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MIF from mussel: Coding sequence, phylogeny, polymorphism, 3D model and regulation of expression

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ABSTRACT

Three macrophage migration inhibitory factor (MIF)-related sequences were identified from a *Mytilus galloprovincialis* EST library. The consensus sequence included a 5′-UTR of 32 nucleotides, the complete ORF of 345 nucleotides, and a 3′-UTR of 349 nucleotides. As for other MIFs, *M. galloprovincialis* ORF does not include any signal or C-terminus extensions. The translated sequence of 115 amino acids possesses a molecular mass of 12,681.4, a pI of 6.27 and a stability index of 21.48. Its 3D structure resembles human MIF except for one shorter α-helix. Although evolutionary separated from ticks and vertebrates, *Mg*-MIF appeared to be closely related to *Pinctada fucata* and *Haliotis*, but not to *Chlamys farreri* and *Biomphalaria glabrata*. Numerous mutation points were observed within the *Mg*-MIF ORF, defining 11 amino acid variants within the mussels from Palavas-France and 14 amino acid variants within the mussels from Palermo-Italy. The 2 major variants from Palavas were identical to 2 of the 4 major variants from Palermo. In all the 18 *Mg*-MIF variants, residues involved in tautomerase and in oxidoreductase activities were conserved. Generally, one mussel expressed 2 *Mg*-MIF amino acid sequences but with different frequencies of occurrence. *Mg*-MIF is constitutively expressed principally in hemocytes and in the mantle. In contrast to other animal models, *Mg*-MIF expression was always down regulated following challenge by bacteria and fungi, confirming previous data obtained with microarray. Down regulation started as soon as 1 h and *Mg*-MIF expression returned to background 9–48 h after the challenge. Exception was regarding the yeast, *Candida albicans*, down-regulation between 9 and 72 h, suggesting yeast and bacteria-filamentous fungi trigger different mechanisms of elimination.

1. Introduction

Cytokines are soluble mediators that play significant roles in immune reactions by means of inter-cellular/molecular processes. Numerous cytokines have been reported from diverse vertebrate species (reviewed by http://www.copewithcytokines.de for instance). The presence of cytokine-like activity or of related mRNA have also been observed in nearly all invertebrate species, including tunicates, echinoderms, annelids, nematodes, arachnids and insects (reviewed by (Beschin et al., 2001)). A recent paper reveals that the expression of a TNF α-like gene is involved in ascidian inflammatory responses (Parrinello et al., 2008). In mollusks, first indirect evidences of cytokines were obtained by cross-reactions with anti-cytokine antibodies, *in vitro* activities of vertebrate cytokines on invertebrate cells and activity of invertebrate sera on vertebrate cells (reviewed by (Canesi et al., 2006; Ottaviani, 2006)). To date, numerous EST-related to cytokines have been reported from the Pacific oyster, *Crassostrea gigas* (Gueguen et al., 2003; Roberts et al., 2008, 2009), the Mediterranean mussel, *Mytilus galloprovincialis* (Venier et al., 2003), the Bay scallop, *Argopecten irradians irradians* (Song et al., 2006) and the Antarctic bivalve, *Laternula elliptica* (Park et al., 2008b). Among these, only 2 cytokines have been analyzed. The first was TNF-α-related factor from the Zhikong scallop, *Chlamys farreri*, the expression of which was up-regulated in hemocytes after 3 h *in vitro* contact with LPS (Yu et al., 2007), and from *C. gigas*, also up-regulated 12 h following *in vivo* challenge with a cocktail of four pathogenic *Vibrio* strains (Park et al., 2008a). The second was IL-17 from *C. gigas*, with rapid accumulation of transcript following challenge by immersion in a cocktail of heat-killed bacteria (Roberts et al., 2008, 2009).

Macrophage migration inhibitory factor (MIF) is a highly conserved protein, which exerts wide-ranged activities in vertebrates.

Abbreviations: EST, expressed sequence tags; *Mg*-MIF, macrophage migration inhibitory factor from *Mytilus galloprovincialis*; ORF, open reading frame; UTR, untranslated region.

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It is a central mediator of innate immunity and has been shown to correlate with regulation of macrophage functions (Onodera et al., 1997), lymphocyte immunity (Abe et al., 2001) and a number of immune and inflammatory diseases (del Vecchio et al., 2000). As a consequence, more than 1000 scientific papers have been published on MIF, which is a ubiquitous mediator functioning as a cytokine, hormone or enzyme (Swope and Lolis, 1999). Its neuroendocrine role as mediator that increases the host response to microbial endotoxins suggested that MIF is at the crossroads between endocrine and immune systems (Bacher et al., 1998; Lafuente et al., 2009; Prieto-Lafuente et al., 2009). Indeed, MIF contributes to broad-spectrum immune and inflammatory responses (Chaisavaneeyakorn et al., 2005; Oddo et al., 2005) and is acting in immune evasion of some parasitic nematodes by modifying the activity of host cells (Pastrana et al., 1998; Wu et al., 2003). In addition, MIF exhibits several unusual features that distinguished this factor from typical cytokines, for example enzymatic thiol-protein oxidoreductase and tautomerase/isomerase activities (Kleemann et al., 1998).

MIF homologues have been detected in numerous animal species, from nematodes, ticks and echinoderms to jawless and jawed fishes, amphibians and chickens (Javeed et al., 2008), suggesting that this molecule has been conserved over 1 billion years of evolution. Only 4 cDNA sequences related to MIF have been reported so far from gastropods (Mitta et al., 2005; Wang et al., 2009a) and 2 from bivalves (Cui et al., 2011; Wang et al., 2009b). In addition, the role of MIF in the immune response against bacteria or parasites has been investigated only in the gastropods, *Haliotis diversicolor* (Wang et al., 2009a) and *Biomphalaria glabrata* (Baeza Garcia et al., 2010), and in the bivalve, *Pinctada fucata* (Cui et al., 2011).

In the present report, we (i) used 3 putative MIF sequences identified from a *M. galloprovincialis* EST library to establish the complete coding sequence and its phylogenetic relationships, (ii) constructed a 3D molecular model of the deduced protein, (iii) searched for polymorphism of *Mg*-MIF within and between *M. galloprovincialis* populations of distant geographic origins, and (iv) quantified the *Mg*-MIF gene expression in various tissues and in hemocytes following *in vivo* challenges of the mussels.

2. Materials and methods

2.1. Mussels and hemocyte collections

Adult mussels, *M. galloprovincialis* (6–7 cm shell length), were purchased in July 2008 from the marine farm Les Compagnons de Maguelone, Palavas-France. Other mussels were purchased from Fazio Vivaio Miticoltura, Palermo-Italy, during July–September 2009. They were acclimated for 24 h in a flow-through system of oxygenated seawater before sampling. Hemolymph (0.8 ml per mussel) was collected from the posterior adductor muscle with a 1 ml syringe containing 0.2 ml of the anti-coagulant modified Alsever's solution buffer (Torreilles et al., 1999). Hemocytes were pelleted by 10 min centrifugation at 800*g*, 4 °C.

2.2. RNA extraction, reverse transcription, PCR and cDNA cloning

Total RNA was extracted according to the Trizol Reagent protocol (Invitrogen), resuspended in 20 μl of sterile distilled water and concentrations were measured on spectrophotometer ND-1000 (NanoDrop Technologies). First strand cDNAs were synthesized from 1 μg of total RNA using hexaprimers (Invitrogen) and murine leukemia virus reverse transcriptase (Promega), and purified with Wizard SV Gel and PCR Clean-up System (Promega) then kept in sterile distilled water at −20 °C until use. Forward F2 and reverse R3 PCR primers were hand-designed according to the consensus sequence constructed based on MGC03559, MGC08770 and MGC08785 (Fig. 1) from the *M. galloprovincialis* EST database Mg_NOR01 (Venier et al., 2009). PCR program included initial denaturing of 35 ng cDNA template by 2 min at 94 °C, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The unique amplicon of 598 nucleotides was cloned in the plasmid pCR II TOPO from the TOPO TA Cloning kit (Invitrogen). Plasmids were isolated using Wizard Plus SV Miniprep (Promega) and specificity of inserts has been confirmed by sequencings performed by Millegen (Labèges-France). Transformed *Escherichia coli* colonies were individually transferred to deep agar containing Luria-Bertoni medium and 50 μg/ml kanamycin, in 96 well microtiter plates and sent to Agowa GmbH (Berlin-Germany) for sequencing using M13 as universal primers. Each clone was double strand sequenced and the sequences corrected accordingly.

Regarding tissue expression patterns, total mRNA was extracted from hemocytes, and from dissected gills, hepatopancreas, mantle, muscle and foot from 12 mussels, and then mixed as 3 pools of 4 mussels for each tissue. Reverse transcription and PCR conditions were performed as above but with primers F5 and R1 (Fig. 1). Resulting amplifications of 202 base pairs were run in 1.5% agarose gel. BET stained bands were photographed using the BioRad Gel-Doc XR and scanned under the AlphaEaseFC software (Alpha Innotech). Results are presented as the arithmetic mean of optical density of the 3 pools ± SD. Data were analyzed with the Student's *t*-test for statistically significant difference ($p < 0.05$).

2.3. Analysis of cDNA and of deduced amino acid sequences

Several adjustments and comparisons have been made: (i) the nucleotides from up-stream and down-stream the primers, including the primer sequences, were removed, (ii) all the sequences were aligned using Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html) and clustered according to nucleotide sequences, (iii) UTRs were removed and coding sequences compared using Multalin, (iv) the different coding sequences were translated into pro-peptides (http://www.expasy.ch/tools/dna.html), and (v) the resulting amino acid sequences compared using Multalin. Isoelectric points and instability index were calculated by http://au.expasy.org/cgi-bin/protparam.html. Signal peptide prediction was analyzed using the SignalP-3.0 Server available at http://www.cbs.dtu.dk/services/SignalP/.

2.4. Phylogenetic relationships and structural analysis

Sequences related to MIF from invertebrates and vertebrates were subjected to multiple alignments using Clustal-W program. Final sequence alignments were done using CLUSTAL-X v.1.81 29, the similarity shaded with GeneDoc v.2.6.002. A phylogenetic tree was constructed by the Neighbor-Joining method after 1000 bootstrap iterations using CLC workbench 6.4. The protein structural model was developed using the SWISS-MODEL and the Swiss-PDB Viewer (Arnold et al., 2006) using the human model as comparison (Sun et al., 1996).

2.5. Challenges with bacteria and fungi

Hemocyte cDNA samples of mussels challenged with one injection of bacteria (*Vibrio splendidus* LGP32, *Vibrio anguillarum*, *Micrococcus lysodeikticus*) were those previously used for analysis of lysozyme gene expression (Li et al., 2008). Fungal challenge consisted of one injection of 50 μl of PBS–NaCl (2 mM KH_2HPO_4,