton X-100. Sections were then incubated overnight at 20 °C in TBS containing mouse anti-cystatin B antiserum (1:400 dilution), 1 % normal goat serum, 1 % ovalbumin and 0.05 % Triton X-100. After two washes in TBS, sections were incubated for 2 h with a goat anti-mouse FITC-tagged antiserum (Jackson Immunoresearch Laboratories) diluted 1:100 in TBS. After two washes, slides were mounted in glycerol containing 25 % TBS and 0.1 % *p*-phenylenediamine. Labelled cells were observed using a Leica laser-scanning microscope (TCS NT) equipped with a Leica (DMIRBE) inverted microscope and an argon/krypton laser. FITC signal was detected using a 488 nm band pass excitation filter and a 575–640 nm pass barrier filter. Images were acquired sequentially as single transcellular optical sections and averaged over 16 scans/frame.

Controls were incubations of anti-cystatin B immunoserum pre-adsorbed by the 14-amino-acid synthesized peptide [corresponding to the potentially immunogenic region of the Tt-CYSB protein (Tyr^7–Val^{21}; see above)].

RESULTS

Transcriptomic approach of leech innate immune response

Seventy-two primer combinations [of the 24 5′-arbitrary oligonucleotides (HAP 1–24) with HT11A, G and C] were used in the present study. Ninety-one differential bands with a size range from 300 to 1500 bp were excised (Figure 1). Among these 91 products, 64 show an increase in amplification signal (positive candidates) following bacterial challenge, whereas 27 show a decrease in this signal (negative candidates) under the same conditions (Table 1). All the candidate genes were submitted to BLAST analysis (Table 1) [15]. Of these, 25 positive and 10 negative products matched with known sequences; 27 positive and 14 negative products are still unknown after BLAST alignment (Table 1). Twelve positive and 3 negative products did not re-amplify after gel extraction because of their high size and/or low amount of eluted DNA (Table 1). Some identified genes, such as those for ribosomal proteins and aldehyde dehydrogenase, and paramyosin genes, are redundant and correspond to highly expressed genes (Table 1).

Because DD RT–PCR is known to generate many false positives [19], a second screening technique was undertaken with DNA microarrays. All DD RT–PCR candidates were spotted on to glass slides. Total RNA from control or challenged animals was used in order to hybridize fluorescent cDNA probes with spotted candidates. Two positive controls were used, namely the cDNAs of *T. tessulatum* antimicrobial peptides named theromacin and theromyzin, which are known to be induced after bacterial challenge [20]. Both antimicrobial peptide genes were selected during our statistical analysis and thus validated the DNA microarrays. The statistical analysis confirmed in part the data obtained

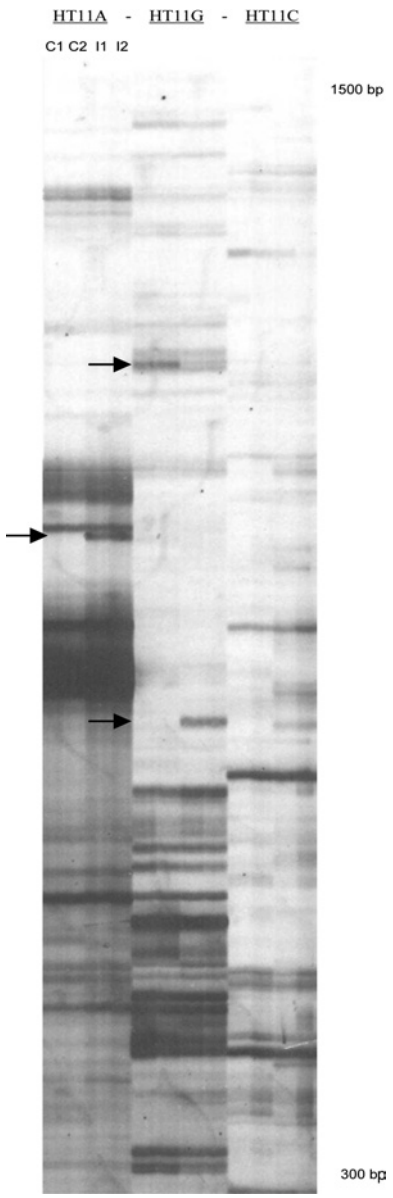

Figure 1 Representative differential display autoradiograph

Seventy-two combinations of primer sets from the RNAimage kit (GenHunter) comprising HT11A, HT11C, HT11G and HAP 1-24 were used to perform DD RT–PCR of mRNA from bacteria injected (I1, I2) or control (C1, C2) leeches. Ninety-one different bands (shown by the black arrows) over 300 bp were excised from the polyacrylamide gels.

by DD RT–PCR (Table 2). All the products selected by the microarray approach correspond to positive candidates in the DD RT–PCR experiments. No negative candidate was selected. Interestingly, three positive differential products from distinct amplification signals correspond to the threonine deaminase gene. They were all the first selected genes in the statistical analysis, confirming the efficiency of the microarrays (Table 2). The other candidates correspond to three unknown genes and five identified genes: malate dehydrogenase, cystatin B, PABP1 (polyadenylate-binding protein 1), ribosomal protein 60 S L3 and α-tubulin genes (Table 2). This analysis shows a relative comparison between two sets of animals, and was performed after normalization using four hop genes as a control. Before each labelling reaction, the four different *in vitro*-synthesized control RNAs (hop genes) were added to the samples. Their relative levels were monitored using the four corresponding cDNAs spotted on to the slide. This normalization allows us to consider whether the experimental changes in expression levels are significant.

Cystatin B gene: a cysteine proteinase inhibitor up-regulated in leech innate immunity

Among the genes induced following septic shock that were detected in the transcriptomic studies, a leech cystatin gene was identified (GenBank® accession number AF542131). In vertebrates, cystatins are known to inhibit the cysteine proteinases belonging to the cathepsin family [21]. In immunity, cathepsins are involved in processing functions of mammalian antigen-presenting cells [22,23]. They allow class II antigenic peptide binding by degradation of Ii chaperone in the initial class II–Ii complex. Moreover, *D. melanogaster* cathepsin L (CP1) is present in small granules of haemocytes, and might play a role in phagocytic events [24]. Thus, owing to the major implication of cathepsins in immunity, we focused on the cystatin gene analysis.

The cystatin gene was partially characterized with the DD RT–PCR analysis and its complete cDNA sequence was obtained by PCR amplification from the Marathon® cDNA library (Clon-Tech). Its sequence showed an open reading frame of 306 nucleotides (Figure 2A). BLAST analysis [15] showed that *T. tessulatum* cystatin possesses 54 % identity with human cystatin B (also named stefin B). The full amino acid sequence of this inhibitor was deduced using the SignalP V1.1 program. The analysis did not reveal the presence of a signal peptide, which is typical of cystatin B. Cystatin B (stefin B) is an intracellular, tight-binding and reversible inhibitor of papain-like cysteine proteinases, such as cathepsins. Leech cystatin, like other cystatin Bs, exhibits an active site characterized by a glycine residue (Gly^5) in the N-terminus and a putative active site located at residues Gln^{47}-Xaa-Val-Xaa-Gly^{51} (QXVXG). Finally, mammalian cystatins B are unique among cystatins in having a free cysteine residue before the Gly^5 residue at the N-terminus. Recent studies have shown that this cysteine is involved in binding to proteinases and seems to be specific for cystatin B [25,26]. The leech cystatin also possesses this cysteine residue. Thus the presence of all these features in the leech inhibitor confirms that we have characterized the leech cystatin B gene (named *Tt-cysb*), and thus *Tt-cysb* is the first cystatin B gene known in invertebrates. The Multalin program presents a comparison of Tt-CYSB protein with some known cystatins B (Figure 2B) [27].

According to DD RT–PCR and cDNA microarray analyses, the *Tt-cysb* gene is overexpressed after a bacterial challenge (Table 2). Tt-cysb mRNA and Tt-CYSB protein localizations were therefore performed using *in situ* hybridization and immunocytochemistry in control and immunostimulated animals (Figure 3). Interestingly, *Tt-cysb* is specifically expressed in large circulating coelomic cells in control and immunostimulated animals (Figures 3A and 3B). These 'large coelomocytes' are only detected in coelomic cavities, and are distributed throughout the whole animal. The sections hybridized with the sense *Tt-cysb* probe were totally devoid of labelling (Figure 3B'). In addition, pre-treatment of sections with RNase A before hybridization abolished the positive staining, providing further evidence of the signal specificity (results not shown). To determine whether *Tt-cysb* could be transcriptionally regulated at the level of the coelomocytes or whether changes of transcript rates could be only the results of changes in cell populations, Tt-cysb mRNA contents were quantified at the cellular level (Figure 3C). Among the tissue sections probed with a ^{35}S-radiolabelled *Tt-cysb* antisense riboprobe,

Table 1 Database search for candidates with significant similarity

The gene expression modulation observed for each candidate after bacterial challenge is indicated: up-regulation (+) or down-regulation (−). Identification column shows the best-matched identity (if any) and the GenBank accession number for every matched differential product.

Candidate	Identification [Genbank accession number]	Modulation	Candidate	Identification [Genbank accession number]	Modulation
1	Not reamplified	+	47	No reamplified	+
2	Actin 2 [CK640381]	+	48	No reamplified	+
3	Cystatin B [AF542131]	+	49	Unknown	+
4	Phosphatidylethanolamine-binding protein [CK640382]	+	50	Unknown	−
5	Unknown	+	51	Unsequenced	+
6	Unknown	+	52	Alpha protein coatomer sub-unit [CK640393]	+
7	Unknown	−	53	Aldehyde dehydrogenase [CK640394]	−
8	Unknown	−	54	Unknown	−
9	Ribosomal protein 40S S18 [CK640383]	+	55	Unknown	+
10	Unknown	−	56	Unknown	+
11	Ribosomal protein 40S S4 [CK640384]	+	57	Peptidyl–prolyl *cis–trans* isomerase [CK640395]	+
12	Unsequenced	−	58	Lipoic acid synthetase [CK640396]	−
13	Ribosomal protein 40S S4 [CK640385]	+	59	Unknown	−
14	Fructose-bisphosphate aldolase 1 [CK640386]	−	60	Unknown	+
15	Unknown	−	61	Unknown	+
16	Unknown	+	62	Unknown	+
17	No reamplified	+	63	Unknown	−
18	Ribosomal protein 40S S4 [CK640387]	+	64	Unknown	+
19	Ribosomal protein 40S S4 [CK640387]	+	65	Unknown	+
20	PABP1 [CK640388]	+	66	Cullin-3 [CK640397]	−
21	Not reamplified	−	67	Unknown	+
22	Unsequenced	−	68	Unknown	+
23	Unknown	−	69	Unknown	−
24	Unknown	+	70	Unknown	−
25	Unknown	+	71	Unknown	−
26	Unknown	+	72	Unknown	+
27	Unknown	+	73	Aldehyde dehydrogenase [CK640398]	−
28	Unknown	+	74	Unknown	+
29	Unknown	+	75	Growth hormone inducible transmb protein [CK640399]	+
30	Unknown	−	76	Unknown	+
31	Unknown	−	77	Growth hormone inducible transmb protein [CK640400]	−
32	Unknown	+	78	Threonine deaminase [CK640401]	+
33	Unknown	+	79	Ribosomal protein 40S SA [CK640402]	+
34	Not reamplified	+	80	α-Galactosidase A [CK640403]	−
35	Ribosomal protein 60S L3 [CK640389]	+	81	Paramyosin [CK640404]	−
36	Ribosomal protein 60S L3 [CK640389]	+	82	Paramyosin [CK640405]	+
37	Elongation factor 1-α 2 [CK640390]	+	83	Unknown	+
38	Malate dehydrogenase [CK640391]	+	84	ATP-dependent RNA helicase [CK640406]	+
39	Ribosomal protein 60S L3 [CK640392]	−	85	Actin 2 [CK640407]	+
40	Not reamplified	+	86	α-Tubulin [CK640408]	+
41	Not reamplified	+	87	Tropomyosin 2 [CK640409]	−
42	Not reamplified	+	88	Threonine deaminase [CK640410]	+
43	Not reamplified	+	89	Threonine deaminase [CK640411]	+
44	Not reamplified	+	90	Unknown	+
45	Not reamplified	+	91	Unknown	+
46	Ribosomal protein 60S L3 [CK640392]	+			

30 large coelomocytes from four individual animals (120 cells) were analysed at each time post-injection. Quantification was expressed as AU (arbitrary units) corresponding to the number of silver grains counted for every large coelomocyte using Biocom autoradiography software. Silver grains are produced by contact of ^{35}S emission with the autoradiographic emulsion. The number of grains is proportional to the hybridization signal. Background level was measured and subtracted for each slide. According to the quantification, an increase of signal between control animals (28.8 AU) and sham-treated animals (33.8 AU) was not significant. In contrast, the increase of silver density between sham-treated animals and bacteria-injected animals (65.8 AU) was significant ($P < 0.05$). These data demonstrate a 2-fold increase in Tt-cysb mRNA levels in the large coelomocytes of challenged leeches (Figure 3C). No other tissue was shown to exhibit a hybridization signal. All these results corroborated and extended the DD RT–PCR and microarray data by identifying the cells implicated in the Tt-cysb mRNA synthesis.

We then examined the time course of *Tt-cysb* induction by Northern blot analysis. Purification of coelomocytes was not possible, because of the high degree of adhesion to the coelomic cavity wall, and thus we performed this study on the whole animal. In spite of the weak representation of coelomocyte mRNA among the total RNA of the whole animal, the analyses nevertheless confirmed the induction of expression of *Tt-cysb* (Figure 3D). The data were normalized with the 18 S rRNA signal. Signals from PBS-injected animals, compared with control signals, showed a slight increase in Tt-cysb mRNA level after PBS injection. Signals from *E. coli*-injected animals, compared with those of PBS-injected animals, revealed a slight increase in Tt-cysb mRNA level 3 h after *E. coli* injection. This increase was more significant 24 h after injection. Signals from *M. luteus*-injected animals, compared

Table 2 DNA microarray analysis of differentially display candidates

All the candidates retained by DNA microarray screening are up-regulated after bacterial challenge. Candidates are listed according to their *t* test result (*p*) and their ratio between control and immunostimulated values.

Candidate	Identification [Genbank accession number]	*P*	Ratio
78	Threonine deaminase [CK640401]	0.0001	2:54247
89	Threonine deaminase [CK640411]	0.0003	2:3981
88	Threonine deaminase [CK640410]	0.0003	2:32888
24	Unknown	0.0003	1:90227
Theromacin	*T. tessulatum* antimicrobial peptide (positive control) [AY434032]	0.0008	1:84867
38	Malate dehydrogenase [CK640391]	0.0019	1:46243
3	Cystatin B [AF542131]	0.0028	1:49705
20	PABP1 [CK640388]	0.0029	1:44028
36	Ribosomal protein 60S L3 [CK640389]	0.0033	1:39137
86	α-Tubulin [CK640408]	0.0039	1:61054
67	Unknown	0.0048	1:42512
Theromyzin	*T. tessulatum* antimicrobial peptide (positive control) [AY434033]	0.0049	1:53785
74	Unknown	0.0050	1:45091

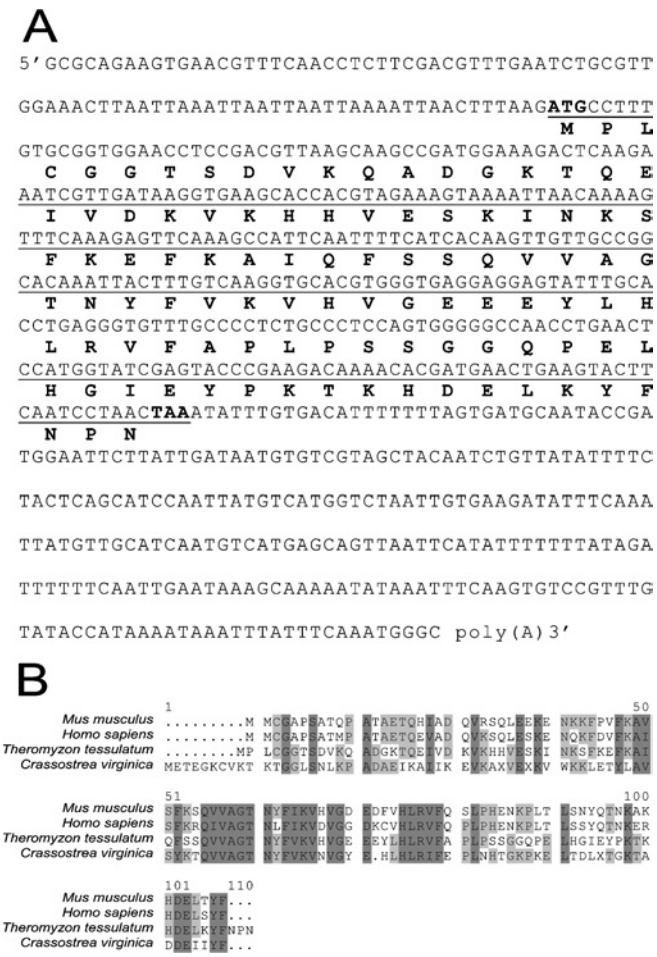

Figure 2 The amino acid and nucleotide sequences of *T. tessulatum* cystatin B

(**A**) Complete molecular cloning of *Ts-cysb* cDNA reveals an open reading frame of 306 nt (show underlined) encoding a protein of 102 amino acids in length (shown by the lettering in bold typeface). The SignalP V1.1 program revealed no signal peptide. (**B**) The Multalin program shows a high identity with known cystatin B (54 % with human cystatin B and 50 % with murine cystatin B), It also shows similarities with a putative cystatin B in the oyster *Crassostrea virginica* (GenBank® accession no. BG624574). However, lack of a cysteine residue (Cys[4] in Ty-CYSB) close to a glycine residue seems to indicate that oyster cystatin is not a form B cystatin [26]. Identical (high consensus) residues are shown on a dark-grey background; residues that are similar (low consensus) are shown on a light-grey background.

with those of PBS-injected animals, showed an increase in Tt-cysb mRNA level 3 h after *M. luteus* injection. As for the *E. coli* injection, this modulation was more important 24 h after *M. luteus* injection. Globally, *Tt-cysb* induction seemed to be stronger after the injection of *M. luteus* than after the injection of *E. coli*. Otherwise, regardless of which bacteria were injected, other analyses were performed 12 h after the injections and did not detect any further significant increase in Tt-cysb mRNA levels other than those obtained 3 h after bacterial injections (results not shown). In addition, for the animals analysed 24 h after injection, we noted that the Tt-cysb mRNA level was more markedly increased (approx. 1.6-fold) after injection with a bacterial mixture (*E. coli* and *M. luteus*) than were those obtained after single bacteria injection (approx. 1.2-fold after *E. coli* injection and approx. 1.4-fold after *M. luteus* injection; Figure 3D).

Tt-CYSB storing sites were investigated by immunocytochemistry on whole-animal paraffin sections. Strong immunoreactivity was observed in large coelomocytes only. Tt-CYSB labelling was detected as intensely fluorescent small spots distributed throughout the cytoplasm and surrounding the nucleus (Figure 3E), suggesting that Tt-CYSB is packed in granules or vesicles.

DISCUSSION

The present study demonstrates that DD RT–PCR and microarrays are, as for other invertebrate models [28], powerful and efficient combined techniques for transcriptomic analysis of gene expression in the leech. Furthermore, this allowed an exploration of biological responses to bacterial challenge [29]. DD RT–PCR generates many candidates with an important number of false positives [19]. The combination of this technique with DNA microarray analysis resolves this problem due to its sensitivity and its large powerful discrimination. As for many other experimental models, the *T. tessulatum* genome remains to be sequenced. However, this approach should allow a large-scale analysis of the transcriptome in the leech, as well as for other invertebrate species [30]. In addition, these combined techniques permit us to undertake kinetic analyses in order to elucidate clustering of immune-system-induced genes.

Among the selected candidates, several appear to be unrelated to the immune response. With regard to plant immunity studies, the threonine deaminase gene is induced by methyl jasmonate or wounding [31,32]. Methyl jasmonate is involved in signalling processes, leading to immune responses [33,34]. Thus we consider, among other candidates, that the threonine deaminase gene represents an interesting molecule for further studies in the understanding of immune mechanisms. Concerning the malate dehydrogenase gene, previous studies have reported its induction in human monocytes and macrophages after LPS (lipopolysaccharide) stimulation [35,36]. These data are comparable with events observed during bacterial challenge in leeches. This enzyme acts in gluconeogenesis, and might be crucial for the energetic requirements of immune cells. As for the ribosomal protein 60 S L4 gene, several studies have shown modulations of these gene families in human monocytes after LPS injection [35,36]. Some authors reported that ribosomal-protein-derived peptides have

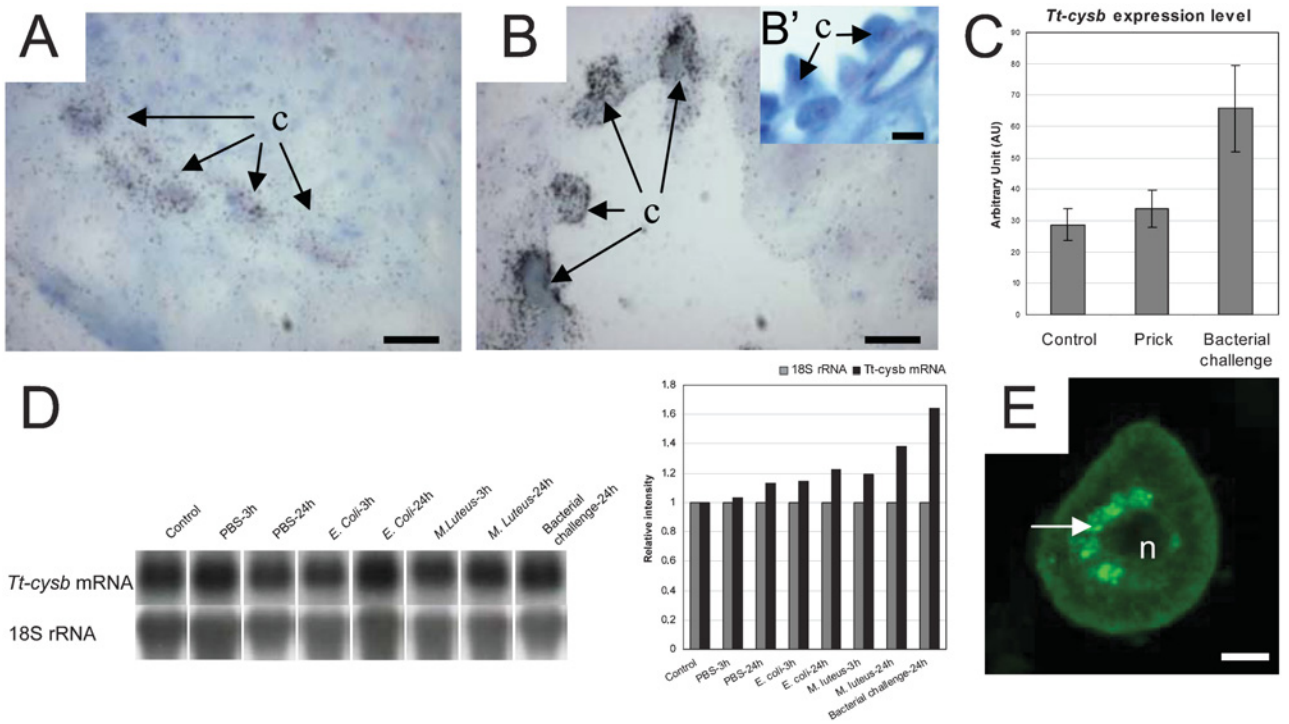

Figure 3 Detection of Tt-cysb mRNA by *in situ* hybridization and Northern blotting, and immunodetection of Tt-CYSB protein in a tissue section of the leech

Paraffin-embedded sections of leeches were hybridized with *Ty-cysb* antisense (**A** and **B**) or sense (**B'**) riboprobes labelled with [^{35}S]UTP radiolabelling appears as dark-silver deposits. Silver grains are seen in the coelomocytes of both non-stimulated (**A**) and stimulated (**B**) animals. Comparatively, 24 h after bacterial challenge, signals in coelomocytes appear stronger. Individual coelomocyte titration for Tt-cysb mRNA levels was performed using a Biocom system. Results are expressed as AU. Tt-cysb mRNA content in coelomocytes of stimulated animals is approximately twice the level of that found in control animals (**C**). After normalization with 18 S RNA, Northern blot analyses show that Tt-cysb mRNA from bacteria injected into the whole body is approx. 1.6-fold the level of control body mRNA (**D**). Sections were investigated for Tt-CYSB protein content by immunodetection with FITC-labelled secondary antibody. Strong immunoreactivity is observed in coelomocytes from non-stimulated leeches (**E**; shown by the white arrow). c, coelomocyte; n, nucleus. Bars represent either 100 μm (**A**, **B** and **B'**) or 30 μm (**D**).

antimicrobial activity [37]. This gene family might then comprise the origin-of-defence molecules [38,39]. Finally, the PABP1 gene is also of interest. A PABP gene is induced in activated T-cells of the mouse by PMA and ionomycin [40]. All these molecules are involved in T-cell activation [41,42]. Moreover, PABPs initiate the translation of specific mRNAs corresponding to molecules involved in the same biological process [43]. We suggest that, in the leech, PABP gene up-regulation might trigger the expression of gene clusters all involved in immune response.

As a result, this transcriptomic study in a lophotrochozoan animal suggests an approach for further experiments that should allow us to elucidate the functions of these induced genes in immunity. Previously, these up-regulated genes have been detected from a comparison between PBS-injected (control) animals and bacteria-injected (experimental) animals. As a single PBS injection or sterile injury can trigger immune mechanisms, we may anticipate further important modulations for these immune-induced genes in basal states. Moreover, we performed our transcriptomic analysis with total RNA from the whole organism. Further differential studies that are focused on specific immune tissues, such as coelomocytes, should identify powerful induced genes with higher modulation values.

Although our results are supported by other studies using ecdysozoans animals, some immune-system-induced genes detected in the present leech analysis are newly identified as having a role in immunity. For example, the existence of a protein related to cystatin B and its potential involvement in immunity are novel features in invertebrates. Based on its known inhibitor function, we have initially chosen to focus the analysis on this immune-system-induced gene.

Tt-CYSB corresponds to the first cystatin B cloned in invertebrates. Cystatin B belongs to the first family of cystatins, i.e. the intracellular stefins [44,45]. Cystatin B is known to inhibit some cathepsins, namely cysteine proteinases [46,47]. In vertebrates, cystatin B has a broad distribution range in cells and tissues, and targets *in vitro* lysosomal cathepsins B, H, K and L [48,49]. The unique pathophysiological role known for cystatin B in humans, despite a lack of putative target proteinases, is a childhood form of epilepsy, Unverricht-Lundborg disease (or EPM1) [50]. The loss-of-function mutations in human cystatin B and deletion of the gene in mice gave rise to progressive myoclonus epilepsy, caused by cerebellar apoptosis [51]. The down-regulation of cystatin B triggers an increase in cathepsin activity in the cytoplasm of proliferating cells in the central nervous system [52]. Recently, some authors reported evidence of cathepsin B activity in EPM1 [53]. The EPM1 disease demonstrates that cystatin–cathepsin interactions are vital. Cystatin B may inhibit cathepsin-dependent apoptotic mechanisms [51]. Indeed, the consequence of a lack of cystatin B is the increased proteolytic activity of cathepsins, leading to apoptosis and glial activation [54].

In leeches, the *Tt-cysb* gene is up-regulated 24 h after a bacterial challenge in large circulating coelomic cells named large coelomocytes (by approx. 2-fold when compared with control animals by *in situ* hybridization). This time of analysis (24 h post-injection) seems to be appropriate for the study of immune-response-regulation processes. Interestingly, these data show that

cystatin B inhibits cathepsins [46]. These enzymes are involved in immune mechanisms. The genetic modulation in *D. melanogaster* after infection with a pathogen has been reported [6–8]. Taken together, these data suggests that Tt-CYSB may regulate leech cathepsins and have a role in leech immune response regulation in these circulating coelomic cells. Indeed, *Tt-cysb* up-regulation and its localization in circulating coelomic cells allow us to hypothesize new immune functions for this proteinase inhibitor. Because cathepsins are implicated in phagosome processing in mammals [55], we suggest that the proteinase inhibitor may regulate some phagocytosis processes via the inhibition of the cysteine proteinases. As for the secreted cystatin family 2 members, various studies have shown involvement of cystatins in the modulation of the immune response [56]. In the present study, we report for the first time the potential involvement of an intracellular cystatin family 1 member, i.e. the leech cystatin B, *Tt-cysb*. In future studies we will consider the characterization of cathepsins in *T. tessulatum* in order to elucidate cystatin–cathepsin-interaction processes in leech immunity.

The present study provides new knowledge of the mechanisms of the innate immune response in a lophotrochozoan, which bears some resemblance with vertebrate, plant and invertebrate immune responses. Leech innate-immune-response studies are still in the early stages, but may bring new insights to the overall study of innate immunity. Our combination of differential analysis and cDNA microarrays in the leech highlights new immune-system-induced genes and the necessity to explore innate immunity in diverse models.

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REFERENCES

1 Lemaitre, B., Kromer-Metzger, E., Michaut, L., Nicolas, E., Meister, M., Georgel, P., Reichhart, J. M. and Hoffmann, J. A. (1995) A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the Drosophila host defense. Proc. Natl. Acad. Sci. U.S.A. **92**, 9465–9469
2 Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M. and Hoffmann, J. A. (1996) The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell **86**, 973–983
3 Medzhitov, R. and Janeway, Jr, C. A. (1997) Innate immunity: impact on the adaptive immune response. Curr. Opin. Immunol. **9**, 4–9
4 Hoffmann, J. A. and Reichhart, J. M. (2002) Drosophila innate immunity: an evolutionary perspective. Nat. Immunol. **3**, 121–126
5 Salzet, M. (2001) Vertebrate innate immunity resembles a mosaic of invertebrate immune responses. Trends Immunol. **22**, 285–288
6 De Gregorio, E., Spellman, P. T., Rubin, G. M. and Lemaitre, B. (2001) Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. Proc. Natl. Acad. Sci. U.S.A. **98**, 12590–12595
7 Irving, P., Troxler, L., Heuer, T. S., Belvin, M., Kopczynski, C., Reichhart, J. M., Hoffmann, J. A. and Hetru, C. (2001) A genome-wide analysis of immune responses in Drosophila. Proc. Natl. Acad. Sci. U.S.A. **98**, 15119–15124
8 Levy, F., Bulet, P. and Ehret-Sabatier, L. (2004) Proteomic analysis of the systemic immune response of Drosophila. Mol. Cell. Proteomics **3**, 156–166
9 Vierstraete, E., Verleyen, P., Baggerman, G., D'Hertog, W., Van den Bergh, G., Arckens, L., De Loof, A. and Schoofs, L. (2004) A proteomic approach for the analysis of instantly released wound and immune proteins in Drosophila melanogaster hemolymph. Proc. Natl. Acad. Sci. U.S.A. **101**, 470–475
10 Hoffmann, J. A. (2003) The immune response of Drosophila. Nature (London) **426**, 33–38
11 Malecha, J., Verger-Bocquet, M. and Tramu, G. (1989) Mise en évidence et évolution, au cours du cycle biologique, de neurones producteurs d'une substance apparentèe à la motiline porcine dans le ganglion supraoesophagien de la sangsue Theromyzon tessulatum. Can. J. Zool. **67**, 636–640
12 Liang, P. and Pardee, A. B. (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science **257**, 967–971
13 Frohman, M. A., Dush, M. K. and Martin, G. R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. U.S.A. **85**, 8998–9002
14 Karlin, S. and Altschul, S. F. (1990) Methods for assessing the statistical significance of molecular sequence features by using general scoring schemes. Proc. Natl. Acad. Sci. U.S.A. **87**, 2264–2268
15 Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**, 3389–3402
16 Schena, M., Shalon, D., Davis, R. W. and Brown, P. O. (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science **270**, 467–470
17 Mitta, G., Vandenbulcke, F., Noel, T., Romestand, B., Beauvillain, J. C., Salzet, M. and Roch, P. (2000) Differential distribution and defence involvement of antimicrobial peptides in mussel. J. Cell Sci. **113**, 2759–2769
18 Munoz, M., Vandenbulcke, F., Saulnier, D. and Bachere, E. (2002) Expression and distribution of penaeidin antimicrobial peptides are regulated by haemocyte reactions in microbial challenged shrimp. Eur. J. Biochem. **269**, 2678–2689
19 Ali, M., Markham, A. F. and Isaacs, J. D. (2001) Application of differential display to immunological research. J. Immunol. Methods **250**, 29–43
20 Tasiemski, A., Vandenbulcke, F., Mitta, G., Lemoine, J., Lefebvre, C., Sautiere, P. E. and Salzet, M. (2003) Molecular characterization of two novel antibacterial peptides inducible upon bacterial challenge in an annelid: the leech Theromyzon tessulatum. J. Biol. Chem. (2004) DOI 10.1074/jbc.M312156200
21 Anastasi, A., Brown, M. A., Kembhavi, A. A., Nicklin, M. J., Sayers, C. A., Sunter, D. C. and Barrett, A. J. (1983) Cystatin, a protein inhibitor of cysteine proteinases. Improved purification from egg white, characterization, and detection in chicken serum. Biochem. J. **211**, 129–138
22 Barrera, C., Ye, G., Espejo, R., Gunasena, S., Almanza, R., Leary, J., Crowe, S., Ernst, P. and Reyes, V. E. (2001) Expression of cathepsins B, L, S, and D by gastric epithelial cells implicates them as antigen presenting cells in local immune responses. Hum. Immunol. **62**, 1081–1091
23 Riese, R. J. and Chapman, H. A. (2000) Cathepsins and compartmentalization in antigen presentation. Curr. Opin. Immunol. **12**, 107–113
24 Tryselius, Y. and Hultmark, D. (1997) Cysteine proteinase 1 (CP1), a cathepsin L-like enzyme expressed in the Drosophila melanogaster haemocyte cell line mbn-2. Insect Mol. Biol. **6**, 173–181
25 Reverter, D., Sorimachi, H. and Bode, W. (2001) The structure of calcium-free human m-calpain: implications for calcium activation and function. Trends Cardiovasc. Med. **11**, 222–229
26 Pol, E. and Bjork, I. (2001) Role of the single cysteine residue, Cys 3, of human and bovine cystatin B (stefin B) in the inhibition of cysteine proteinases. Protein Sci. **10**, 1729–1738
27 Corpet, F. (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. **16**, 10881–10890
28 Takeuchi, H., Fujiyuki, T., Shirai, K., Matsuo, Y., Kamikouchi, A., Fujinawa, Y., Kato, A., Tsujimoto, A. and Kubo, T. (2002) Identification of genes expressed preferentially in the honeybee mushroom bodies by combination of differential display and cDNA microarray. FEBS Lett. **513**, 230–234
29 Green, C. D., Simons, J. F., Taillon, B. E. and Lewin, D. A. (2001) Open systems: panoramic views of gene expression. J. Immunol. Methods **250**, 67–79
30 Crawford, D. L. (2001) Functional genomics does not have to be limited to a few select organisms. Genome Biol. **2**, interactions 1001.1–1001.2
31 Samach, A., Broday, L., Hareven, D. and Lifschitz, E. (1995) Expression of an amino acid biosynthesis gene in tomato flowers: developmental upregulation and MeJa response are parenchyma-specific and mutually compatible. Plant J. **8**, 391–406
32 Hildmann, T., Ebneth, M., Pena-Cortes, H., Sanchez-Serrano, J. J., Willmitzer, L. and Prat, S. (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell **4**, 1157–1170
33 Thomma, B. P., Eggermont, K., Tierens, K. F. and Broekaert, W. F. (1999) Requirement of functional ethylene-insensitive 2 gene for efficient resistance of Arabidopsis to infection by Botrytis cinerea. Plant Physiol. **121**, 1093–1102
34 Feys, B. J. and Parker, J. E. (2000) Interplay of signaling pathways in plant disease resistance. Trends Genet. **16**, 449–455
35 Suzuki, T., Hashimoto, S., Toyoda, N., Nagai, S., Yamazaki, N., Dong, H. Y., Sakai, J., Yamashita, T., Nukiwa, T. and Matsushima, K. (2000) Comprehensive gene expression profile of LPS-stimulated human monocytes by SAGE. Blood **96**, 2584–2591
36 Hashimoto, S., Suzuki, T., Dong, H. Y., Yamazaki, N. and Matsushima, K. (1999) Serial analysis of gene expression in human monocytes and macrophages. Blood **94**, 837–844

37 Lee, D. G., Park, Y., Kim, H. N., Kim, H. K., Kim, P. I., Choi, B. H. and Hahm, K. S. (2002) Antifungal mechanism of an antimicrobial peptide, HP (2–20), derived from N-terminus of Helicobacter pylori ribosomal protein L1 against Candida albicans. Biochem. Biophys. Res. Commun. **291**, 1006–1013
38 Putsep, K., Branden, C. I., Boman, H. G. and Normark, S. (1999) Antibacterial peptide from H. pylori. Nature (London) **398**, 671–672
39 Putsep, K., Normark, S. and Boman, H. G. (1999) The origin of cecropins; implications from synthetic peptides derived from ribosomal protein L1. FEBS Lett. **451**, 249–252
40 Yang, H., Duckett, C. S. and Lindsten, T. (1995) iPABP, an inducible poly(A)-binding protein detected in activated human T cells. Mol. Cell. Biol. **15**, 6770–6776
41 Miller, R. A., Garcia, G., Kirk, C. J. and Witkowski, J. M. (1997) Early activation defects in T lymphocytes from aged mice. Immunol. Rev. **160**, 79–90
42 Krasnow, S. W., Zhang, L. Q., Leung, K. Y., Osborn, L., Kunkel, S. and Nabel, G. J. (1991) Tumor necrosis factor-alpha, interleukin 1, and phorbol myristate acetate are independent activators of NF-kappa B which differentially activate T cells. Cytokine **3**, 372–379
43 Sachs, A. B. and Deardorff, J. A. (1992) Translation initiation requires the PAB-dependent poly(A) ribonuclease in yeast. Cell **70**, 961–973
44 Rawlings, N. D. and Barrett, A. J. (1990) Evolution of proteins of the cystatin superfamily. J. Mol. Evol. **30**, 60–71
45 Barrett, A. J., Fritz, H., Grubb, A., Isemura, S., Jarvinen, M., Katunuma, N., Machleidt, W., Muller-Esterl, W., Sasaki, M. and Turk, V. (1986) Nomenclature and classification of the proteins homologous with the cysteine-proteinase inhibitor chicken cystatin. Biochem. J. **236**, 312
46 Thiele, U., Auerswald, E. A., Gebhard, W., Assfalg-Machleidt, I., Popovic, T. and Machleidt, W. (1988) Inhibitorily active recombinant human stefin B. Gene synthesis, expression and isolation of an inhibitory active MS-2 pol-stefin B fusion protein and preparation of Des[Met1,2(2)]stefin B. Biol. Chem. Hoppe Seyler **369**, 1167–1178
47 Turk, B., Colic, A., Stoka, V. and Turk, V. (1994) Kinetics of inhibition of bovine cathepsin S by bovine stefin B. FEBS Lett. **339**, 155–159

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48 Cimerman, N., Mesko Brguljan, P., Krasovec, M., Suskovic, S. and Kos, J. (2001) Serum concentration and circadian profiles of cathepsins B, H and L, and their inhibitors, stefins A and B, in asthma. Clin. Chim. Acta **310**, 113–122
49 Kos, J. and Lah, T. T. (1998) Cysteine proteinases and their endogenous inhibitors: target proteins for prognosis, diagnosis and therapy in cancer. Oncol. Rep. **5**, 1349–1361
50 Pennacchio, L. A., Lehesjoki, A. E., Stone, N. E., Willour, V. L., Virtaneva, K., Miao, J., D'Amato, E., Ramirez, L., Faham, M., Koskiniemi, M. et al. (1996) Mutations in the gene encoding cystatin B in progressive myoclonus epilepsy (EPM1). Science **271**, 1731–1734
51 Pennacchio, L. A., Bouley, D. M., Higgins, K. M., Scott, M. P., Noebels, J. L. and Myers, R. M. (1998) Progressive ataxia, myoclonic epilepsy and cerebellar apoptosis in cystatin B-deficient mice. Nat. Genet. **20**, 251–258
52 Riccio, M., Di Giaimo, R., Pianetti, S., Palmieri, P. P., Melli, M. and Santi, S. (2001) Nuclear localization of cystatin B, the cathepsin inhibitor implicated in myoclonus epilepsy (EPM1). Exp. Cell. Res. **262**, 84–94
53 Houseweart, M. K., Pennacchio, L. A., Vilaythong, A., Peters, C., Noebels, J. L. and Myers, R. M. (2003) Cathepsin B but not cathepsins L or S contributes to the pathogenesis of Unverricht-Lundborg progressive myoclonus epilepsy (EPM1). J. Neurobiol. **56**, 315–327
54 Lieuallen, K., Pennacchio, L. A., Park, M., Myers, R. M. and Lennon, G. G. (2001) Cystatin B-deficient mice have increased expression of apoptosis and glial activation genes. Hum. Mol. Genet. **10**, 1867–1871
55 Muno, D., Kominami, E. and Mizuochi, T. (2000) Generation of both MHC class I- and class II-restricted antigenic peptides from exogenously added ovalbumin in murine phagosomes. FEBS Lett. **478**, 178–182
56 Schierack, P., Lucius, R., Sonnenburg, B., Schilling, K. and Hartmann, S. (2003) Parasite-specific immunomodulatory functions of filarial cystatin. Infect. Immun. **71**, 2422–2429