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Deciphering the Immune Function and Regulation by a TLR of the Cytokine EMAPII in the Lesioned Central Nervous System Using a Leech Model1,2

David Schikorski,* Virginie Cuvillier-Hot,* Céline Boidin-Wichlacz,* Christian Slomianny,† Michel Salzet,‡* and Aurélie Tasiemski3*

A highly conserved ortholog of the human complex p43/endothelial monocyte-activating polypeptide II (EMAPII) was characterized in the CNS of the leech Hirudo medicinalis. As observed in mammals, the leech complex is processed to release the cytokine HmEMAPII. Taking advantages of these similarities, we have attempted to elucidate the role of EMAPII in the CNS using the leech model. Although EMAPII is considered a modulator of inflammatory reactions within the peripheral innate immune response in humans, its function in CNS immunity has yet to be described. Chemotaxis assays were conducted, revealing the ability of EMAPII to exert a chemoattractant effect on both leech and human microglial cells, indicating a novel function of this cytokine in the human brain. Quantitative RT-PCR analysis together with in situ hybridization and immunohistochemistry approaches showed that bacterial challenge induced the expression of HmEMAPII at the lesion site where microglial cells accumulated. Moreover, gene silencing experiments have demonstrated that the gene expression of HmEMAPII is under the control of a signaling pathway associated with the TLR HmTLR1, newly characterized in the CNS of our model. To the best of our knowledge, this is the first report showing evidence for (1) the chemoattractant properties of EMAPII on leech and human microglial cells, (2) the regulation by a TLR of the expression of a gene encoding a cytokine in the CNS of an invertebrate, and (3) an immune function of a TLR in a lophotrochozoan model.


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2 The nucleotide sequence of p43/EMAPII reported in this paper has been submitted to the GenBank/European Bioinformatics Institute under the accession number FJ462720.

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nerves known as connectives. Segmental ganglia consist of midbody segmental ganglia linked to each other by longitudinal processes to a negative degeneration fate (7). Infiltration of the CNS by peripheral immune cells makes the intrinsic immune response of the mammalian CNS even more complex to dissect. By offering simple systems, invertebrates represent a great alternative for studying the immune function of the CNS. It is now well established that invertebrates possess an efficient immune response; however, only very few of them have a CNS organization that is as well described as that of the medicinal leech Hirudo medicinalis (14). This lophophorozoa has been extensively used as a model organism in cellular analyses of nervous system function, ranging from the neural basis of behavior to studies of ion channel function (15). The nerve cord of the leech is comprised of a fixed number of midbody segmental ganglia linked to each other by longitudinal nerves known as connectives. Segmental ganglia consist of ~400 neurons, many of which are very well characterized developmentally, anatomically, physiologically, and neurochemically, along with a large population of microglial cells (16). Of particular significance, the medicinal leech has been used to study the repair of the nervous system at the level of the identified nerve cells, an approach that is currently difficult or not possible with more complex nervous systems (17). The nerve cord of the leech can be easily removed from the animal and maintained in culture for weeks in the absence of peripheral immune system components and blood cells that might infiltrate the nerve cord after injury. This allows focused studies on the intrinsic immune responses developed by the leech nervous system. Unlike mammals, the leech CNS has demonstrated the capacity to repair itself and to restore its function after injury (18). The process of regeneration is accompanied by a rapid activation of microglial cells, which leads to their accumulation at the lesion site where they phagocytose the damaged tissues (19). Interestingly, our group recently showed that the leech nerve cord uses a common panel of proteins to initiate an antimicrobial response and a regrowth program (20). We demonstrated that microglial challenge promoted the regenerative process of the injured CNS of the medicinal leech by inducing, in neurons and microglia, the synthesis of antimicrobial peptides that exert neurotrophic properties. The injured leech nerve cord also appeared to mount an intrinsic antimicrobial response by discriminating between pathogen components. The perception mode of microbial substances by the leech CNS may implicate different sensing receptors, among which a TLR called HmTLR1, which has been newly characterized in vertebrates and microglial cells was filtered with a nylon membrane having a 5-μm-diameter mesh. The eluted medium containing microglial cells was centrifuged at 2500 rpm for 10 min. The supernatant was discarded and 60 μl of fresh complemented L-15 medium per nerve cord was added.

Isolation of neurons

Nerve cords were placed individually in 35-nm petri dishes containing 500 μl of complemented L-15 medium. Each ganglion was manually and delicately opened with microdissection instruments under sterile conditions. Neurons were extruded from each ganglion and then completely dissociated by passing the solution through a large pipette tip several times.

Purification of microglial cells

After cell dissociation, the complemented L-15 medium containing neurons and microglial cells was filtered with a nylon membrane having a 5-μm-diameter mesh. The eluted medium containing microglial cells was centrifuged at 2500 rpm for 10 min. The supernatant was discarded and 60 μl of fresh complemented L-15 medium per nerve cord was added.

Biochemical characterization of Hmp43/EMAPII

Protein extraction. Samples (10 nerve cords or three entire leeches) were crushed with a mortar in liquid nitrogen. The powder was resuspended in lysis buffer (9.5 mM urea, 2% Triton X-100, 65 mM 2-ME, and 1.25% SDS). The protein concentration was determined by the Bradford method using BSA as a standard (21). SDS-PAGE. Electrophoresis was performed according to the technique described by Tastet et al. (22). The running gel was composed of 10% acrylamide with 150 g of Tris/0.0 N HCl and the stacking gel was composed of 4% acrylamide with 120 g of Tris/0.8 N HCl. Migration was performed using a cathode buffer (0.6% Tris base, 2.5% taurine, and 0.1% SDS) and an anode buffer (0.6% Tris base, 2.8% glycine, and 0.1% SDS). A total of 50 μg of protein was loaded in Laemmli buffer (120 g of Tris/0.8 N HCl, 50% glycerol, 10% SDS, and 7.7% DTT). Gels were run at 70 V for 15 min and then at 120 V for 45 min.

Western blotting. The SDS-PAGE gel was transferred to a nitrocellulose membrane (Hybond-C Extra; Amersham Biosciences) by electroblotting. After transfer, the membrane was blocked for 1 h in PBS containing 0.05% Tween 20 and 0.7% casein and then probed with the commercial donkey polyclonal anti-HEMAPII Ab (1/1200 dilution; PeproTech) in blocking solution overnight at 4°C. After intensive washes with PBS/0.05% Tween 20, the immunolabeled bands were detected using a peroxidase-conjugated anti-donkey secondary Ab (1/5000; 90 min at room temperature). An ECL Western blotting kit (Amersham Biosciences) was used for chemoluminescence visualization with Kodak X-Omat AR film.

Migration assays for leech and human microglial cells

Migration assays for leech microglia were assessed using the double-P assay according to the method of Kühnert et al. (23) with some minor modifications. Briefly, petri dishes (35-mm diameter) were filled with 1 ml of 0.5% agar and 1% gelatin in PBS and cooled for 2 h at room temperature. Two 6-mm-diameter containers were excised, with each comprising a parallel individual channel 0.75-mm wide and 20-mm long and spaced 15 mm apart. One container was filled with 50 μl of purified leech microglial cells (1×10⁶), and the second was filled with 50 μl of human recombinant EMAPII at 0.01 ng/ml, 1 ng/ml, 100 ng/ml, or 1 μg/ml. A thin perpendiculicular incision was made with a clean coverslip between the two parallel channels to create a bridge equidistant to the two containers. Migrating cells present in the chemoattractant containers were collected after 30 min and counted on a Malassez slide. The chemoattractant effect of the EOP slide was also assessed for the human microglial cell line CHME3. Cells were cultured at 37°C in a humidified 5% CO₂ atmosphere in DMEM (Invitrogen) supplemented with 2 mM l-glutamine, 10% heat-inactivated fetal calf
medium, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The medium was replenished every 3 days. Migration assays were performed using the Thincerts transwell system (8-μm pore size; Greiner Bio-One). A total of 300 μl of 3 × 10^5 cells/ml was placed in the upper chamber and 700 μl of serum-free medium containing various concentrations of EMAPII (0.01 ng/ml, 1 ng/ml, 100 ng/ml, or 1 μg/ml) was placed in the lower chamber. Serum-free medium alone and a filtered (4°C) solution of Ag or microgram of Ab) were used as negative controls. Medium containing 100 ng/ml IP-10 (CXCR-10; PeproTech) was used as a positive control. To determine the role of the CXCR3 receptor in the migratory response, human or leech microglia cells incubated for 30 min in medium containing 10 μg/ml anti-CXCR3 Ab (R&D Systems) were also evaluated. The migration chamber was incubated for 6 h at 37°C. After scoring the upper face of the filter on an inverted bud, the filters were washed three times in PBS and fixed for 10 min at 4°C in methanol. Filters were stained for 15 min with Hoechst, and cells present on the lower surface of the filters were counted with a fluorescence microscope. All experiments were performed in triplicate. The results are expressed as the mean cell number ± SD. Statistical analyses were performed using a two-tailed Student’s t test.

cDNA cloning of Hmp43/EMAPII

For EST bank screening, the partial sequence encoding the putative protein Hmp43/EMAPII, which is highly similar to the human synthetase complex p43/EMAPII, was retrieved from the leech H. medicinalis nervous system database EST from the Hirudinea Genomics Consortium (Genoscope: www.cns.fr/externe/English/Projects/Project_PE/PE.html). The complete nucleotide sequence of Hmp43/EMAPII was obtained from 3’ and 5’ RACE using the detailed protocol below. Rapid amplification of 3’ and 5’ cDNA ends (RACE) and cloning were carried out in QiaGel by shaking the mixture with 1.4-mm ceramic beads (2 × 45 s, 6500 rpm) in a Precellys 24 homogenizer (Bertin; distributed by Ozyme). RNA extractions were performed according to the manufacturer’s instructions, and samples were treated for DNA contamination with RQ1 DNase1 (Promega). Specific sense (5’-CTCAAC TCAGTTACTCCTACTCA-3’) and antisense (5’-CGGCGTGATCTGGT-3’) primers were deduced from the 3’ and 5’ extremities of the cDNA sequence and cloned encoding the putative leech protein Hmp43/EMAPII. For 3’ RACE PCR, DNA (3 μg) was transcribed into single-stranded cDNA using the oligo(dT)_{18} adaptor primer (5’-CGATGCACATCTGCTG(TCA)_3’) and the SuperScript II kit (Invitrogen/BRL, manufacturer’s protocol). One-fourth of the reaction volume was amplified by PCR using the oligo(dT)_{18} adaptor primer and specific sense oligonucleotide. For 5’ RACE PCR, a 5’ cDNA library was constructed from RNA using the BD Smart Race cDNA amplification kit (Clontech, manufacturer’s protocol). A total of 5 μl of the 5’ cDNA was amplified by PCR using a 5’ terminal universal primer and an antisense-specific oligonucleotide. In each case, PCR’s were performed for 30 cycles using an Advantage 2 polymerase enzyme (Clontech) with an elongation time of 2 min. All PCR products were subcloned into a pGEM-T easy vector (Promega), and cDNA clones were sequenced on an ABI Prism 310 genetic analyzer (Applied Biosystems).

Gene expression analysis

Gene expression in purified cells. The ganglia from six isolated nerve cords were carefully decapsulated by removing the collagen layer that envelopes the nerve cord with microscissors. Neurons (>10 μm) and microglial cells (5 μm) were mechanically dissociated and resuspended in 200 μl of complete L-15 medium. The cells were then filtered through a 70-μm filter (70 μm pore size; Greiner Bio-One). A total of 40–100 ng per slide) were hybridized as previously described (24). As a control, antisense riboprobes were replaced with sense riboprobes.

Antisera

Because of the high conservation between the human and leech EMAPII, a commercially available anti-human EMAPII produced in donkey was used for our analysis (PreproTech).

Whole mount immunohistochemistry

Nerve cords, depleted or not of microglial cells, were fixed overnight at 4°C in 4% paraformaldehyde at different times after bacterial exposure. Immunohistochemistry was performed as previously described (20). Samples were inoculated with 1 ng/ml EMAPII or Ag at 1 ng/ml or 1 μg/ml. Nerve cords were fixed in 4% paraformaldehyde solution. Cells were then centrifuged on slides (12,000 neurons/slide) and fixed for 30 min at 4°C by addition of ice-cold 4% paraformaldehyde for the synthesis of the probes. Antisense and sense riboprobes were generated from linearized cDNA plasmids by in vitro transcription using an RNA labeling kit (Roche). Labeled riboprobes (40–100 ng per slide) were hybridized as previously described (24). As a control, antisense riboprobes were replaced with sense riboprobes.

Immunochemistry

Neurons dissociated from nerve cords incubated for 6 h with bacteria were fixed for 30 min at 4°C by addition of ice-cold 4% paraformaldehyde solution. Cells were then centrifuged on slides (12,000 neurons/slide) and immerses for 10 min in TBS (0.1 mol/L Tris (pH 7.5) and 0.9% NaCl). After fixation, the neurites were incubated in a solution containing 3% normal goat serum. Neurons were then incubated in TBS containing 3% normal goat serum, cells were incubated overnight at 20°C with mouse anti-HmTLR1 (1/4000) or goat anti-EMAPII Abs (1/100) in TBS containing 2% normal goat serum and 0.01% Triton X-100. Next, the cells were rinsed three times with TBS and incubated for 2 h at room temperature with Alexa Fluor 546 (red)-conjugated goat anti-mouse secondary Ab (Invitrogen) diluted 1/2000 in AB solution and Alexa Fluor 488 (green)-conjugated rabbit anti-donkey secondary Ab in AB solution (1/4000) for 1 h at 37°C. As a control, the immunolabeling procedure was conducted using the preimmune serum for HmTLR1 and a filtered (4 μm) solution of goat anti-hEMAPII Ab previously saturated with the hEMAPII recombinant protein overnight at 4°C (1 μg of Ag per microgram of Ab). To examine the cell distribution at sites of injury, nuclear staining was performed by incubating the slices for 30 min with Hoechst 33342 dye. Slices were then mounted in Glycergel and examined using a confocal microscope (Zeiss LSM 510) or an inverted microscope (Leica DMI2RE).

HmTLR1 RNA interference

HmTLR1 gene silencing was adapted from the procedure described by Baker et al. in the leech embryo (26). After 4 days of culture, ganglia from the nerve cords of three animals with the same treatment (control or 10 μg of HmTLR1 dsRNA) were pooled, rapidly transferred into 1 ml of Qiazol (Qiagen) solution. Cells were then centrifuged on slides (12,000 neurons/slide) and frozen for 30 min at −80°C until use. As the HmTLR1 gene is induced upon microbial challenge, gene silencing was performed in the nerve cords incubated with 5 μl of a mix of heat-killed Gram-positive (Micrococcus nishinomiyaensis) and Gram-negative (Aeromonas hydrophila) bacteria at 3 × 10^7 CFU/ml. Bacteria were added 6 h before the end of the culture period. RNA extraction, cDNA synthesis, and quantitative RT-PCR were performed as previously described (13). For Northern blot analysis, cDNA (1 μg) was transcribed from total RNA, and the probes used for HmTLR1 quantification were specifically designed to recognize a zone of the gene outside of the dsRNA sequence (forward primer, 5’-CGAGCAAACTCGCTGCCAACAC-3’ and reverse primer, 5’-TTGGAGTCTATCATGCGG-3’). The 18S RNA was used as a reference gene (18S forward, 5’-TGGCTTAGTTCTGGTTCTGT-3’ and reverse, 5’-TCAGCCGTGATCTGGT-3’). Real-time reactions were conducted on an Applied Biosystems 7500 using a hot start, followed by 40 cycles at 94°C for 15 s, 56°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 3 min. Analysis of the relative gene expression data was performed using the ΔΔCt method. For each primer pair, a plot of the log cDNA dilution vs ΔCt, was generated to validate the quantitative PCR results (data not shown).
ATTAGACCGGCAA-3'; reverse primer, 5'-CTGCGGTTAAATGCTTGC-3'. Primers used for HmEMAPII and 18S rRNA (internal standard) detection have been previously described. The slopes of the regression lines for HmTLR1 and HmEMAPII were 0.0664 and 0.0166, respectively, suggesting equivalent amplification efficiencies compared with the 18S gene (data not shown).

Visualization of HmTLR1 protein levels by Western blot. Proteins were extracted from the protein platelet that forms during RNA extraction, according to the manufacturer's instructions. The protein extracts were subjected to electrophoresis in a 12% SDS-polyacrylamide gel and electrophoretically transferred onto a nitrocellulose membrane. Samples were probed with a mouse polyclonal anti-HmTLR1 Ab (1/4000, overnight at 4°C). Immuno-reactive bands were detected with a peroxidase-conjugated goat anti-mouse secondary Ab (1/5000, 1 h at room temperature; Jackson ImmunoResearch Laboratories) and the SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Scientific).

Results

**Molecular evidence of HmAp3, a p43-related molecule, in the leech CNS**

The complete nucleotide sequence of HmAp3 was obtained using a two-step PCR approach to amplify from the cDNA library prepared from the leech CNS (Fig. 1A). Analysis of the amino acid sequence deduced from the cDNA showed the presence, as in mammals, of a tRNA-binding domain in the leech sequence (residue 157–258), confirming that HmAp3 belongs to the aminoacyl ARNt synthetase family. As unexpected, phylogenic analysis revealed that the leech p43 is evolutionarily closer to the sequences of p43 characterized in deuterostomians, such as echinoderms, amphibians, or mammals, than those described in protostomians (Fig. 2B). Among the p43 proteins identified in protostomians, including insects and nematodes, the leech HmAp3 sequence therefore appears to be the highest conserved ortholog to mammalian molecules.

**HmEMAPII is processed from HmAp3 in the leech CNS**

Interestingly, alignments denote that the most conserved part of HmAp3 is located at the C-terminal region, which corresponds to a proinflammatory cytokine designated EMAPII in mammals (Fig. 2A). To determine whether, as in vertebrates, HmEMAPII could be detected from HmAp3, Western blot analysis of leech extracts was performed using the Multalin software (www.bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html). B, The phylogenic tree deduced from the alignment of the p43 sequences obtained using the phylogeny software web service available at www.phylogenic.fr.

**FIGURE 1.** Characterization and processing of the leech HmAp3/EMAPII to release HmEMAPIII. A, Nucleotide sequence of HmAp3 cDNA: Duced amino acid sequence of the open reading frame corresponds to the 318-aa HmAp3/EMAPII precursor. The characteristic t-RNA binding domain is underlined, and HmEMAPII is indicated in bold. B, Western blot analysis was performed using total protein extracts from the leech nervous system or entire animals (whole body). Immunostaining with an anti-EMAPII Ab revealed two bands of ~18 and 35 kDa, corresponding to HmEMAPII and HmAp3 mass predictions, respectively. Equivalent well loading was assessed by a generic protein coloration of the gel (Coomassie blue).

**FIGURE 2.** Alignment and phylogenic analysis of HmAp3/EMAPII. A, HmAp3 was compared with the p43 sequences identified in the protostomians Caenorhabditis elegans (Q20970) and Drosophila melanogaster (NP610426) and in the deuterostomians Strongylocentrotus purpuratus (XP10923337), Xenopus laevis (NP001080110), Mus musculus (P31230), and Homo sapiens (Q129720). Alignment was performed using the Multalin software (www.bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html). B, The phylogenic tree deduced from the alignment of the p43 sequences obtained using the phylogeny software web service available at www.phylogenic.fr.

Interestingly, alignments denote that the most conserved part of HmAp3 is located at the C-terminal region, which corresponds to a proinflammatory cytokine designated EMAPII in mammals (Fig. 2A). To determine whether, as in vertebrates, HmEMAPII could be detected from HmAp3, Western blot analysis of leech extracts was performed using an anti-EMAPIII Ab. As presented in Fig. 1B, two bands were identified at molecular masses of 35 kDa and 18 kDa. These are consistent with the predicted masses deduced from the cDNA of the HmAp3 precursor (35,185.37 Da) and the cleaved form HmEMAPIII (18,746.70 Da), respectively, indicating for the first time in an invertebrate the release of HmEMAPII from its precursor.

Initially, the cleavage and release of EMAPII in mammals was proposed to be mediated by enzymes synthesized during apoptosis, such as caspases (27). Our data for the leech suggest that the maturation process does not require the occurrence of any apoptotic conditions, since the mature form of EMAPII was immunodetected...
in the whole body of the leech as well as in untreated and bacterially challenged CNS (Fig. 1B).

The gene expression site of Hmp43/EMAPII

To better understand the physiological role of Hmp43/EMAPII, localization of the gene expression site was investigated by ISH and RT-PCR on purified neural cells (Fig. 3). Interestingly, Hmp43/EMAPII was expressed in neurons and in microglial cells.

The chemoattractant role of HmEMAPII on microglial cells

EMAPII as well as p43 have been described to participate in the recruitment of vertebrate polymorphonuclear leukocytes and mononuclear phagocytes (28). Microglial cells are monocyctic cells that form a unique population of brain-resident macrophages. We hypothesized that EMAPII could exert a chemoattractant effect on microglial cells similar to its effect on monocytes. As presented in Fig. 4, the ability of this molecule to recruit leech or human microglial cells was investigated by conducting chemotaxis assays with different concentrations of EMAPII protein. The high amino acid conservation between leech and human EMAPII convinced us to use the human recombinant EMAPII for all chemotaxis experiments. The experiments demonstrated that EMAPII dose-dependently enhanced the migration of purified leech microglial cells (Fig. 4A) and a human microglial cell line (Fig. 4B) at a concentration comparable to that required for monocyte recruitment. The chemoattractant effect of EMAPII was blocked when an anti-EMAPII Ab was added to the culture medium, confirming the participation of EMAPII in this biological process. These findings demonstrate for the first time the ability of EMAPII to exert a chemotactic effect on microglial cells, suggesting that this cytokine participates in the recruitment of these cells in the injured CNS of both leeches and mammals. In the mammalian CNS, the inducible chemokine receptor CXCR3, reported to bind IP-10/CXCL10, has been implicated in the migratory response of microglial cells (29). Interestingly, at the peripheral level, EMAPII is known to induce the migration of endothelial progenitor cells by activating this receptor. Our data presented in Fig. 3 demonstrate a chemoattractant property of IP-10 comparable to that of EMAPII on both leech and human microglia. This observation was corroborated by the observed inhibition of the chemoattractant properties of EMAPII on microglia upon addition of an anti-CXCR3 blocking Ab to the culture medium, allowing us to establish a novel relationship between EMAPII and CXCR3 expressed by microglial cells.

The Hmp43/EMAPII gene is inducible following trauma and bacterial challenge of the leech CNS

The level of Hmp43/EMAPII transcript was quantified by real-time RT-PCR in the injured leech CNS (Fig. 5). In a first set of experiments, axotomized nerve cords maintained in culture up to 8

FIGURE 3. Analysis of Hmp43/EMAPII and HmTLR1 gene expression sites in nerve cords and in isolated neural cells. FISH with Hmp43/EMAPII (A and B) and HmTLR1 antisense riboprobes (C and D) from glia deficient nerve cords (A and C) and from isolated neural cells (B and D). Nerve cords depleted of microglial cells were obtained by opening the capsule surrounding the ganglia, which makes the neurons visible. Transcripts were detected in the cell bodies of neurons “n” and in microglia “µ”. No signals were detected with the Hmp43/EMAPII (E) and HmTLR1 (F) sense riboprobes. Hmp43/EMAPII and HmTLR1 transcripts were amplified by RT-PCR from the CNS, from purified neurons, and from purified microglial cells confirming the data obtained by FISH (G); “c” corresponds to the nonamplification control of the PCR.

FIGURE 4. Dose-dependent chemoattractive effect of EMAPII on leech (A) and human microglial cells (B). A Student’s t test confirmed the significant migration of microglial cells in response to EMAPII at the optimal concentration of 100 ng/ml for both leech and human cells. Serum-free medium (L-15 or DMEM) and anti-EMAPII Ab were used as negative controls. The implication of CXCR3 in the cell recruitment was estimated through the use of IP-10, a CXCR3 agonist, and an anti-CXCR3 Ab. Results are presented as the percentage increase above control ± SD (n = 5).
gene expression was significantly increased when axotomized nerve cords were incubated for 6 h with a controlled number of Gram-negative or Gram-positive bacteria. No variation was observed in the presence of mannan or zymosan, a yeast component. In contrast, LPS, a motif expressed on the surface of Gram-negative bacteria, and muramyl dipeptide, a peptidoglycan constituent of both Gram-positive and Gram-negative bacteria, appear to be inducers of Hmp43 gene expression, suggesting that the induction is microorganism-specific. Altogether, these results suggest that the up-regulation of Hmp43/EMAPII gene expression could result from two responses, a response triggered by microorganisms that takes place a few hours postchallenge and a bacteria-independent response that requires more time after injury to be activated (Fig. 5C).

Accumulation of Hmp43/EMAPII at the lesion site of the bacterially challenged CNS

To clarify the quantitative PCR data, the localization of the production site was also investigated by immunohistochemical approaches. Double staining of challenged nerve cords was performed at $t = 0$ h and $t = 6$ h postaxotomy using Hoechst 33258 and the anti-EMAPII polyclonal Ab (Fig. 5A). In contrast to the CNS fixed immediately after dissection (Fig. 6Aa) or with nerve cords cultured for 6 h under aseptic conditions (Fig. 6D), nerve cords fixed 6 h after septic challenge displayed a strong immunoreactivity at the lesion site (Fig. 6G), along with an accumulation of microglial cells as revealed by nuclear staining with Hoechst 33258 (Fig. 6I). Microglial cells are resident cells that are evenly distributed in leech ganglia and in the bundle of axons that connect them. After damage to the CNS, these cells have been shown by Müller and colleagues to migrate to the site of the lesion, where they accumulate (18). There, microglial cells phagocytose damaged tissues and produce laminin as well as antimicrobial peptides, which are known to promote the regenerative process (30). Immunodetection of Hmp43/EMAPII at the axotomized site, where microglial cells accumulate, is consistent with the chemoattractant properties of this molecule on this cell population.

To verify whether the accumulated Hmp43/EMAPII is synthesized by the recruited microglial cells themselves and/or produced by neurons and axonally transported to the lesion site, whole mount immunohistochemistry was performed on leech CNS-depleted of microglial cells (Fig. 7). Under these conditions, the accumulation of microglial cells, which is normally apparent (Figs. 6, F and I, and 8B) at the axotomized site, failed to occur (Figs. 7, F and I, and 8C). As presented in Fig. 7G, this depletion appeared to significantly reduce but not completely abolish the accumulation of Hmp43/EMAPII at the site of axotomy. Confocal microscopy analyses (Figs. 6G and 7G) more precisely revealed the presence of Hmp43/EMAPII in the cell bodies of neurons and in the axons of the injured connectives, suggesting a neuronal production that is presumably followed by the axonal transport of the chemotactant substance to the lesion site. The decrease of the signal suggests that under normal conditions, Hmp43/EMAPII may be produced by the recruited microglial cells. This hypothesis was confirmed by double staining with both the anti-tp43/EMAPII Ab and the mAb 9A8, a marker for leech glial cells (Fig. 8, A and B).

Thus, the presence of HmEMAPII at the axotomized site indicates a neuronal synthesis and a production by microglial cells.

The microbial induction of Hmp43/EMAPII is under the control of the HmTLR1 signaling pathway

Having determined the gene expression site and demonstrated the conditions for its up-regulation, we next sought to characterize the danger signal receptor associated with HmEMAPII gene induction.

### Diagrams

**FIGURE 5.** Quantification of the levels of expression of Hmp43/EMAPII in leech nerve cords by real-time PCR analysis using the $\Delta\Delta C_{t}$ method. A. Analyses of RNA levels were assessed during the neural repair of the leech CNS under sterile conditions. A significant and persistent induction of the Hmp43/EMAPII gene beginning 24 h postaxotomy was observed. B. Analysis of the RNA levels was also evaluated in axotomized nerve cords cultured for 0 and 6 h in the presence or not (control) of various microbial components. The results showed a significant induction of the Hmp43/EMAPII gene in response to LPS stimulation. The graphs show the mean ± SEM of triplicate samples (*, $p < 0.05$; $t$ test). Reference (18S) and targets were amplified in separated wells ($n > 10$ in all cases). LTA indicates lipoteichoic acid; MDP, muramyl dipeptide. C. Hypothetical scheme illustrating the two responses implicated in the up-regulation of Hmp43/EMAPII gene expression in the leech CNS.

The experiment was conducted with leech axotomized nerve cords incubated for 6 h in the presence of different microbial components (Fig. 5B). This time point was selected to distinguish the effects due to the presence of microorganisms from those related to the trauma itself. As illustrated in Fig. 5B, Hmp43/EMAPII days postaxotomy were cultured under sterile conditions (Fig. 5A). Quantitative PCR data indicated a persistent enhancement of Hmp43/EMAPII transcript levels starting 24 h postaxotomy up to 8 days. No variation was observed 6 h posttrauma. Thus, in absence of microorganisms, the gene induction of Hmp43/EMAPII appears to be controlled by physiological events, requiring >6 h to react to CNS trauma.

The same experiment was conducted with leech axotomized nerve cords incubated for 6 h in the presence of different microbial components (Fig. 5B). This time point was selected to distinguish the effects due to the presence of microorganisms from those related to the trauma itself. As illustrated in Fig. 5B, Hmp43/EMAPII accumulation of microglial cells, which is normally apparent (Figs. 6, F and I, and 8B) at the axotomized site, failed to occur (Figs. 7, F and I, and 8C). As presented in Fig. 7G, this depletion appeared to significantly reduce but not completely abolish the accumulation of Hmp43/EMAPII at the site of axotomy. Confocal microscopy analyses (Figs. 6G and 7G) more precisely revealed the presence of Hmp43/EMAPII in the cell bodies of neurons and in the axons of the injured connectives, suggesting a neuronal production that is presumably followed by the axonal transport of the chemotactant substance to the lesion site. The decrease of the signal suggests that under normal conditions, Hmp43/EMAPII may be produced by the recruited microglial cells. This hypothesis was confirmed by double staining with both the anti-tp43/EMAPII Ab and the mAb 9A8, a marker for leech glial cells (Fig. 8, A and B).

Thus, the presence of HmEMAPII at the axotomized site indicates a neuronal synthesis and a production by microglial cells.

The microbial induction of Hmp43/EMAPII is under the control of the HmTLR1 signaling pathway

Having determined the gene expression site and demonstrated the conditions for its up-regulation, we next sought to characterize the danger signal receptor associated with HmEMAPII gene induction.
We recently characterized a TLR in the leech nervous system called HmTLR1 (31) present in the neural cell types, which expressed Hmp43/EMAPII (Fig. 3, C, D, and G). Immunohistochemical procedures (Fig. 6) performed on axotomized nerve cords incubated with bacteria showed an increase in the amounts of HmTLR1 and Hmp43/EMAPII present in the axotomized sites of the CNS (Fig. 6H, g and h). No changes were observed in lesioned nerve cords cultured under sterile conditions (Fig. 6E, d and e). These observations suggest that bacterial activation of this receptor may trigger the gene expression of Hmp43/EMAPII.

To confirm the role of HmTLR1 in Hmp43/EMAPII induction, the gene encoding HmTLR1 was silenced (Fig. 9). Incubation of the nerve cords with HmTLR1 dsRNA reduced the expression of HmTLR1 at both the transcriptional (Fig. 9A) and translational levels (Fig. 9B), as shown by RT-PCR and Western blot analysis, respectively. The efficiency of the gene silencing was also quantified in the lesioned CNS incubated for 6 h with bacteria. It was indeed necessary to check whether the gene induction of HmTLR1 observed in the presence of bacteria was abolished to evaluate the link between HmTLR1 and the bacterially induced gene expression of Hmp43/EMAPII. Real-time RT-PCR results demonstrated a reduction of the HmTLR1 messenger to a quantity comparable to the control samples (Fig. 9C). We found that when the HmTLR1 gene expression was silenced, Hmp43/EMAPII gene induction was abolished, since the level of expression in bacterially challenged CNS was similar to that of the controls (6 h vs 6 h + bacteria), thereby confirming the success of the gene silencing even under conditions of induction. The expression level of Hmp43/EMAPII was then investigated in the knock-down samples (Fig. 9D). We observed that when the HmTLR1 gene expression was silenced, Hmp43/EMAPII gene induction was abolished, since the level of expression in bacterially challenged CNS was similar to that of the controls (6 h vs 6 h + bacteria). Taken together, these data suggest that Hmp43/EMAPII gene expression is linked to the activation of an HmTLR1 pathway. This novel finding indicates a link between a danger signal receptor and an infection- and lesion-inducible cytokine that exerts chemoattractant activity.

Discussion

The data presented herein demonstrate the existence of an infection- and lesion-inducible cytokine that exerts chemoattractant activity.
properties toward microglial cells in the CNS of an invertebrate. Interestingly, we found that under septic conditions, *Hmp*43/EMAPII gene expression is under the control of the HmTLR1 signaling pathway.

Using molecular approaches, an ortholog of the vertebrate complex p43/EMAPII, *Hmp*43/EMAPII, was fully characterized from the leech CNS. Phylogenic studies showed that *Hmp*43/EMAPII was more closely related to its deuterostomian than to its protostomian counterparts. Interestingly, sequence alignment of the *Hmp*43/EMAPII protein also revealed an important conservation between the leech and human molecules at the C-terminal region, which corresponds to the EMAPII domain. Our data indicate that, as in mammals, the leech p43 is cleaved to form free EMAPII. Even if p43 proteins are widespread in the animal kingdom, this result constitutes the first evidence of the release of the mature form, EMAPII, in an invertebrate. The detection of the cleaved HmEMAPII suggests the existence of an enzymatic activity in the leech that is implicated in this maturation process. In mice, some IL-converting enzymes (ICE), notably caspases 3 and 7, have initially been proposed to be responsible for the cleavage of p43 during the apoptotic cascade (13, 32). Controversially, Schwarz and colleagues first demonstrated that human p43 is not a substrate for caspase cleavage and that this cleavage is likely due to the activation of the cysteine protease cathepsin L (33, 34). These findings led the authors to think that mature EMAPII is not a byproduct of apoptosis. In the medicinal leech, the processing also appears not to require any apoptotic conditions, as it was observed byproduct of apoptosis. In the medicinal leech, the processing also appears not to require any apoptotic conditions, as it was observed in vivo under basal conditions and appears to be likely due to a cysteine protease based on the lysyl residues present in the N-terminal region of the mature HmEMAPII. Interestingly, analysis of the EST library of the leech CNS revealed the presence of a highly conserved cathepsin L-like cysteine peptidase. Future investigations will be conducted to determine the implications of the cathepsin L-like cysteine peptidase in the release of HmEMAPII in the leech CNS.

The biological function of HmEMAPII in the leech CNS was then investigated. In mammals, EMAPII is considered to be a cytokine that exerts chemoattractant activity toward macrophagic cells (13). Given that the microglia constitutes the resident macrophagic cell population in the leech and mammalian CNS, the role of EMAPII was examined by conducting chemotaxis assays using *Hirudo* and human microglia. Our data revealed a dose-dependent migratory response of both populations in response to the human hEMAPII recombinant protein, with a maximal effect at the same concentration. The maximum migratory response of both leech and human microglia to the same concentration of human EMAPII was examined by conducting chemotaxis assays using *Hirudo* and human microglia. Our data revealed a dose-dependent migratory response of both populations in response to the human hEMAPII recombinant protein, with a maximal effect at the same concentration. The maximum migratory response of both populations in response to the human hEMAPII recombinant protein, with a maximal effect at the same concentration. The maximum migratory response of both populations in response to the human hEMAPII recombinant protein, with a maximal effect at the same concentration. The maximum migratory response of both populations in response to the human hEMAPII recombinant protein, with a maximal effect at the same concentration.
detected in a leech CNS EST library (our unpublished data). Additional experiments will be performed to verify whether the receptor identified in our model is implicated in the microglia recruitment by EMAPII. To our knowledge, these data are the first to show the ability of hEMAPII to exert a chemotactic effect on leech and human microglial cells, thus attributing a function to this cytokine in the human brain that is consistent with the reported accumulation of EMAPII in microglial cells of the injured human brain (11). There is evidence that other molecules serve as chemotactic properties of EMAPII. ATP, NO, and the molecule C1q have been shown to be responsible for activating the movement of microglia in the leech CNS (35–37). Due to the redundancy of such molecules, blocking EMAPII by the addition of the anti-EMAPII Ab did not have any apparent impact on the neural repair (data not shown).

A significant and persistent increase in the level of Hmp43/EMAPIII transcript starting 24 h postaxotomy was observed during the course of neural repair, suggesting that Hmp43/EMAPII plays a role throughout the regenerative process. Interestingly, in the presence of a controlled number of bacteria, the amount of messenger was more rapidly enhanced. Altogether, these observations suggest that the up-regulation of Hmp43/EMAPIII gene expression is the result of two distinct responses, one triggered by microorganisms, which takes place a few hours postchallenge, and the other as part of a response to cellular damage and requiring more time after injury to become activated. This observation is similar to the distinct innate immune responses to infection and wounding of the epidermis of C. elegans recently shown by Pujol et al. These authors showed that the up-regulation of antimicrobial peptide gene expression upon infection is separate from that upon injury, but that both responses converge in a common pathway (38).

Having determined the gene up-regulation upon microbial challenge, we then sought to further characterize the implication of HmEMAPIII in neural repair by localizing its production site. Immunohistochemistry analyses indicated an increase in the amounts of HmEMAPIII in the challenged CNS. This enhancement occurred principally at the axotomized sites, but only in the presence of bacterial components. No change was observed under sterile conditions at 6 h. HmEMAPII is produced at the lesion site where microglial cells accumulate. This accumulation is known to be the result of cell migration and does not reflect microglial cell division in response to leech CNS injury (39). Consequently, the production of Hmp43/EMAPIII at the site where microglial cells are massively recruited supports the theory that this cytokine may exert its chemoattractant properties toward microglia in vivo. Recruited microglia may also favor the migration process by producing the cytokine themselves, as revealed by the immunocytochemistry data. The detection of Hmp43/EMAPIII in the microglial cells accumulated at the injured site of the leech CNS emphasizes some similarities of the inflammatory response of the nerve cord in our model with that of the human brain (11). In the leech, however, neuronal cells also contribute to the production of HmEMAPIII, as demonstrated in the preparation of leech CNS depleted of microglial cells. By favoring the recruitment of microglial cells to the axotomized site, EMPIII indirectly contributes to neural repair and to the antimicrobial response of the leech CNS. Indeed, recruited microglial cells have been described to participate in the phagocytosis of damaged tissues and in the regeneration process by producing laminin, an extracellular matrix molecule known to promote neurite outgrowth and antimicrobial peptides, which exert neurotrophic activities (20, 40).

In many species, including invertebrates and vertebrates, molecular components of a particular group of pathogens can be recognized by TIR-domain containing receptors (TLRs) that act as immune sentinels. Their activation triggers an intracellular signaling pathway, followed by the up-regulation of defense genes. Interestingly, our group has recently characterized the TLR HmTLR1, which was the first TLR characterized in H. medicinalis. The complete amino acid sequence of HmTLR1 presents a high percentage of homology with the mouse TLR13, the functions of which are still unknown. Silencing of the HmTLR1 gene in the CNS demonstrated that upon microbial challenge, this receptor is involved in the induction of the gene encoding Hmp43/EMAPIII. These data are reminiscent of some observations of rat microglial cells, which have been reported to produce EMAPII after systemic injections of TLR agonists, such as polyinosine-polycytidylic acid (a TLR3 ligand) and R848 (a TLR7/8 ligand) (33).

The regulation of EMAPII by a TLR in both leech and mammals reinforces the great conservation between these two models. Moreover, the existence of an immunity mediated by a TLR in the leech CNS shows for the first time an immune function of a TLR in a non-ecdysozoan model (i.e., in an invertebrate model that is different from C. elegans and D. melanogaster). This functional information, recently described as lacking in important lineages such as cnidarians or lophotrochozoans by Leulier and Lemaitre, could help to draw a scenario of the emergence of TLRs as sensors of microorganisms in invertebrates (41).

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**Disclosures**

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**References**


