Characterization of a defensin from the oyster Crassostrea gigas. Recombinant production, folding, solution structure, antimicrobial activities, and gene expression.

Yannick Gueguen, Amaury Herpin, André Aumelas, Julien Garnier, Julie Fievet, Jean-Michel Escoubas, Philippe Bulet, Marcelo Gonzalez, Christophe Lelong, Pascal Favrel, et al.

To cite this version:

Yannick Gueguen, Amaury Herpin, André Aumelas, Julien Garnier, Julie Fievet, et al.. Characterization of a defensin from the oyster Crassostrea gigas. Recombinant production, folding, solution structure, antimicrobial activities, and gene expression.. Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology, 2006, 281 (1), pp.313-23. 10.1074/jbc.M510850200 . hal-00286204

HAL Id: hal-00286204
https://hal.archives-ouvertes.fr/hal-00286204
Submitted on 31 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Copyright
Characterization of a Defensin from the Oyster *Crassostrea gigas*

RECOMBINANT PRODUCTION, FOLDING, SOLUTION STRUCTURE, ANTIMICROBIAL ACTIVITIES, AND GENE EXPRESSION

Received for publication, October 5, 2005, and in revised form, October 20, 2005. Published, JBC Papers in Press, October 24, 2005, DOI 10.1074/jbc.M510850200

Yannick Gueguen1,2, Amayur Herpin1,3, André Aumelas1, Julien Garnier1, Julie Fievet1, Jean-Michel Escoubas1,4, Philippe Bulet1,5, Marcelo Gonzalez1,2, Christophe Lelong5, Pascal Favrel3, and Evelyne Bachère6

From the 1Ifremer, CNRS, Université de Montpellier II, UMR 5171, Génomé Populations Interactions Adaptation, 2 Place E. Bataillon, CC80, F-34095 Montpellier cedex 5, France, the 2Laboratoire de Biologie et Biotechnologies Marin, Institut de Biologie Fondamentale et Appliquée, UMR 101 Ifremer, Université de Caen, Physiologie et Ecophysiology des Mollusques Marin, 14032 Caen cedex, France, the 3Centre de Biochimie Structurale, CNRS UMR 5048, INSERM U554, CNRS UMR5048, Université Montpellier I, 29 rue de Navacelles, F-34090 Montpellier Cedex, France, **Atheris Laboratories, Case Postale 314, CH-1233 Bernex-Geneva, Switzerland, and the *SARS International Centre for Marine Molecular Biology, High Technology Centre, 5008 Bergen, Norway

In invertebrates, defensins were found in arthropods and in the mussels. Here, we report for the first time the identification and characterization of a defensin (Cg-Def) from an oyster. Cg-def mRNA was isolated from *Crassostrea gigas* mantle using an expressed sequence tag approach. To gain insight into potential roles of Cg-Def in oyster immunity, we produced the recombinant peptide in *Escherichia coli*, characterized its antimicrobial activities, determined its solution structure by NMR spectroscopy, and quantified its gene expression in vivo following bacterial challenge of oysters. Recombinant Cg-Def was active in vitro against Gram-positive bacteria but showed no or limited activities against Gram-negative bacteria and fungi. The activity of Cg-Def was retained in vitro at a salt concentration similar to that of seawater. The Cg-Def structure shares the so-called cystine-stabilized α-β motif (CS-αβ) with arthropod defensins but is characterized by the presence of an additional disulfide bond, as previously observed in the mussel defensin (MGD-1). Nevertheless, despite a similar global fold, the Cg-Def and MGD-1 structures mainly differ by the size of their loops and by the presence of two aspartic residues in Cg-Def. Distribution of Cg-def mRNA in various oyster tissues revealed that Cg-def is mainly expressed in mantle edge where it was detected by mass spectrometry analyses. Furthermore, we observed that the Cg-def messenger concentration was unchanged after bacterial challenge. Our results suggest that Cg-def gene is continuously expressed in the mantle and would play a key role in oyster by providing a first line of defense against pathogen colonization.

Antimicrobial peptides (AMPs) are important components of the innate immune system that have been conserved during evolution (1). They constitute a first line of host defense against pathogens in plants and animals (2, 3). We estimate that more than 1000 antimicrobial peptides have been described at the level of their primary structure (2). They are gathered in the Antimicrobial Sequence Database (www.bbcm.units.it/~tossi/amsdb.html). AMPs can be classified into three major groups: (i) linear peptides that form amphipathic α-helices, (ii) cyclic peptides containing cysteine-residue engaged in disulfide bonds, and (iii) peptides with an overrepresentation in certain amino acids (proline, arginine, glycine, or histidine). Despite their great diversity in terms of size, primary structure, amino acid composition, and mode of action, most AMPs are characterized by the preponderance in cationic and hydrophobic amino acids (2). In most of the cases, this amphipathic character is considered as crucial for the interaction of the effective peptide with the membrane of sensitive microorganisms. This first interaction seems to be essential whatever the exact mode of action: (i) through disruption of their negatively charged cytoplasmic membranes or (ii) through killing following translocation into the bacteria without membrane lyses and binding to a specific target protein (4). Depending on their tissue distribution, AMPs ensure either a systemic or a local protection of the organism against pathogens.

Among the AMPs, defensins represent an important peptide family. They are abundant and widely distributed in human and animal tissues that are involved in host defense against microbial infection. Defensins are compact cationic peptides, ~3–5 kDa in size, containing three or four disulfide bridges, and are active against a wide range of bacteria and...
fungi (5). The vertebrate defensins can be grouped into three subfamilies, the α-defensins and β-defensins, which are distinguished on the basis of the connectivity of their six cysteine residues, and more recently the cyclic θ-defensins, (6). The α-defensins are produced constitutively and stored in neutrophils of many animals and in human Paneth cells. β-Defensins, which have been identified in many cell types, including epithelial cells and neutrophils, were reported to be inducible or constitutively expressed (3, 7, 8). In mammals, apart from their antimicrobial activities, defensins play an important role in inflammation, wound healing, and regulation of specific immunity reactions (9). In contrast with the classification of the vertebrate defensins, based on their secondary structure, the grouping in clear distinct subfamilies of the invertebrate defensins is based on their biological properties, antibacterial versus antifungal (2). The invertebrate defensins differ from the vertebrate defensins by their disulfide bridging, (10). Defensins are the most widespread family of invertebrate AMPs, and >70 different defensins have been isolated in arthropods (insects, ticks, spiders, and scorpions) and mollusks (2). Most of the insect defensins were isolated from the hemolymph of experimentally infected animals, whereas in scorpions, termites, and mollusks, defensins are present in granular hemocyes of non-infected animals (2). In mollusks, AMPs have only been reported in bivalves, such as in the mussels Mytilus edulis (11) and Mytilus galloprovincialis (12). Interestingly, defensins from the Mediterranean mussels have been found to display sequence homology to defensins from arthropods, even though they belong to distant phylogenetic groups. Until now, in oyster, antimicrobial activities have been detected in the hemolymph of some species, however, AMPs have never been fully characterized despite many attempts to purify, from hemolymph and other tissues, such molecules by using RP-HPLC approaches (13).

In the present study, we report the characterization of the first AMP in oyster. The Crassostrea gigas defensin (Cg-Def) mRNA has been isolated from mantle edge using the expressed sequence tag approach. Cg-Def displays sequence homology with members of the arthropod defensin family and shares with the defensins (MGD-1 and -2) from the mussel *M. galloprovincialis*, a fourth pair of cysteine residues. To shed light on the evolutionary conservation of defensins in invertebrates and the function of this peptide in the defense reactions of the oyster, we expressed Cg-Def in *Escherichia coli* and determined, using the refolded peptide, its spectrum of activity against a panel of bacteria and fungi. To draw some structure-function features, the three-dimensional structure of the Cg-Def was determined in aqueous solution by 1H NMR spectroscopy and molecular modeling, and then compared with MGD-1, its counterpart from mussels. Finally, the Cg-Def gene expression was analyzed in response to experimental bacterial challenge of oysters.

**EXPERIMENTAL PROCEDURES**

*Animals and RT-PCR Amplification of Cg-def—*Adult oysters, *C. gigas*, were purchased from a local oyster farm in Normandie (France) or Palavas (Gulf of Lion, France) and kept in seawater at 15 °C. A cDNA encoding part of a putative defensin was identified by randomly sequencing clones from a *C. gigas* mantle edge cDNA library constructed in A-ZAP II (University of Caen). Specific primers were then used to isolate the full-length Cg-def cDNA from the same library. Briefly, the 5′ missing end of the transcript was identified using the oriented C. gigas mantle edge cDNA library as template and using a gene-specific oligonucleotide (Cg-DefR1: 5′-ACCAGAGCGTGGCT-GACATCAC-3′) and a vector-specific oligonucleotide (T3: 5′-AAT-TAACTCCATACAAAGG-3′) as primers. PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gel. Fragment of the expected size was extracted from the gel using a Qiagen kit, cloned into the pGEM-T easy vector using a TA cloning kit (Promega, Madison, WI), and sequenced.

*Screening of C. gigas Genomic Library—*A genomic library of *C. gigas* was constructed in λ-DASH II (Stratagene). A total of 1.8 × 10⁹ independent clones was recovered. After amplification to 4.5 × 10⁹ plaque-forming units/ml, 2.5 × 10⁹ recombinant λ-DASH phages were plated at 5 × 10⁶ per dish, adsorbed to nitrocellulose membranes, and screened at high stringency with digoxigenin-11-dUTP-labeled 322-bp encoding the full-length Cg-def cDNA. Positive clones were purified (QiaGen kit, Courtaboeuf, France) and subjected to restriction analysis and Southern blot hybridization using the original probe to confirm that the λ-DASH clones contained Cg-def-specific sequence. Genomic organization of positive clones was subsequently determined by direct sequencing. Exon/intron boundaries were determined by comparing the genomic sequence to the corresponding cDNA one.

*Determination of Cg-def Expression in Different Tissues—*Total RNA was isolated from adult tissues using Tri reagent (Sigma-Aldrich) according to the manufacturer’s instructions. After treatment during 20 min at 37 °C with 1 unit of DNase I (Sigma-Aldrich) to prevent genomic DNA contamination, 1 µg of total RNA was reverse transcribed using 1 µg of random hexanucleotides primers (Promega), 0.5 mM dNTPs, and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega) at 37 °C for 1 h in the appropriate buffer. The reaction was stopped by incubation at 70 °C for 10 min. Then, quantitative RT-PCR analysis was performed using the iCycler apparatus (Bio-Rad). iQ™ SYBR Green supermix PCR kit (Bio-Rad) was used for real-time monitoring of amplification (5 ng of template cDNA, 40 cycles: 95 °C/15 s, 60 °C/15 s) with the following primers: QsDefF 5′-CCCAATACGTCTGATCACATCA-3′ and QsDefR 5′-TTCTCTACCCATAGTGT-3′, primers, a parallel amplification of oyster GAPDH transcript (EMBL CIG548886) was carried out to normalize the expression data of the Cg-def transcript. The relative level of Cg-def expression is calculated for 100 copies of the GAPDH housekeeping gene following the formula: n = 100 × 2^(Ct GAPDH − Ct Cg-def).

*Recombinant Expression of Cg-def—*Recombinant Cg-Def was expressed in *E. coli* as an N-terminal His₉-tagged fusion protein using the pET-28a system (Novagen, Madison, WI). By PCR amplification, a Met-coding trideoxynucleotide was incorporated 5′ of the Cg-def cdNA and cloned in-frame with the N-terminal His₉ in the EcoRI/Sall sites of pET-28a. The Cg-def coding cdNA sequence was amplified by PCR using forward primer DefFw1 (5′-GGCCGAATTCATGGATT-TGGGTGTCCGGG) paired with reverse primer DefRv1 (5′-ATATATGTCGACTTACTTCTTTCCATTACAATCGG). The reaction was performed by incubating the reaction mixtures at 94 °C for 4 min, followed by 15 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min for 26 cycles using Isi™ DNA polymerase (Qbiogene). The underlined codon in the forward primer denotes a Met codon to incorporate a CNbr cleavage site immediately upstream of the N terminus of the designed peptide according to the method described for recombinant mouse α-defensin production (14).

Recombinant Cg-Def was expressed in *E. coli* Rosetta (DE3) pLysS cells (Novagen) transformed with the pET-28a/Cg-Def construct. The cells were grown at 37 °C to A₆₀₀ 0.9 in Luria-Bertani (LB) medium (10 g of bacto-Tryptone, 5 g of bacto yeast extract, and 10 g of NaCl) supplemented with 50 µg/ml kanamycin. Expression of fusion proteins was induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside. After

---

**Note:** The content is from a scientific journal article and is presented in a natural text format without additional analysis or annotations. The text is focused on the isolation, characterization, and expression of a defensin from the oyster *Crassostrea gigas*. It includes details on methods for cloning and expression, as well as initial characterization of the defensin. The article is likely discussing its potential role in the oyster’s defense mechanisms. The text is technical and specific to the field of molecular biology and immunology.
growth at 37 °C for 3 h, bacterial cells were harvested by centrifugation and stored at −20 °C. The cells were lysed by resuspending bacteria pellets in 6 mM guanidine HCl in 100 mM Tris-HCl, pH 8.1, followed by sonication at 40% amplitude for 2 min using a Vibra cell Sonifier 450 (Branson Ultrasonics, Ammanesse, France). The lysate was clarified by centrifugation in a Sorvall SA-600 rotor at 10,000 × g for 30 min at 4 °C prior to protein purification.

**Purification and Folding of Recombinant Cg-Def**—His-tagged Cg-Def fusion protein was purified by affinity chromatography by incubating cell lysates with nickel-nitrilotriacetic acid resin (Novagen) at a ratio of 25:1 (v/v) in 6 mM guanidine HCl, 20 mM Tris-HCl (pH 8.1) for 4 h at 4 °C. Fusion proteins were eluted with two column volumes of 6 mM guanidine HCl, 1 M imidazole, 20 mM Tris-HCl (pH 6.4), dialyzed against 5% acetic acid (HOAc) in SpectraPor dialysis membranes (Spectrum Laboratories Inc., Rancho Dominguez), and lyophilized. The methionine residue introduced at the Cg-Def N terminus was subjected to CNBr cleavage by dissolving the lyophilized His6 fusion proteins in 50% formic acid, addition of solid CNBr to 10 mg/ml (final concentration), and incubation of the mixtures for 8 h in the darkness at 25 °C. The cleavage reaction was terminated by adding 10 volumes of water, followed by freezing and lyophilization. Then, the cleaved fusion peptide mixture was reduced in 100 mM Tris-HCl buffer at pH 8 in the presence of 100 mM dithiothreitol. The reaction mixture was purified to homogeneity using a C8 reverse-phase high performance liquid chromatography (RP-HPLC), and the fractions of interest were lyophilized. The peptide was resolved by RP-HPLC step using the same column and eluting conditions as mentioned above. Alternatively, the cleaved fusion peptide mixture was directly folded at pH 8.1 in a buffer solution containing 0.1 M NaHCO3 and 3 mM reduced and 0.3 mM oxidized glutathione in the presence of 2 mM urea and 25% N,N-dimethylformamide (15). Then, the folded Cg-Def was purified to homogeneity by RP-HPLC using a C8 column, as described above. Peptide purity was controlled by acid-urea PAGE, and the peptide concentration was estimated by amino acid analysis and UV absorption at 280 nm based on the extinction coefficients of the molecule. Molecular masses of the purified peptides were determined using matrix-assisted laser desorption ionization mode mass spectrometry (MALDI-TOF MS) and electrospray ionization mass spectrometry (ESI-MS).

**Antimicrobial Assays**—Antimicrobial activity of recombinant Cg-Def was assayed against several bacteria, including the Gram-positive Micrococcus lysodeikticus, Staphylococcus aureus, S. hemolyticus, Bacillus megaterium, Brevibacterium flavum, Microbacterium maritimum, the Gram-negative E. coli 363, Vibrio alginolyticus, and Salmonella typhimurium. The activity of the peptide was also investigated against the following filamentous fungi Fusarium oxysporum, Botrytis cinerea, and Penicillium crustosum. Minimum inhibitory concentrations (MICs) were determined in duplicate by the liquid growth inhibition assay based on the procedure described by Hetru and Bulet (16). Poor broth (PB: 1% bacto-Tryptone, 0.5% NaCl w/v, pH 7.5) nutrient medium was used for standard bacteria, and saline peptone water (1.5% peptone, 1.5% NaCl, pH 7.2) was used for marine bacteria. Antifungal assay was performed in potato dextrose broth (Difco, Sparks, MD) at half strength supplemented with tetracycline (10 µg/ml final concentration). Growth was monitored spectrophotometrically at 620 nm on a Multiscan microplate reader colorimeter (Dynatech).

Bactericidal or bacteriostatic effect was measured by CFU counting following a 15-h incubation at 30 °C. A bactericidal kinetic was assayed with M. lysodeikticus. Ten microliters of purified peptide, at a concentration 10 times over the MIC value, was mixed with 90 µl of an exponential phase culture of M. lysodeikticus at a starting A600 of 0.01 in PB nutrient medium and incubated at 30 °C. Aliquots of 10 µl were plated after 0 min, 2 min, 10 min, 90 min, and 15 h of incubation on nutrient agar plates. The number of CFUs was established after a 15-h incubation at 30 °C. As a control, the bacterial culture was incubated with 10 µl of sterile water.

**NMR Spectroscopy**—NMR samples of Cg-Def were prepared either in a 95:5 H2O:D2O mixture (v/v) or in 99.98% D2O to yield a 0.6–0.8 mM solutions. The pH was adjusted to the desired values by addition of deuterated HCl or NaOD and measured at room temperature with a 3-mm electrode. They are given uncorrected for the deuterium isotopic effect. Proton chemical shifts were referenced with respect to sodium 4,4-dimethyl-4-silapentane-1-sulfonate according to the IUPAC recommendations. 1H NMR experiments were performed on a Bruker Avance 600 spectrometer equipped with a triple resonance cryoprobe, and spectra were recorded in the temperature range of 10–30 °C. In all experiments, the carrier frequency was set at the center of the spectrum at the water frequency. Double-quantum filtered-correlated spectroscopy (DQF-COSY) (17, 18), z-filtered total-correlated spectroscopy (z-TOCSY) (19, 20), and nuclear Overhauser effect spectroscopy (NOESY) (21) spectra were acquired in the phase-sensitive mode using the States-TPPI (time proportional phase incrementation) method (22). For spectra recorded in H2O, the water resonance was suppressed by the WATERGATE method (23), except for the DQF-COSY spectra where a low power irradiation was used. The z-TOCSY spectra were obtained with a mixing time of 90 ms and NOESY spectra with mixing times of 100, 150, and 300 ms. Data were processed by using the XWINNMR software. The full sequential assignment was achieved using the general strategy described by Wüthrich (24). To identify the slow exchanging amide protons, the sample was dissolved in D2O at 25 °C, and first a TOCSY (80 mn) and then an NOESY (180 mn) experiment was recorded for their identification.

**Structure Calculation and Analysis**—The NOESY cross-peaks were measured from two NOESY spectra (pH 3.25, at 20 and 25 °C) with a mixing time of 150 ms, and subsequently divided into five classes, according to their intensities. Very strong, strong, medium, weak, and very weak NOEs were then converted into 2.5, 3.0, 3.5, 4.0, and 5.0 Å distance constraints, respectively. The ϕ angle restraints were derived from the 3JHN-CaH coupling constants, and the χ1 angle restraints were derived from the combined analysis of the 3JHα-Hβ,β′ coupling constants and intra-residues NOEs, respectively. To calculate the three-dimensional structures, distance and dihedral angle restraints were used as input in the DYANA program that uses simulated annealing combined with molecular dynamics in torsion angle space (25). In the first stage of the calculation, an initial ensemble of 20 structures was generated from a template structure with randomized ϕ,ψ dihedral angles and extended side chains. In preliminary calculations, neither hydrogen bond nor disulfide bond was used as restraint. Hydrogen bonds were considered as present if the distance between heavy atoms was less than 3.5 Å and the donor-hydrogen-acceptor angle was greater than 120°. Finally, to calculate the Cg-Def three-dimensional structure, 456 NOE-derived distances and 13 dihedral constraints, including the cis conformation of the Cy3–Pro5 amide bond (ω = 0°) and the disulfide bonds were used as input. A calculation of 60 conformers was carried out, and the resulting 10 structures with a minimum of restrained violations (no violation of >0.3 Å) were analyzed with INSIGHT 97.
C. gigas Oyster Defensin

ulation Inc., San Diego, CA). The Ramachandran analysis was performed with PROCHECK (26), and the limits of the secondary structure elements and the van der Waals surfaces were determined with STRIDE (27). The chemical shifts and coordinates of Cg-Def are deposited in the BioMagResBank (BMRB entry: 6849) and the Protein Data Bank (PDB entry: 2B68), respectively.

Analyses of C. gigas Mantle by RP-HPLC and MALDI-TOF MS—Mantle tissue from 200 bacteria-challenged C. gigas oysters was collected, rapidly frozen with liquid nitrogen, and ground to fine powder. Then, the mantle sample was diluted in 10% acetic acid, homogenized using an ultra-turax, and left at 4 °C under gentle stirring for 15 h. Extracts were centrifuged at 8000 × g for 20 min at 4 °C, and the supernatant was prepurified by solid-phase extraction on Sep-Pak C18 cartridges (Waters Associates) equilibrated with acidified water (0.05% trifluoroacetic acid). After washing with acidified water, peptides were eluted with 100% acetonitrile containing 0.05% trifluoroacetic acid. The Sep-Pak fraction was then lyophilized, reconstituted in ultrapure water, and loaded onto a C8-reversed-phase high performance liquid chromatography (RP-HPLC) on an UP10C8 column (Interchorm modulocart uptisphere 10 C8, 250 × 10 mm), and elution was performed using a 0–55% acetonitrile gradient developed over 30 min at a flow rate of 5 ml/min. Fractions were hand collected, lyophilized, resuspended in 100 µl of ultrapure water, and then assayed for antimicrobial activity. Finally, the active fractions were purified in a third step by C18 RP-HPLC, on a Symmetry Shield RP18 column (Waters, 5 µm, 250 × 250) using a 0–45% acetonitrile gradient developed over 45 min at a flow rate of 1 ml/min. The corresponding fractions were then analyzed by MALDI-TOF MS.

Bacterial Challenge and Quantification of Cg-def Gene Expression—Adult C. gigas oysters were stimulated by bath with heat-killed bacteria (5 × 106 bacteria/liter). The stimulation was performed with three bacterial species, M. lysodeikticus, Vibrio splendidus, and V. anguillarum. The bacterial strains were grown separately overnight, in saline peptone water at 25 °C for Vibrio strains and in Luria-Bertani (LB) medium for M. lysodeikticus (30 °C). Bacteria were collected by centrifugation (10,000 × g, 5 min) and suspended in fresh growth medium. Bacteria concentration was calculated from the optical density at 550 nm (1 unit of A550 corresponds to 5 × 108 bacteria/ml). Mantle tissue samples (100–300 mg) were then collected at two times post-stimulation (24 and 48 h) and washed in sterile seawater, cut into small pieces, and incubated overnight at 4 °C in TRIzol reagent (1 ml/100 mg of tissue). Total RNAs were extracted following manufacturer instructions (Invitrogen) and treated with DNase Turbo (Ambion). The experiments were done in triplicate and, to minimize individual variability, at least ten oysters were used in each experimental condition.

Following heat denaturation (70 °C for 5 min), reverse transcription was performed using 1 µg of total RNA prepared with 50 ng/µl oligo(dT)12–18 in a 50-µl reaction volume containing 1 mM dNTPs, 1 unit/µl RnaseOUT™ (Invitrogen), and 200 units/µl murine mammary tumor virus reverse transcriptase in reverse transcriptase buffer. PCR amplifications were performed with the LightCycler® (Roche Applied Science) in the presence of SYBR-Green™ (Master SYBR Green™) with the following primers: Defm1, 5′-GATGTTTCTCG-AGACATGG-3′ and Defm2, 5′-CACAGTGACCCCCGTCTCACA-3′ as sense and antisense primers, respectively. The gene encoding the elongation factor (EF, GenBank™ AB122066) was used as internal control. For EF the forward and reverse primers were EF (5′-ATGCA-CCAGGCGTGCAAGAAA-3′) and EFR (5′-TCCGACGATTCTTTGGGATGT-3′), respectively. Samples were run under the following conditions: 95 °C (10 min); then 39 cycles of 95 °C (10 s), 62 °C (5 s), and 72 °C (15 s). For further expression level analysis, the crossing points were determined for each transcript using the LightCycler software. Specificity of RT-PCR product was analyzed on agarose gel and melting curve analysis. The copy ratio of each analyzed cDNA was determined as the mean of three replicates. The relative expression ratio of Cg-def was calculated based on the crossing points deviation of each RT-PCR product of RNA extracted from stimulated oyster versus the appropriate control sample and expressed in comparison to the reference gene EF. The relative expression ratio of Cg-Def was calculated based on the delta-delta method for comparing relative expression results (28).

RESULTS

Cg-def cDNA Cloning and Primary Structure Analyses—The complete cDNA of C. gigas defensin (Cg-Def) was obtained by 5′ amplification of cDNA ends-PCR on a C. gigas mantle edge cDNA library, as described under “Experimental Procedures” (GenBank™ AJ565499). The oyster Cg-def cDNA contained 323 bp, comprising a coding region of 195-bp, a 110-bp 3′ untranslated region containing a polyadenylation consensus sequence (AAATAA) at position 66–72 of the 3′ UTR, and a poly(A) tail. The 195-bp coding region encoded a 65-amino acid propeptide (GenBank™ CA192980). The deduced amino acid sequence starts with a 22-residue signal peptide, and the cleavage site for signal peptidease is most likely located after the alanine residue preceding the glycine in position 23 as predicted by SignalP 3.0 software (data not shown). Cg-Def is not synthesized as a precursor with a C-terminal extension nor an N-terminal pro-region as observed with the Mediterranean mussel or the Drosophila defensins, respectively. The amino acid sequence of the mature peptide was aligned with other defensins from the “arthropod defensin family” available in GenBank™ and ExPASy, that contains defensins from arthropods, mollusks, and fungi (Fig. 1, A and B). As already observed for the mussel defensins MGD-1 and MDG-2, Cg-Def is an original member of this family due to the presence of two extra cysteine residues. Cg-Def shares 50% of identity with MGD-1 from M. galloprovincialis, but has a unique doublet of lysine residue at the C-terminal part of the molecule (Fig. 1A). Less identity was shared with defensin from the scorpion Androctonus australis (46%) and the tick Ixodes scapularis (44%). The selected members of the “arthropod defensin family” belonging to the so-called primitive defensin family show a high degree of conservation with both Cg-Def and the different mussel defensins (Fig. 1A) (29). Interestingly, very recently, a 40-amino acid AMP named pectacin (GenBank™ number CA183768) with marked homologies to the arthropod and mollusk defensins has been isolated from the saprophytic fungus Pseudoplectania nigrella.

Genomic Organization and Analysis of the Promoter Region—Cg-def gene (EMBL AM050547) harbors a unique intron whose splice junction follows the AG-GT rule (Fig. 1C). The genomic organization of Cg-def is similar to that of the mussel and scorpion defensin genes (29). It also displays the features of the other arthropod and mollusk defensin genes studied so far that demonstrate a genetic relatedness: (i) the exon encoding the mature defensin is never split by any intron, (ii) the intron flanking the exon encoding the mature defensin shows for all genes a strict level of phase conservation (phase 1) (29). Analysis of the 3.6-kb genomic DNA sequence upstream from the putative ATG start codon identified several consensus sequences for transcription factors commonly observed in promoters of cellular housekeeping genes (Sp1), genes expressed during early embryogenesis (AP2, Gata1, and Gata2), development (Zeste), cell cycle regulation (H1TF2, H4TF1, and H4TF2), and cell differentiation (Gata2, Iki, Ik2, Ik3, IRF2, and Oct1, -2, -4, -6, and -9) and genes expressed in a tissue-specific manner (Pit-1 and SRF).
Cg-Def was expressed in E. coli Rosetta (DE3) pLysS cells transformed with the PET-28a/Cg-Def construct. Recombinant His$_6$-tagged fusion protein was purified by affinity chromatography from the bacteria cell lysates. After chemical cleavage with CNBr and refolding, Cg-Def was purified by RP-HPLC, and its purity was judged by analytical RP-HPLC, MALDI-TOF, and ESI-MS mass spectrometry analyses (Fig. 2, A and B). The oxidative folding of Cg-Def was assessed under two different conditions. First, after complete reduction with dithiothreitol, purified denatured Cg-Def was refolded during 48 h at room temperature in the presence of a Tris-HCl, 100 mM, pH 8, buffer. The refolding process was followed by RP-HPLC revealing at 48 h an additional more hydrophobic peak eluting at 35 min (Fig. 2A, asterisk). By ESI-MS analyses, this hydrophobic fraction was found to contain a molecule with a molecular mass of 4,634.10 Da (see Fig. 2B1). The value measured by ESI-MS is in perfect agreement to the calculated average molecular mass of 4,634.34 Da. This suggests that the recombinant Cg-Def has eight cysteine residues paired. Alternatively, as described by Wu et al. (15), the use of N,N-dimethylformamide and urea was shown to enhance 5-times the folding efficiency of Cg-Def (data not shown). Using this protocol, the purified recombinant Cg-Def was also found to have a molecular mass measured by ESI-MS that is in perfect agreement to the calculated average molecular mass (see Fig. 2B1). The purified refolded peptide was then submitted to antimicrobial assays and NMR spectroscopy studies.

Antimicrobial Activity of Cg-Def—The antimicrobial activity of the recombinant Cg-Def was determined against a panel of microorganisms, including Gram-positive and Gram-negative bacteria and filamentous fungi. The MIC values obtained are reported in Table 1. The peptide was active at very low concentration against most of the Gram-positive bacteria, including M. lysodeikticus, S. aureus, and the marine bacteria Brevibacterium stationis and Microbacterium maritpicum. However, Cg-Def was not active at 20 μM against B. megaterium. Cg-Def showed no activity at 20 μM against the Gram-negative bacteria V. alginolyticus and S. typhimurium. At higher concentration (35 μM), the peptide was active against E. coli. Cg-Def displayed antifungal activity against F. oxysporum at relatively high concentrations (9 μM; Table 1). Experiments were conducted to examine the bacterial effects of Cg-Def. The bactericidal activity of the peptide was assessed by plating cultures, and the number of CFUs was counted after overnight incubation at 30°C. Cg-Def exerted bactericidal effects against all the Gram-positive bacteria tested, excepted M. maritpicum (Table 1). When M. lysodeikticus was incubated with Cg-Def at concentration 10 times higher than the MIC value, all bacteria were killed in few minutes (Table 2).

To determine if the high salinity of the seawater might modify the efficacy of Cg-Def in vivo, the effect of the peptide on bacterial growth was tested in vitro at NaCl concentrations ranging from 85 mM to 1 M. Cg-Def is highly active against M. lysodeikticus and B. stationis even at 600 mM NaCl, a concentration closed to the value measured in seawater. Interestingly, the MIC value is unchanged throughout the NaCl con-
concentration range tested (85–600 mM) (0.01 μM for M. lysodeikticus and 0.2 μM for B. stationis; Table 3). At 1 M NaCl, M. lysodeikticus did not grow in the control, and a low bacterial growth can be observed for the marine bacteria B. stationis. In contrast to most AMPs, Cg-Def seems to conserve its antibacterial activity at such a high salt concentration (30, 31).

NMR Structural Study—The two-dimensional NMR spectra (TOCSY, DQF-COSY, and NOESY) of Cg-Def were recorded at different temperatures, ranging from 17 to 32 °C. The identification of all the spin systems of Cg-Def was obtained by analysis and comparison of DQF-COSY, TOCSY, and NOESY spectra according to the strategy described by Wüthrich (24). Nevertheless, we noticed that the two-dimensional spectra showed, for each residue of the D38CNGK42 sequence, two spin systems approximately equally populated. This suggests a heterogeneity for this C-terminal part inferred to result from the partial deamidation to yield the Asp40 and isoAsp40 mixture. The Asn-Gly sequence (Asn40-Gly41) has been shown to experience the highest propensity for the deamidation process through the succinimide intermediate (32). Indeed, such a deamination process is known to occur and depend upon primary sequence, three-dimensional structure, and solution properties such as pH, temperature, ionic strength, and buffer ions (33). The two spin systems were assigned to the initial molecule (Asn40) and to the Asp40 analogue. These two similar spin systems were identified by recording TOCSY spectra at several pH values in the 2–5 pH range to monitor the ionization state change of the carboxyl group. The spin system sensitive to the ionization state change was assigned to Asp40. The other one, unaffected in this pH range, was assigned to Asn40. Only a small amount of the third expected compound (isoAsp40) was formed. It was detected in the TOCSY experiment but not fully characterized in the NOESY.

Chemical shifts of the recombinant Cg-Def are reported in the Table S1 provided as supplementary materials. Both amide and α protons

![Figure 2](http://www.jbc.org/)

**FIGURE 2.** Quality control of recombinant C. gigas defensin (Cg-Def) and detection of the natural form in acidic extracts of mantle tissue. 

A, following reduction, refolding of the recombinant peptide was performed at room temperature in the presence of Tris-HCl buffer. The refolding was monitored by RP-HPLC using an acetonitrile gradient in acidified water (for details see “Experimental Procedures”). The gray and black lines correspond to the linear and to the refolded (asterisk) recombinant peptides, respectively. The molecular mass of the refolded peptide was measured by ESI-MS (8) and MALDI-TOF MS (82). The molecular mass (average value) measured at 4,634.10 Da is in perfect agreement with the calculated average molecular mass at 4,634.34 Da. A difference of 3 Da was observed between the molecular masses measured using MALDI-TOF MS (m/z 4638.46, see B2) versus the one detected in ESI-MS at 4,634.10 Da (m/z 4,635.10). C and D, following purification by RP-HPLC of an acidic extract of mantle tissue, the two fractions active against the tested microorganisms were analyzed by MALDI-TOF MS. The first fraction (see the arrowhead in C) eluting at the same retention time as the recombinant-refolded Cg-Def contains ions of m/z 4,638.96 corresponding to the one measured by MALDI-MS for the recombinant peptide. In the second bioactive fraction (see the arrowhead in panel D), ions of m/z 4,631.45 were detected.
defined and displays the cystine stabilized-α-β motif that consists of an helical structure and two β strands cross-linked by three disulfide bonds (34) and sometimes by a fourth disulfide bond (35–37). The backbone atoms of the ten conformers were overlaid for the well defined regions spanning residues 4–39 resulting in a pairwise average r.m.s.d. of 0.43 ± 0.09 and 0.98 ± 0.16 Å for the backbone and heavy atoms, respectively. The Ramachandran plot of all residues (except for the glycine and proline residues) of the ten best conformers indicated that 89.7% were located in the most favored and the additional allowed regions, and 5.8% in the generously allowed regions. A total of 4.4% of the residues were found in the disallowed regions. Limits of secondary structure elements indicated that the Cg-Def structure mainly consisted of a helical part (8–18 residues), two β-strands (residues 22–26 and 33–37), and three loops (residues 1–7, 19–21, and 27–32 for loops L1, L2, and L3, respectively) (Figs. 1B and 3). Three successive type IV turns were identified in L1 (1–4, 3–6, and 4–7) and L3 (27–30, 28–31, and 29–32 loops), and a type I turn was found for the 26–29 sequence. It is interesting to note that the Cys8-Pro5 amide bond adopted the unusual cis conformation. Finally, the global fold is highly constrained by the 4–25, 11–34, 15–36, and 20–39 disulfide bonds. The Cg-Def helix exhibits an amphiphilic character with a hydrophobic side contributing to the hydrophobic core of the molecule and a hydrophilic side accessible to the solvent, including mainly, Lys10, Asn13, His14, Lys16, and Ser17 residues. Nevertheless, the Leu9 and Ile18 hydrophobic residues are solvent-exposed. The hydrophobic core mainly includes the four disulfide bonds, the Ala22 side chain and is extended to the L1 (Pro5) and L3 hydrophobic loops (Ala27, Ala28, Leu30, Trp31, and Leu32). As expected, the positively (Lys9 and Ile18) hydrophobic residues are solvent-exposed. The hydrophobic side mainly includes the four disulfide bonds, the Ala22 side chain and is extended to the L1 (Pro5) and L3 hydrophobic loops (Ala27, Ala28, Leu30, Trp31, and Leu32). As expected, the positively (Lys9, Lys16, Arg21, Arg33, Lys42, and Lys43) and negatively charged (Asp26 and Asp39) residues are solvent-exposed.

The Cg-Def structure was compared with that of MGD-1 (PDB entry: 1FJN) whose three-dimensional structure was previously determined (37). Their superimposition showed that the helical and β-strands elements were very conserved (Fig. 4).

Cg-def mRNA and Peptide Tissue Localization—To investigate the tissue distribution of Cg-def expression, we analyzed by real-time RTPCR mRNA content in various oyster tissues. Low or no Cg-def mRNA amount was measured in most of the organs analyzed, including hemocytes, heart, digestive gland, and gills, whereas a high mRNA level, ~20–60 fold the level measured in the remaining animal tissues, was detected in mantle (Fig. 5). In non-stimulated C. gigas oysters, the mantle appears as the main tissue expressing Cg-def transcripts.

To demonstrate the presence of native Cg-Def in mantle, an acidic extract of this tissue was prepared, subjected first to RP-HPLC and then to antibacterial activity screening. Following solid-phase extraction of the acidified extracts from oyster mantle, RP-HPLC
revealed the presence of two fractions with antimicrobial activity. These two fractions were subjected to molecular mass fingerprint analysis by MALDI-TOF MS. To ascertain the molecular mass measured, the well folded recombinant Cg-Def was used as external calibrant (see Fig. 2B2). A difference of 3 Da was observed between the molecular mass measured by ESI-MS (4,634.10 Da, panel B1) and the one measured by MALDI-TOF MS (4,637.46 Da, arrow in panel B2). The less hydrophobic fraction (see Fig. 2C) contains a component with a molecular mass at 4637.96 corresponding to that measured by MALDI-TOF MS for the recombinant folded Cg-Def. This peptide with antimicrobial activity against M. lysodeyiticus might likely correspond to the native form of Cg-Def. Interestingly, the second active fraction (more hydrophobic), that has not yet been identified, showed a close but different molecular mass of 4631.45 Da (see Fig. 2, arrow).

Real-Time PCR Analyses of Cg-def Transcript Levels after Oyster Bacterial Challenges—To determine the expression pattern of Cg-def during bacterial challenge, two batches of oysters were selected. In the first one, oysters were stimulated by bath with killed bacteria (see “Experimental Procedures”) and in the second one, non-stimulated oysters were used as control. Then, quantitative RT-PCR analyses were performed with total RNA extract from mantle collected at two times post challenge (24 and 48 h). No striking discrepancies were observed for Cg-def expression between naive and bacteria challenged oysters at the different times analyzed (Fig. 6). These preliminary analyses revealed that Cg-def mRNA are present in naive C. gigas oyster and that the level of transcript was unaffected by the bacterial-challenge performed.

**DISCUSSION**

Efficient host defense mechanisms are needed to neutralize microbial invasions to which living organisms are exposed. In higher vertebrates, innate and adaptive immunity are present. In contrast, invertebrate and plant defense against pathogens takes place exclusively through mechanisms that are part of the innate immunity. The involvement of AMPs in natural resistance to infection is sustained by their strategic location in phagocytes, in body fluids, and at the epithelial level, i.e. at interfaces between the organisms and its environment. In oyster, even if antimicrobial activities have been detected in the hemolymph of some species (38), no AMP has been fully characterized despite many attempts to purify such molecules by biochemical approaches from hemolymph and other tissues (13). In this report, and for the first time in an oyster, we describe the characterization of an AMP isolated from mantle tissue. Using molecular approaches, a new member of the widespread defensin family, which is present in animal and plant kingdoms, was identified. Usually, three disulfide bonds characterize this defensin family (Fig. 1A). The amino acid sequence of the mature C. gigas defensin displays interesting homologies with defensins from the “arthropod family,” including the mussel’s defensins. Moreover, as observed with the defensins MGD-1, MGD-2, and MGD-2b from M. galloprovincialis, Cg-Def has four disulfide bonds. This additional bridge was proposed to render the peptide more stable in high osmolarity media such as in seawater (37). However, this fact is not common to all marine mollusk defensins, because the mussel M. edulis defensins do not bear this additional disulfide bond (Fig. 1A) (11). Interestingly, Cg-Def possesses an N-terminal pro-segment presumed to be a signal sequence for translocation to the lumen of the rough endomembrane reticulum but has neither a C-terminal extension nor an N-terminal pro-region as observed in the mussel or the Drosophila defensins, respectively (39). This property would suggest a different way of processing for the oyster defensin.

To investigate its biological properties, Cg-Def was produced in E. coli and refolded in vitro. This recombinant approach was efficient, yielding enough material (~1 mg of well folded pure peptide per liter of culture) for functional assays and structural characterization. A challenging aspect for the production of Cg-Def in E. coli was the presence of

**FIGURE 3. Structure of Cg-Def.** Stereoview of the ten best conformers of Cg-Def. The 4–39 heavy atoms of the backbone were used for the superimposition. The mean pairwise r.m.s.d. is of 0.43 ± 0.09 Å. The four disulfide bridges are labeled and displayed as dashed lines. Hydrophobic, positively, and negatively charged side chains are colored in green, blue, and red, respectively.
eight cysteine residues in the mature peptide. Indeed, the correct refolding of proteins with several disulfide bonds is a general problem. As discussed by Harder et al., only few reports describe the production of AMP in bacteria, a fact that reflects the difficulty of expressing and folding such cysteine rich molecules in a bacterial host system (6). Previous reports described the production of human $\alpha$- and $\beta$-defensins in E. coli (6, 40). We report here for the first time the efficient production in a bacterial host of an AMP with four cysteine bridges that can be properly refolded \textit{in vitro}. The activity spectrum of the recombinant Cg-Def was evaluated against a panel of bacteria and fungi. Consistent with studies on invertebrate defensins (2), Cg-Def was active \textit{in vitro} against Gram-positive bacteria but showed no or limited activities against Gram-negative bacteria and fungi. Most interestingly, Cg-Def kept its antibacterial properties in the presence of high NaCl concentrations (up to 1 M). This favors the hypothesis that Cg-Def retains its activity in \textit{in vivo} conditions (seawater) and would play a role in the antimicrobial defense of the oyster C. gigas. For these reasons, Cg-Def represents a model of choice for structure-activity relationship studies to design a lead antibacterial drug to treat infections of bacteria in a salt-rich environment (41). This is particularly interesting for cystic fibrosis, for which it has been suggested that the primary genetic defect increases the salt content of fluid lining the airway, which reversibly inactivates antimicrobial molecules (42, 43).

As expected from the disulfide bridges array, the global fold of Cg-Def includes the CS-$\alpha\beta$ motif observed in the defensins isolated from mussels (37), insects (34), and plants (44). The Cg-Def and MGD-1 sequences were aligned, and their structures were compared (Figs. 1B and 4). This alignment revealed 50% of identity (23 residues with three gaps), including eight cysteines (45). Cg-Def and MGD-1 share the fourth disulfide bond that contributes to the stability and to the rigidity of their three-dimensional structure. The identical residues were mainly gathered in four segments (residues 1–5, 14–18, 23–25, and 32–36).
Beyond their sequence comparison the Cg-Def structure was compared with that of MGD-1 (PDB entry: 1FJN) (37). Their superimposition showed that the helical and α-strand elements were rather conserved and the best fit was obtained for the backbone superimposition of the residues 1–4, 7–17, 21–25, and 34–36 of Cg-Def with residues 1–4, 5–18, 21–25, and 34–36 of MGD-1 (23 residues). An r.m.s.d. of 1.04 Å was measured for the backbone. Moreover, the two structures share the Cys³-Pro⁵ cis-amide bond and a similar disulfide bond pattern. Nevertheless, some differences were observed for the Cys²⁰–Cys³⁰ disulfide bond and for the length of the three loops. Although the three equivalent disulfide bonds (4–25, 11–34, and 15–36 for Cg-Def and 4–25, 11–34, and 14–35 for MGD-1) well overlaid, the fourth one (Cys³⁰–Cys³⁹ for Cg-Def and Cys²¹–Cys³⁶ for MGD-1) is significantly different mainly due to the translation of Cys³⁰ by one residue toward the center of the molecule. This is certainly due to the shorter L3 loop (one residue less) joining the helix and the first β-strand. The consequence is a shift of this disulfide bridge that locates on the opposite side of the β-sheet (“above” in Cg-Def and “below” in MGD-1 as displayed in Fig. 4). Conversely, the L1 and L3 loops of Cg-Def display one additional residue. Whereas the sequences of the L1 loops, including the Cys³–Pro⁵ cis-amide bond are well conserved (Fig. 4), the sequences of the L3 loops are totally different. This latter is much more hydrophobic (³²GDIRKL³² for Cg-Def) than that of MGD-1 (³²GWIRKL³²) that contains two positively charged residues. Nevertheless, due to the Trp positions in the sequence (at the end for Cg-Def and at the beginning for MGD-1), it appeared that they have opposite location with regard to the loop. In contrast, the Leu³⁵(Cg-Def)/Leu³¹(MGD-1) side chains were similarly located (Fig. 4B). Because it has been shown that the L3 loop of MGD-1 is responsible for a large part of the antibacterial activity (46), this significant structural difference is worth being noted.

It is commonly admitted that the surface distribution of hydrophobic and hydrophilic side chains in AMPs is essential for their antimicrobial activity. The alignment showed that several of them (Phe³, Pro⁵, Ile⁵⁸, Tyr¹⁴, Leu¹⁵, and His¹⁹, Lys³⁳, and Arg³⁷) were conserved (Fig. 1 and see below). However, the two antimicrobial sequences mainly differ by the presence of two aspartic residues (Asp³⁰ and Asp³⁶) and by the addition of a dibasic peptide (Lys³⁵ and Lys³⁶) at the C terminus. Although, the Cg-Def and MGD-1 surfaces display significant differences mainly due to the two aspartic acids, the hydrophobic L3 loop and, by the “above”/“below” locations of the Cys²⁰–Cys³⁹/Cys²¹–Cys³⁶ disulfide bonds, several hydrophilic (Phe²/², Pro⁵/⁵, Leu⁵/⁵, Tyr¹⁴/¹⁴, Tyr²⁴/²⁴, Leu²⁵/²⁵, and charged residues (Lys¹⁰/¹⁰, Arg¹²/¹², His¹⁴/¹⁴, Arg²¹/Lys¹⁵, Arg³³/³³, Lys³⁵/³⁵, and Lys³⁷/³⁷) are similarly located at the surface giving rise to a comparable location of hydrophobic and hydrophilic clusters (Fig. 4B).

Our results showed that in non-stimulated animals, the mantle appears as the main tissue expressing Cg-Def. Although we were not able to isolate by HPLC significant amount of pure defensin from the oyster, we clearly detected its presence by mass spectrometry analyses of mantle tissue (Fig. 2C). The results presented here are in contrast with those reported for the mollusk, M. galloprovencialis. In the mussel, AMPs are only produced in hemocytes where they are stored and released following bacterial challenges (45). In the well studied insect model, Drosophila melanogaster, AMPs are predominantly produced in the fat body (the functional equivalent of mammalian liver) and secreted into the blood in response to a microbial challenge, which characterizes the systemic response in insects. Otherwise, the expression of these peptides is also locally regulated in the surface epithelia of several Drosophila tissues (47), as also observed for the expression of peptides in mammalian epithelia. For example, Drosophila defensin is expressed in the labeled glands and in the seminal receptacle and spermatheca (for a review see Imler and Bulet (48)). Additionally, in mammals, AMPs are constitutively produced by blood cells (49). Apart from insects, most of the AMPs reported in the different groups of invertebrates have been isolated from hemocytes (13). Our preliminary results would suggest that Cg-Def is continuously expressed in the oyster mantle, i.e. at epithelial surface, indicating that this AMP has an important function in host protection against environmental microbes. Indeed, in oyster, the mantle is a site of intense exposure to external environment that represents a dynamic ecosystem for numerous bacterial species, including commensals and potential pathogens. A more detailed study of Cg-def gene expression in response to various microbial challenges will be performed to gain insight into the role of Cg-Def in oyster immunity. In addition, AMPs isolation and characterization must be investigated from other oyster tissues. These data on AMPs are of great interest to understand how the oyster immune system interacts with the commensal flora and how it responds to environmental pathogens.

Acknowledgments—We are very grateful to Dr. A. J. Ouellette for excellent advice during defensin recombinant expression. We also thank B. Romestand, J. de Lorgeril, G. Desserre, and M. Leroy for helpful discussion and assistance.

REFERENCES
1. Yang, D., Biragyn, A., Kwak, L. W., and Oppenheim, J. J. (2002) Trends Immunol. 23, 291–296
