Gambicin: A novel immune responsive antimicrobial peptide from the malaria vector *Anopheles gambiae*

Jacopo Vizioli*, Philippe Bulet*, Jules A. Hoffmann*, Fotis C. Kafatos†‡, Hans-Michael Müller†, and George Dimopoulos†§

*Institut de Biologie Moléculaire et Cellulaire, 15 Rue René Descartes, 67084 Strasbourg Cedex, France; and †European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany

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A novel mosquito antimicrobial peptide, gambicin, and the corresponding gene were isolated in parallel through differential display-PCR, an expressed sequence tag (EST) project, and characterization of an antimicrobial activity in a mosquito cell line by reverse-phase chromatography. The 616-bp gambicin ORF encodes an 81-residue protein that is processed and secreted as a 61-aa mature peptide containing eight cysteines engaged in four disulfide bridges. Gambicin lacks sequence homology with other known proteins. Like other *Anopheles gambiae* antimicrobial peptide genes, gambicin is induced by natural or experimental infection in the midgut, fatbody, and hemocyte-like cell lines. Within the midgut, gambicin is predominantly expressed in the anterior part. Both local and systemic gambicin expression is induced during early and late stages of natural malaria infection. *In vitro* experiments showed that the 6.8-kDa mature peptide can kill both Gram-positive and Gram-negative bacteria, has a morphogenic effect on a filamentous fungus, and is marginally lethal to *Plasmodium berghei* ookinetes. An oxidized form of gambicin isolated from the cell line medium was more active against bacteria than the nonoxidized form from the same medium.

Anopheles gambiae | antimicrobial peptide | innate immunity | malaria

The mosquito *Anopheles gambiae* is the major vector of *Plasmodium falciparum* in sub-Saharan Africa, causing 2 to 3 million deaths yearly. The capacity of mosquitoes to transmit malaria from one host to another is determined by numerous factors such as their longevity, feeding preference, and permissiveness to parasite development (1). Not all mosquito-parasite strain combinations are compatible: some *Plasmodium* strains are unable to develop in certain refractory mosquito strains because of innate immune responses that can kill all ingested *Plasmodia* (2, 3). Parasite killing in the mosquito is not exclusively restricted to these extreme cases of total refractoriness: large losses of the parasite during certain stages of its life cycle also occur in fully susceptible mosquito strains (4). These losses are correlated with transcriptional activation of immune genes by malaria infection during invasion of epithelial tissues and translocation to the salivary glands (5–7). Some of the components involved in parasite elimination may be antimicrobial peptides, which are key factors in fighting off infections in insects (8).

In *Drosophila melanogaster*, seven distinct antimicrobial peptide families are induced on microbial challenge (9). These families include defensin and cecropin, which are the only classes of antimicrobial peptides so far reported in mosquitoes (10–14). Mosquitoes also contain genes encoding lysozyme (15–17). In *A. gambiae* three antimicrobial peptides, one defensin (11) and two isoforms of cecropin A (14), have been characterized. Recently, a second cecropin gene (cecropin B) has been identified in a pilot gene discovery project based on mosquito cell line expressed sequence tags (ESTs) (6). *Anopheles* defensin shows antibacterial and antifungal activities at physiological concentrations (18) and its expression is strongly up-regulated on infection by bacteria or *Plasmodium* (7, 19). Similarly, *Anopheles* cecropin A and its mRNA are inducibly expressed in cell lines and mosquitoes, and both amidated and nonamidated isoforms of this cecropin are active against a broad spectrum of microorganisms (14). The *Anopheles* defensin and cecropin are expressed in midgut, thorax, and abdominal tissues of naive mosquitoes. These peptides are preferentially expressed in the anterior part of the midgut (14, 19).

Here we present a novel *A. gambiae* gene, gambicin, encoding a mature 61-residue cysteine-rich immune inducible peptide. Mature gambicin peptide is active against Gram-positive and Gram-negative bacteria, a filamentous fungus, and the ookinete stage of the malaria parasite. The gene expression pattern of gambicin is similar to those of cecropin and defensin, showing predominant expression in the anterior midgut compartment, thorax, and abdomen. In contrast to the previously characterized *A. gambiae* antimicrobial peptides, gambicin does not share sequence similarity with other database entries and is thus a novel antimicrobial peptide.

Methods

Preparation of Serum-Free Cell Conditioned Growth Medium. Cells of the *A. gambiae* cell lines 4a-3A and 4a-3B (20) were seeded in 75-cm^2 tissue culture flasks (Greiner, Nurtingen, Germany) containing 25 ml of Schneider's medium (Sigma) supplemented with 10% FCS, Penicillin (100 units/ml), and Streptomycin (100 μg/ml), and grown at 27°C. At 80% confluence, cell layers were rinsed three times with 10 ml of FCS-free/antibiotic-free Schneider's medium. Cultures were grown for two more weeks at 27°C in 15 ml of the same medium. The conditioned medium was cleared by centrifugation, passed through a 0.22-μm filter, and stored at 4°C.

Purification of Antibacterial Peptides from Cell Culture Medium. Culture medium (control) and cell line-conditioned medium (50 ml) were acidified and prepurified by solid phase extraction on Sep-Pak C_{18} Cartridge (Waters). Proteins were eluted with 60% acetonitrile (ACN), acidified with 0.05% trifluoroacetic acid (TFA), and subjected to RP-HPLC on an Aquapore RP-300 C_8 column (250 × 7 mm, Brownlee Lab) using a linear gradient of ACN. Collected fractions were lyophilized and suspended in ultrapure water and aliquots equivalent to 6 ml of conditioned medium were assayed for antibacterial activities, as indicated below.

Gambicin Isolation and Characterization. *Gambicin purification.* Peptides in an aliquot of conditioned medium prepurified as above

Abbreviations: RT, reverse transcription; EST, expressed sequence tag; MALDI-TOF, matrix-assisted laser desorption ionization–time-of-flight.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database [accession no. AJ237664 (gambicin)].

‡To whom reprint requests should be addressed. E-mail: kafatos@embl-heidelberg.de.

§Present address: Department of Biological Sciences, Centre for Molecular Microbiology and Infection, Imperial College of Science, Technology and Medicine, London SW7 2AZ, United Kingdom.

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were separated by size exclusion chromatography on two serially linked HPLC columns (Ultraspherogel SEC 3000 and SEC 2000, 7.5 × 300 mm, Beckman) and eluted with 30% acidified ACN. Fractions with antibacterial activity were further purified by RP-HPLC on an analytical Aquapore OD-300 column (220 × 4.6 mm, Brownlee Lab), using a linear gradient of ACN. Purified products eluted at 26% ACN were characterized by matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry (see below) and Edman degradation, performed on a pulse liquid automatic sequencer (model 473A, Perkin–Elmer Applied Biosystems).

Reduction and S-pyridylethylation. An aliquot of the purified gambicin was subjected to reduction and alkylation as described (21). The *S*-pyridylethylated peptide was desalted by RP-HPLC.

Enzymatic digestions. Purified gambicin (3 μg) was treated with thermolysin (Boehringer Mannheim) at an enzyme/substrate ratio of 1:3 as described (22). Digestion products were separated by microbore RP-HPLC. A thermolysine fragment and the *S*-pyridylethylated peptide were subjected to Asp-N digestion according to the manufacturer's protocol (Takara Shuzo, Tokyo). Digestion products were purified and characterized as above.

MALDI-TOF mass spectrometry. MALDI-TOF MS analysis was performed on a Bruker (Bremen, Germany) BIFLEX III mass spectrometer in a positive linear mode. Samples were prepared by the sandwich method (23). Briefly, 0.5 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA, Sigma) in acetone was placed on the probe tip, followed by 0.5 μl of 5% TFA deposited on a matrix crystallized bed. Samples (0.5 μl) were loaded and covered with 0.5 μl of a saturated solution of HCCA in 50% ACN. After drying, the target was washed briefly with 2 μl of 0.1% TFA and dried again under vacuum.

Bioassays. Antimicrobial activities were determined by liquid growth-inhibition assays against *Micrococcus luteus*, *Escherichia coli* SBS363, and *Neurospora crassa*. Bioassays and determination of Minimal Inhibitory Concentrations (MIC) were performed as described (24), except that bacterial cultures were tested at a starting $mOD_{595} = 0.1$.

Antiparasitic assays were performed against *Plasmodium berghei* ookinetes, cultured *in vitro* and purified as described (25). Parasite viability was assessed by staining with the fluorescent dyes calcein AM and ethidium homodimer (Molecular Probes; ref. 26). Briefly, parasites were incubated in PBS with or without gambicin (10–100 M) for 30–180 min at 21°C. Live and dead parasites were counted by using a fluorescence microscope.

Differential Display PCR, cDNA Cloning, and Cytogenetic Mapping. Differential RNA display between naive and bacterially challenged female mosquitoes and subsequent cloning of the differentially expressed product were performed as described (27), using the 10-mer primer (5′-GGTAGGAATTACT-3′, Operon Technologies, Alameda, CA). The PCR product and a corresponding cDNA clone from an abdominal cDNA library (5) were sequenced, leading to identification of gambicin cDNA. The cDNA clone was mapped cytogenetically by *in situ* hybridization to *A. gambiae* Suakoko strain polytene chromosomes (28).

Expression Analysis. Reverse transcription (RT)-PCR expression analysis was performed as described (27). To estimate expression levels in tissues and infected mosquitoes, the electrophoresed PCR products were resolved in agarose gels stained with SYBR Green I nucleic acid gel stain (Molecular Probes) according to the manufacturer's instructions, and quantified by using a phosphorimager. The ribosomal protein S7 gene (29) was used for normalization of compared templates. In each experiment the PCR cycle numbers were chosen empirically to avoid saturation and attain comparable PCR product amounts of S7. The primers used were as follows: S7A (5′-GGCGATCATCATCTACGT-3′) and S7B (5′-GTAGCTGCTGCAAACTTCGG-3′), and GambicinA (5′-AACCGGAAGGGCGTTTCGTG-3′) and GambicinB (5′-CGTCTGGCACTGATTAAACC-3′). Primers for Antryp 2 (TY2) and Antryp 4 (TY4) were as described (30).

Production of Recombinant Gambicin and Antibody Production. A 204-bp PCR fragment, amplified from the gambicin cDNA clone by using the sense primer Gam-*Eco*RI (5′CCCCGAATTCATGGTGTTTGCTTACGCGCCG3′) and the antisense primer Gam-*Hin*dIII (5′GGGGAAGCTTACAAGAAACATTCTGTAATAAA3′), was subcloned into the *Eco*RI/*Hin*dIII polylinker sites of the expression vector pDS56/RBSII,6×His/E$^-$(−2). Expression of the amino-terminal 6× His-tagged recombinant protein in *E. coli* and purification by nickel-chelate affinity chromatography were performed as described (30). The eluted urea fractions were dialyzed against PBS. Three rats and one rabbit were immunized with 100 μg and 200 μg recombinant gambicin, respectively, using the RIBI Adjuvant System according to the manufacturer's instructions (Ribi Immunochem). The animals were boosted three more times with the same amount of protein and bled 2 weeks following the last injection.

Electrophoresis, Immunoblotting, and Immunofluorescence. Gambicin was resolved electrophoretically by using the tricine-SDS/PAGE system (acrylamide:bisacrylamide, 48:1.5) with a 4% stacking gel, with a 10% spacer gel and a 16.5% separation gel (31). Samples were loaded in conventional Laemmli buffer (125 mM Tris, pH 6.8/20% glycerol/3% SDS/3% β-1-mercaptoethanol) after boiling for 5 min. Polypeptides contained in lysates of dissected strain 4a r/r *A. gambiae* midguts (67 anterior and 15 posterior midguts), and in 4a-3A and 4a-3B cell line conditioned serum-free medium were electrophoretically separated as above. Comparable samples of anterior and posterior midguts as estimated by Ponceau-S staining were loaded on the gel. Recombinant gambicin (30 ng) was loaded as control. Immunoblots on nitrocellulose filters (Protran, Schüll) were performed as described (20), using rabbit anti-gambicin serum diluted 1:1000. Bound antibodies were detected by secondary goat anti-rabbit IgG (H + L) conjugated to alkaline phosphatase (Promega). Immunofluorescence detection of gambicin in 4a-3B cells was performed by using a rat anti-gambicin serum or preimmune serum diluted 1:250 in 1% BSA/PBS as described (20). Midguts from 4–6-day-old naive mosquitoes were dissected, fixed, and stained as described (32), using the rabbit anti-gambicin antibody at 1:3000 dilution. Samples were examined with a Zeiss LSM 510 confocal microscope.

Mosquito Immunization and Hemolymph Collection. Four-day-old *A. gambiae* 4a r/r strain females were infected with bacteria as described (19) and allowed to recover 20 h at 25°C. Hemolymph was collected and purified by RP-HPLC as reported (33). Fractions at the retention time of gambicin were concentrated by ZipTip (Millipore) and analyzed by MALDI-TOF MS.

Results

Gambicin Peptide Purification and Characterization. Peptides present in the conditioned medium of the Anopheles hemocyte-like cell line 4a-3B and control culture medium were separated by RP-HPLC and assessed for antibacterial activity. Several active fractions were exclusively observed in the conditioned medium (Fig. 1*A*). Identical results were obtained when using cell line 4a-3A (data not shown). Selected active fractions were further purified and several previously characterized antibacterial peptides such as lysozyme (15), defensin (11), and cecropin (14) were identified by mass spectrometry and/or Edman degradation. To characterize the molecule(s) responsible for the activity eluted at 26% ACN (Fig. 1*A*, peak G), a new aliquot of

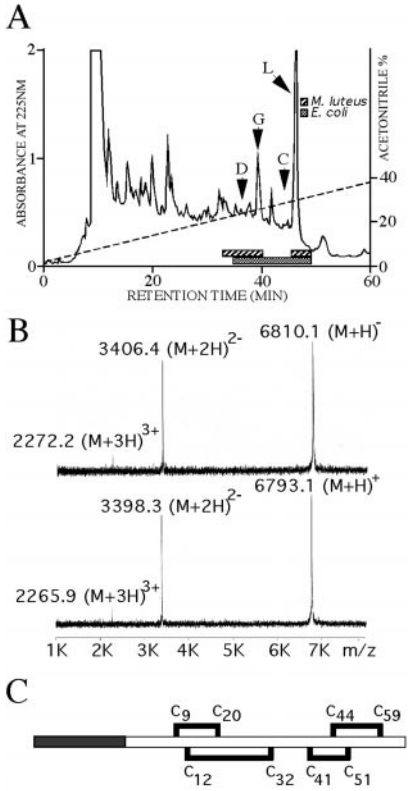

Fig. 1. (*A*) RP-HPLC of *A. gambiae* 4a-3B cell line conditioned medium. Fractions exhibiting antimicrobial activity against *M. luteus* and *E. coli* are indicated. Arrows indicate the fractions containing defensin (D), cecropin (C), gambicin (G), and lysozyme (L). (*B*) MALDI-TOF MS analysis of the purified oxidized (*Upper*) and nonoxidized (*Lower*) gambicins. Gambicin molecular mass values are expressed in m/z. Supplementary peaks corresponding to double- and triple-charged gambicin are indicated. (*C*) Primary structure of the 61-aa mature gambicin disulfide bridge array. The cysteine pairing is indicated in bold lines. The20-residue signal peptide–propeptide region is shown in black.

conditioned medium was purified by size exclusion chromatography before RP-HPLC. Two compounds at 6810.1 m/z and 6793.1 m/z, respectively (Fig. 1*B*), were resolved and found to be active against *M. luteus* and *E. coli*.

The molecule at 6810.1 m/z was subjected to Edman degradation, yielding a partial sequence of 57 residues: MVFAY-APTXARXKSIGARYXGYGYLNRKGVSXDGQTTINSX-EDXKRKFGRXSDGFIT. Sequencing after reduction, *S*-pyridylethylation and mass spectrometry indicated that gambicin contains eight cysteines, at the residues noted with X plus one more (data not shown). To complete the sequence, the pyridylethylated peptide was subjected to endoproteinase Asp-N digestion and a peptide with a molecular mass at 1182.9 m/z was subjected to Edman degradation. The 9-aa sequence obtained (DGFITECFF) corresponded to the C-terminal part of the native peptide (see above) and included the predicted eighth cysteine. Databank search (blast analysis of the data in TrEMBL, Swiss-Prot, and the *Drosophila* genome sequence) showed that the peptide has no significant similarity with known peptides. Therefore, this novel 61-residue cysteine-rich antimicrobial peptide from *A. gambiae* was named gambicin.

The measured molecular mass of the native 61-residue peptide (6810.1 m/z) exceeded by 16.3 Da the calculated molecular mass of the sequenced molecule (6793.8 m/z), even accounting for the formation of four disulfide bridges. This difference suggests the presence of an oxidized amino acid in the sequenced peptide.

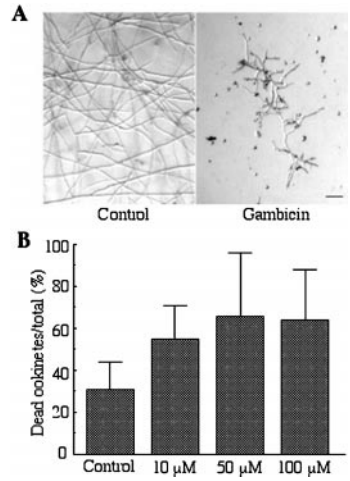

Fig. 2. (*A*) Antifungal assay. Light microscopy observation of *N. crassa* hyphal growth after 24 h of incubation with water (Control) or 100 μM gambicin. (Scale bar, 100 μm.) (*B*) Antiparasitic assay. Percentage of dead ookinetes after incubation with PBS (Control) or gambicin, tested at different concentrations.

Enzymatic cleavage with endopeptidase Lys-C and mass spectrometry analysis of the proteolytic fragments indicated that the oxidized amino acid is located within the first 13 N-terminal residues (data not shown). The calculated molecular mass of the full-length nonoxidized gambicin (6793.8 m/z) corresponds to the second isolated active molecule (see above). Partial N-terminal sequencing of the purified 6793.1 m/z peptide confirmed this hypothesis. Thus, the two isolated peptides correspond to an oxidized and a nonoxidized form of gambicin.

To determine the scaffold of the eight cysteines (Fig. 1*C*), native gambicin was subjected to thermolysine digestion and two proteolytic fragments, at molecular masses of 1745.9 m/z and 2543.6 m/z, respectively, were sequenced by Edman degradation (data not shown). The sequencing results for the first product pointed to the connection of fragment Ala-4–Cys-9 to fragment Ile-15–Tyr-24 through a disulfide bridge between Cys-9[1] and Cys-20[3]. The second fragment comprised the C-terminal part of gambicin, from Ile-38 up to Cys-59. Because two connectivities are possible between the four cysteines of this region, this peptidic fragment was subjected to endoproteinase Asp-N digestion to establish the correct disulfide bridge array. Mass spectrometry analysis of the specifically generated fragments defined the connectivity Cys-41[5]–Cys-51[7] and Cys-44[6]–Cys-59[8] (data not shown). By elimination, the fourth gambicin disulfide bridge corresponds to the link between Cys-12[2] and Cys-32[4].

Antimicrobial and Antiparasitic Activity Assays. Both oxidized and nonoxidized natural forms of gambicin were purified from one liter of cell conditioned medium and tested against the bacteria *M. luteus* and *E. coli*, and the filamentous fungus *N. crassa*. The oxidized form inhibited the growth of *E. coli* and *M. luteus* at Minimal Inhibitory Concentrations (MICs) of 6.25–12.5 μM and 25–50 μM, respectively. Nonoxidized gambicin was significantly less active by this test: it was most effective against Gram-negative bacteria (MIC 25–50 μM), but showed only partial growth inhibition of *M. luteus* at 100 μM. At the highest concentration tested (100 μM), both forms of gambicin delayed *N. crassa* spore germination, causing reduced elongation of hyphae and increased branching (Fig. 2*A*).

Both forms of gambicin enhanced the lethality of *P. berghei* ookinetes approximately 2-fold relative to the control (30.2% $\pm$ 13.5%). The parasite mortalities-associated peptide concentra-

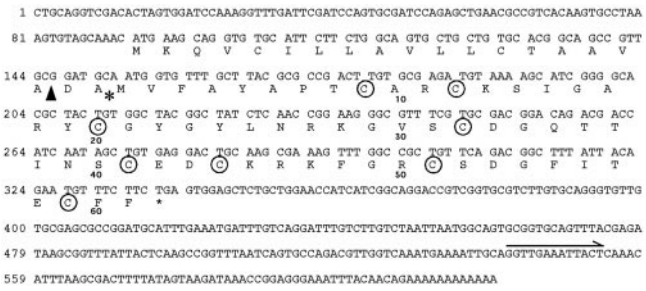

Fig. 3. Nucleotide and encoded amino acid sequence of the gambicin cDNA. The priming site for the random 10-mer primer used in the differential display amplification of the 3′ UTR gambicin fragment is indicated by a horizontal arrow. The predicted signal peptide cleavage site according to von Heine and the beginning of the mature peptide are marked with an arrowhead and asterisk, respectively. Circles mark the 8 cysteines.

tions were 10 μM (54.6% ± 15.8%), 50 μM (65.2% ± 30.2%), or 100 μM (63.1% ± 24.4%), respectively (Fig. 2*B*).

Gambicin DNA Sequence and Chromosomal Location. A 164-bp fragment of the 3′ gambicin UTR was isolated as a differentially amplified PCR fragment from adult 12-h-challenged *A. gambiae* females, using a random 10-mer primer in combination with an oligo dT primer as described (27). The differentially amplified fragment was used as probe for screening an abdominal cDNA expression library (5). A 616-bp full-length cDNA clone was isolated and contained a 243-bp ORF encoding an 81-residue protein. The deduced protein sequence has a combined signal peptide and propeptide region of 20 residues (see *Discussion*), followed by the 61-aa sequence of authentic gambicin (Fig. 3). Furthermore, the complete coding region of gambicin was obtained independently as an EST sequence (designated IRSP for infection responsive short peptide) in a recently implemented gene discovery project (6). The cDNA clone was used as a hybridization probe and located the gene at a single locus at division 31A of chromosome 3R (data not shown).

Expression and Induction of Gambicin Transcripts. Gambicin transcript levels at various developmental stages and in different body parts, and transcriptional regulation on bloodfeeding, bacterial, or malarial immune challenge in hemocyte-like cell lines and adult mosquitoes, respectively, were assayed by RT-PCR. The ribosomal protein S7 transcript was used as normalization standard as in previous studies (5). During development, gambicin mRNA is highly abundant in pupae and adults and is produced at much lower levels in larvae; no transcripts are detectable in the embryo (Fig. 4*A*). Expression levels in naive and unfed adult female and male mosquitoes is similar (data not shown). Gambicin is highly expressed in the midgut, thorax, and abdominal carcass; within the midgut it is predominantly present in the anterior part (Fig. 4*B*). No significant fluctuations of mRNA levels are detected at 6, 30, and 60 h after blood feeding, indicating that gambicin transcription is not significantly affected by blood feeding in contrast to the digestive trypsins, Antryp 2 (TY2) and Antryp 4 (TY4) (Fig. 4*C*).

The modulation of gambicin transcription by immune challenge was assessed by RT-PCR in cell lines treated with heat-inactivated bacteria or bacterial surface components, and in malaria-infected adults at different stages of infection. Unchallenged mosquitoes and cell lines were used as controls. The temporal transcription profile of gambicin was first tested in the highly immune competent cell line 4a-3B (20) between 4 and 24 h after challenge with a mixture of heat-killed *E. coli* and *M. luteus* (Fig. 5*A*). The gambicin gene quickly responded to this challenge with transcriptional up-regulation, ranging between 2.4- and 3.0-fold increase between 4

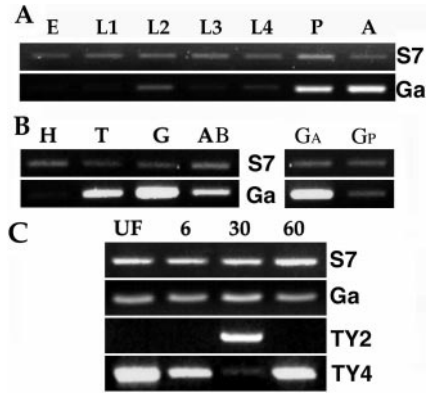

Fig. 4. RT-PCR expression analysis of gambicin mRNA. cDNA templates were normalized by using a ribosomal protein S7-specific primer pair. (*A*) Developmental expression profile assayed in embryo (E), four larval stages (L1–L4), pupae (P), and adult females (A). (*B*) Gambicin expression in head (H), thorax (T), midgut (G), abdomen (AB), anterior midgut (G_A), and posterior midgut (G_P) of 4-day-old female adult mosquitoes. (*C*) Expression of gambicin in unfed (UF) and blood-fed adult female mosquitoes at 6, 30, and 60 h after feeding. Expression of the blood feeding regulated digestive trypsins, Antryp 2 (TY2), and Antryp 4 (TY4) were also assayed in the same samples as controls.

and 12 h and subsiding to 1.6-fold by 24 h. In a second experiment the same cell line was challenged with heat-killed *E. coli*, *M. luteus*, lipopolysaccharide (LPS), or lipoteichoic acid (LTA) for 8 h before the gambicin transcription assay (Fig. 5*B*). Induction ranged from 2.4- to 3.7-fold and appeared to be marginally stronger with *E. coli*/LPS than with the Gram-positive *M. luteus*/LTA; the maximal difference in induction levels was observed between LPS and LTA. Comparable induction was documented for IRSP, the EST that corresponds to gambicin (6).

The effect of malaria infection on gambicin expression was tested at the early and late critical transition stages of the *Plasmodium* life cycle in the vector mosquito (5). Transcription of the gambicin gene was significantly up-regulated both in the carcass and in the midgut

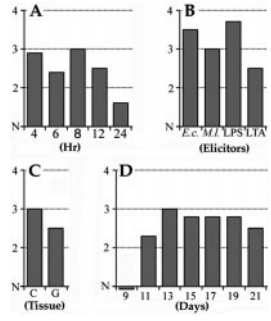

Fig. 5. Infection inducibility of gambicin transcription in bacteria-challenged cell lines and malaria-challenged mosquitoes. The RT-PCR products were analyzed on Cybr-Green-stained agarose gels and quantified by using a phosphorimager. The templates were normalized for expression of the ribosomal protein S7 gene and the relative expression levels for gambicin were arithmetically adjusted for equal S7 expression in the compared templates. Experiments were repeated three to four times and the average fold regulation was estimated for three experiments and indicated in the figure. Variations between experiments ranged between 0.1- and 0.6-fold regulation. The *y* axes indicate fold regulation below or above the naive (N) expression level. Transcriptional induction of gambicin in: (*A*) the 4a-3B cell line at 4, 6, 8, 12, and 24 h after challenged with a mixture of *E. coli* and *M. luteus*; (*B*) the 4a-3B cell line at 6 h after challenge with *E. coli*, *M. luteus,* LPS, and LTA; (*C*) malaria-infected mosquito carcass (C) and midgut (G) at 24 h after feeding on a *P. berghei*-infected mouse; (*D*) malaria-infected mosquitoes at 9, 11, 13, 15, 17, 19, and 21 days after feeding on a *P. berghei*-infected mouse.

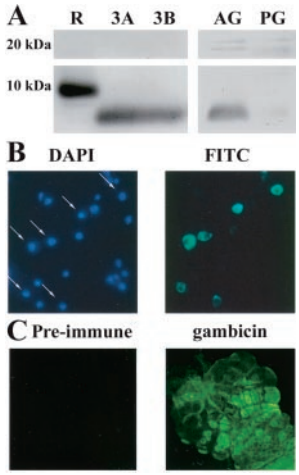

Fig. 6. (*A*) Immunoblot analysis of gambicin protein content in anterior (AG) and posterior (PG) midgut tissue extracts and 4a-3A (3A) and 4a-3B (3B) cell line conditioned medium. Recombinant gambicin (R) was used as a control. (*B*) 4′,6-diamidino-2-phenylindole (DAPI) and FITC (gambicin immunofluorescence) staining of 4a-3B cells. A subset of the nuclear DAPI-stained cells exhibit strong gambicin protein content. (*C*) Whole-mount antibody staining of anterior midgut giving high signals for gambicin in cells of the cardia. Control panel shows midgut staining with rabbit preimmune serum.

at 24 h when the ookinetes invade the midgut epithelium (Fig. 5*C*). Transcription subsided at 9 days postinfection and resumed at 11–21 days, when sporozoites are released into the hemocoel and invade the salivary glands (Fig. 5*D*).

Protein Expression. The induction of Gambicin transcription in hemocyte-like cell lines and the presence of gambicin protein in the conditioned medium suggested that this peptide may be secreted into the hemolymph. Indeed MALDI-TOF MS analysis showed that the hemolymph of bacteria-infected mosquitoes is enriched in gambicin of a molecular mass 6807.3 m/z, corresponding to the oxidized form (data not shown). Rat and rabbit anti-gambicin polyclonal antisera were used to monitor gambicin by immunoblotting and immunofluorescence. Endogenous gambicin has an approximate molecular weight of 6.8 kDa, whereas the recombinant protein is larger (8.2 kDa) because of a 6× His-tag plus four amino acids encoded by the polylinker *Bam*HI and *Eco*RI restriction sites. Gambicin of the same size as in the hemolymph and conditioned medium of the cell lines is detected in the midgut and shows substantial enrichment in the anterior part, correlating with the mRNA expression levels (Fig. 6*A*) and the results of tissue localization. Gambicin is constitutively expressed in midgut cells of the anterior part (Fig. 6*C*) but not the posterior part of the organ (data not shown).

Direct antibody staining of the cell line 4a-3B revealed that gambicin is highly enriched in a subset of cells constituting ≈20% of the total cell population (Fig. 6*B*).

Discussion

The present study reports both the isolation and characterization of a novel immune-induced antimicrobial peptide, and the characterization of the nucleotide sequence of a cDNA (corresponding to an EST and a differential display PCR product) that encodes the peptide precursor. The mature peptide, which we have named gambicin, contains eight cysteine residues that form four intramolecular disulfide bridges. Gambicin does not exhibit compelling sequence similarity with genes or proteins from other organisms. The sequence features of the cDNA-encoded product and authentic gambicin suggests that the precursor undergoes a two-step maturation process: (*i*) elimination of an 18-aa signal peptide and (*ii*) removal of the adjacent Asp–Ala dipeptide (Fig. 3) by a dipeptidyl aminopeptidase. This latter mechanism has been reported for other antimicrobial peptides such as cecropin (34) or penaeidin (35). Gambicin exists in an oxidized and a nonoxidized form; the former shows significantly higher bacteriocidal activity. The results obtained by enzymatic digestion and mass spectrometry indicate that methionine at position 1 is the best candidate for oxidation. A similar posttranslational oxidation of methionine residues has been described for a fragment of proenkephalin A, a protein with antibacterial activity isolated from bovine chromaffin granules (36). Oxidation of gambicin but not of proenkephalin A influences antimicrobial activity. We speculate that gambicin is modified by oxidative reactions activated during the immune response, thus increasing the effectiveness of the molecule.

Gambicin is present in dissected midgut and carcass, hemocyte-like cell lines and their conditioned medium, suggesting that it functions as a systemic antimicrobial peptide. Its transcriptional level is high in the pupal stage, as is common for other antimicrobial peptide genes in various Diptera (37, 11), possibly providing protection during the hatching of the soft adult. The gene is also expressed in the fat body-containing abdominal and thoracic part, and in the midgut as is characteristic for *A. gambiae* antimicrobial peptide genes. The pattern of expression primarily in the anterior midgut is strikingly similar to that of defensin and cecropin genes (7, 14, 19). Production of antimicrobial peptides in the digestive tract has also been observed in other insects (38–40) and in mice (41). If they are secreted into the gut lumen, they may represent a rapid, local immune response against potential exogenous pathogens brought in during feeding. It is tempting to hypothesize that secretion into the anterior midgut would favor action and neutralization in the ingested food before it enters the main posterior part, thus protecting the posterior symbiotic flora that may be important for digestion. The bacterial midgut flora fluctuates greatly during the lifetime of a mosquito and increases up to 16,000-fold after blood feeding (42). This expansion of the endogenous flora does not appear to induce antimicrobial peptide expression, and gambicin transcription is unaffected after bloodfeeding, in contrast to its induction by malaria infection.

Gambicin is induced by immune challenge in both cell lines and mosquitoes. The continuous production and secretion of active gambicin in the immune-competent cell lines, and its rapid transcriptional up-regulation on infection, indicate that gambicin is an acute phase response component of the mosquito innate immune system. This small protein exhibits a wide spectrum of antimicrobial activities, with highest potency against a Gram-negative bacterium, significant activity against a Gram-positive bacterium, and a morphogenic effect against a filamentous fungus similar to that described for the cysteine-rich termicin (22) and plant defensins (43). The observed enhancement of ookinete lethality by gambicin is modest but intriguing. It raises the question whether this activity may be potentiated by additional components of the midgut lumen or epithelial cytoplasm. The lack of sequence similarity with other sequences in the databases opens the possibility that gambicin may have evolved specifically against mosquito-specific *Plasmodium* parasites. Such a possibility remains to be further examined through *in vitro* combinatorial antiparasitic assays and *in vivo* antiparasitic assays by transgenic silencing and overexpression of gambicin in mosquitoes during malaria infection.

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