Therostasin, a Novel Clotting Factor Xa Inhibitor from the Rhynchobdellid Leech, Theromyzon tessulatum*

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Therostasin is a potent naturally occurring tight-binding inhibitor of mammalian Factor Xa (Kᵢ, 34 pm), isolated from the rhynchobdellid leech Theromyzon tessulatum. Therostasin is a cysteine-rich protein (8991 Da) consisting of 82 amino acid residues with 16 cysteine residues. Its amino acid sequence has been determined by a combination of techniques, including Edman degradation, enzymatic cleavage, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) on the native and s-β-pyridylethylated compound. Sequence analysis reveals that it shares no significant homology with other Factor Xa inhibitors except for the putative reactive site. Moreover, it contains a signature pattern for proteins of the endothelin family, potent vasoconstrictors isolated in mammal and snake venom. Therostasin cDNA (825 bp) codes for a polypeptide of 82 amino acid residues preceded by 19 residues, representing a signal peptide sequence. As for the other known inhibitors of Factor Xa, therostasin is expressed and stored in the cells of the leech salivary glands.

During blood vessel injury, the recruitment of platelets necessary for the clot depends on the local production of thrombin. A vascular lesion stimulates the production of thrombin by initiating both a tissue factor and an intrinsic activation pathway of Factor Xa, one of the final proteinases of the blood initiating both a tissue factor and an intrinsic activation pathway. Clinically, the specific inhibition of Factor Xa is necessary for the clot to depend on the local production of thrombin. Anticoagulant therapy, because it does not present any anticoagulation cascade.

1 The abbreviations used are: HPLC, high pressure liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; bp, base pair(s); DIG, digoxigenin.
Isolation and Characterization of Therostasin—After anesthetizing the animals using 0.01% chloroethane, the anterior parts of starred *T. tessulatum* stage 2 were excised (8). They were frozen immediately in liquid nitrogen and stored at −70 °C. Eight gram aliquots were thawed and placed in 25 ml of 20 mM Tris-HCl, pH 8.4 (200 mM NaCl), and homogenized at 4 °C with a Polytron (Bioblock Scientific, Villeneuve d’Ascq, France) (five 15-s bursts on setting 9). After centrifugation (30 min, 10,000 × g on a Beckman JA-20 rotor at 4 °C), the pellet was re-extracted twice with Tris/NaCl. Supernatants were combined, concentrated in a vacuum centrifuge (Savant), and filtered on nitrocellulose membranes (0.45 μm pore size, Millipore). The extract was applied onto a fast protein liquid chromatography column (Superdex G75, 16/60, Amersham Pharmacia Biotech; equilibrated with Tris/NaCl at a flow rate of 1 ml/min and eluted with the same buffer. The column effluent was monitored by UV absorbance (Beckman) at 280 nm. All column fractions (1 ml) were assayed for protease inhibitor activity against Factor Xa. Pooled active fractions were loaded onto a Mono Q column (fast protein liquid chromatography, HR 5/5, Amersham Pharmacia Biotech) equilibrated with 20 mM Tris-HCl (pH 8.8). The column was washed with the same buffer and eluted with a discontinuous linear gradient of 0.2 to 1.5 M NaCl over 60 min at a flow rate of 1 ml/min.

Active fractions were applied to a C₄ Lichrospher RP100 column (250 × 4.6 mm, Merck) with a linear gradient of 1% acetonitrile/min in water acidified with 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. All HPLC purifications were performed with a Beckman Gold HPLC system equipped with a Beckman 168 photodiode array detector.

To follow the purification, chromogenic assays were used. Enzyme assays were carried out at room temperature in 96-well microtiter plates (Dynatec). The color developed from the hydrolysis of peptide-nitroanilide (pNA) substrates was monitored at 405 nm on a Dynatec MR-5000 microtiter reader. The concentration of the purified Factor Xa inhibitor was estimated by the Bradford method using β-globulin as a standard (9). Typically, the assay included 3 μM proteolytic enzyme in 20 mM Tris-HCl, pH 8.4 (0.2 × 2 mM NaCl), and an aliquot of selected column fractions in a total volume of 100 μl. After 15 min of incubation, substrate was added for 5 min and then stopped with 50% acetic acid, and the residual activity was determined. At different stages of purification of the final crude solution of pyridylethylated protease was performed on a Beckman Gold HPLC system equipped with a Beckman 168 photodiode array detector.

| Step | Inhibitor | Specific activity | Protein purification factor |
|------|-----------|------------------|----------------------------|
| Lecch extract | 80 | 0.015 | 300 | 1 |
| Superdex G75 | 0.86 | 0.83 | 2.2 | 53 |
| Mono Q | 0.41 | 0.41 | 1.6 | 387 |
| Reversed phase | 0.00082 | 78 | 0.01 | 5200 |

Molecular Cloning of Therostasin—Total RNA was extracted from anterior parts of *T. tessulatum* using the guanidium isothiocyanate/cesium chloride centrifugation method (11). First-strand cDNAs were prepared from these total RNAs using standard procedures (11). A probe homologue used for screening an agt11 library, synthesized from anterior parts of *T. tessulatum* at stage 2, was prepared by polymersed chain reaction (PCR) with first-strand cDNA as a template. The standard PCR condition involves initial heating at 94 °C for 5 min followed thereafter by 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 48 °C for 2 min, and primer extension at 72 °C for 2 min. The cycles were followed by a final extension at 72 °C for 5 min. The final PCR mixtures (10 μl) were analyzed on 2% (w/v) agarose gel. The PCR products were ligated in the PCR-II vector according to the manufacturer’s instructions (Invitrogen). Dideoxy sequencing reactions of the recombinant plasmids were analyzed with the T7 sequencing kit from Amersham Pharmacia Biotech. The homologous probe was then used to clone the complete cDNA from the agt11 library. The nucleotide sequences were analyzed by the dye-sequencing chain termination method.

In situ Hybridization—A HindIII/BamHI fragment of approximately 900 bp, containing the entire coding region of therostasin, was used to prepare cRNA probes. Digoxygenin-11-UTP-labeled antisense and sense riboprobes were generated by in vitro transcription using the DIG RNA labeling kit and T3 RNA polymerase (Roche Molecular Biochemicals). BamHI plus T3 polymerase gave a sense probe, and HindIII with T7 polymerase produced the antisense probe. The synthesis was carried out for 2 h at 37 °C followed by a 15-min incubation with 20 units of RNase-free DNase I to remove the template. The transcript was lithium chloride-precipitated and pelleted by centrifugation, and the pellet was washed with 70% ethanol, dried, and resuspended in RNase-free water. The probe concentration was evaluated on a 0.8% agarose gel.

Animals were anesthetized with 0.01% chloroethane. They were fixed overnight at 4 °C by immersion in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, embedded in paraffin wax, and serially sectioned at 8 μm. Sections were collected on polysine-coated slides and stored at 4 °C until used for in situ hybridization. After removal of paraffin, sections were placed into 0.1 M glycine (0.2 M Tris-HCl, pH 7.4), for 10 min prior to treatment with proteinase K (1 μg/ml in 100 mM Tris, pH 8.0, 50 mM EDTA) for 15 min at 37 °C. Slides were then rinsed in water followed by fixation in 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min at 20 °C. Slides were treated with 1% triethanolamine (pH 8.0) for 10 min followed with 0.25% acetic anhydride for 10 min. The sections were rinsed again in water, dehydrated by graded alcohols, and allowed to air dry. The digoxigenin-
labeled riboprobes were diluted in hybridization buffer (approximately 20–50 ng RNA/section). The hybridization buffer contained 50% formamide, 10% dextran sulfate, 10% Denhardt’s solution, 0.5 mg/ml Escherichia coli tRNA, 100 mM dithiothreitol, and 0.5 mg/ml salmon sperm DNA. Tissue sections were apposed to the diluted probe with coverslips and placed in a hybridization chamber containing Whatman filter paper moistened with 4× SSC (sodium saline citrate buffer) and 50% formamide. The hybridization boxes were then sealed and placed in a 55 °C oven overnight. The slides were then washed twice (2×30 min) with 2× SSC. After treatment with RNase A (20 µg/ml in 2× SSC) for 30 min at 37 °C, sections were subsequently rinsed in 1× SSC containing 0.07% 2-mercaptoethanol, in 0.5× SSC, 0.07% 2-mercaptoethanol, and in 0.1× SSC 0.07% 2-mercaptoethanol (10 min each). Sections were then immersed in 0.1× SSC containing 2-mercaptoethanol (2×30 min) at 55 °C and rinsed in 0.1× SSC at room temperature. Sections were washed in DIG buffer (100 mM Tris-HCl, pH 7.5, 100 mM NaCl) for 10 min at room temperature and then incubated in the same buffer containing 0.05% Triton X-100 and 2% normal sheep serum for 1 h at room temperature. After two washes with DIG buffer for 10 min, the slides were incubated with alkaline phosphatase-conjugated sheep antidigoxigenin antibody (1:1000, Roche Molecular Biochemicals). This was carried out in DIG buffer containing 1% normal sheep serum and 0.05% Triton X-100 in a humid chamber for 16 h at 20 °C. The slides were washed sequentially with DIG buffer (3×10 min) and 100 mM Tris-HCl, pH 9.5, 50 mM MgCl₂ (1×10 min). Bound antibody was visualized by incubation with a chromogen solution containing 100 mM Tris-HCl (pH 9.5), 50 mM MgCl₂, 100 mM NaCl, 375 µg/ml nitro blue tetrazolium, 188 µg/ml 5-bromo-4-chloro-3-indolyl phosphate, 1 mM levamisole, and 1.3% dimethyl sulfoxide for 6 h in the dark at room temperature. The chromogen reaction was halted by rinsing the slides in DIG buffer (2×15 min). The slides were then rapidly dehydrated, rinsed in toluene, and covered with coverslips using XAM (Merck) mounting medium. Replacing the antisense riboprobe with the sense riboprobe carried out control for in situ hybridization.

FIG. 1. Elution profiles of therostasin following purification steps. A, elution profile on Mono Q column. B, final step of purification on C₈ reversed-phase column. C, a photomicrograph of the fractions containing the therostasin separated by SDS-PAGE under reducing conditions: lane a, crude extract; lane b, after Superdex G75 column separation; lane c, after Mono Q column separation; lane d, after the final step of purification on C₈ reversed-phase column.
RESULTS

Biochemical Characterization of Therostasin—*T. tessulatum* takes three blood meals during its life. After the third blood meal, apoptosis of the salivary gland occurs (8, 15). To obtain the maximum amount of Factor Xa inhibitor present in *T. tessulatum*, an extract of the anterior part of hungry leeches was prepared from animals at stage 2 of their life cycle (Table I). The crude extract was then fractionated on a gel filtration column (Superdex G75, 16/60, Amersham Pharmacia Biotech). In the collected fractions, inhibitory activity toward Factor Xa was found for proteins with a molecular size of less than 30 kDa as determined by SDS-PAGE under reducing conditions (Fig. 1C, lane b). The inhibitor was then purified by anion exchange chromatography on a Mono Q column using a stepwise gradient of NaCl from 0.2 to 1.5 M. The Factor Xa inhibitor was eluted from the column at a NaCl concentration ranging between 0.5 and 0.75 M (Fig. 1A). SDS-PAGE controls revealed several protein bands at a molecular mass of around 15 kDa (Fig. 1C, lane c). Finally, this peptide was purified to homogeneity by reversed-phase HPLC on a C8 Lichrosphere column using a linear gradient of acetonitrile in acidified water (Fig. 1B). Most of the inhibitory activity eluted in one peak at 31% acetonitrile with a molecular mass of 14 kDa in SDS-PAGE (Fig. 1C, lane d). Mass measurement in MALDI-TOF MS of the purified fraction confirmed a single protein with a mass of 8991 kDa (data not shown). Comparison of the mass of this native molecule with that of *s*-pyridylethylated (8991 Da) indicates the position of the acetylation site. The peptide contains 82 amino acid residues with a molecular mass of 8990 Da, which is in perfect agreement with the molecular mass measured by MALDI-TOF MS (8991 Da). This Factor Xa inhibitor, designated therostasin, is a cysteine-rich peptide without post-translational modifications.

Sequence Comparison—Compared with other inhibitors characterized from *T. tessulatum* (16), therostasin shows 70 and 47% sequence identity with theromin, a thrombin inhibitor (16), and tessulin (17), a trypsin-chymotrypsin inhibitor, respectively.

A comparison of therostasin with other leech Factor Xa inhibitors revealed about 20% sequence identity with the inhibitory potency domain of ghilanten (domain I) (2) and antistasin (domain I) (1). Moreover, therostasin shows 31% sequence identity with the internal repeats 4 and 5 of *Hydra* antistasin, a Factor Xa inhibitor isolated from *Hydra* (18). In addition, therostasin shows 26–37% sequence identity with antistasin-type proteins isolated from leeches including guamerin (19), hirustasin (20), and piguamerin (21). Sequence alignment of therostasin with these peptides revealed that identity is observed mainly in the C terminus of these inhibitors (Fig. 3). By sequence similarity with antistasin, the putative active site (P1-P1') of therostasin is expected to be at position 36–37 and would be RI versus RV for antistasin.

Biological Activity—Therostasin is highly specific for Factor Xa (K$_\text{a}$: 34 pm). As antistasin, it also inhibits trypsin in a weaker manner (K$_\text{a}$: 7 nm) but does not inhibit other serine proteases such as chymotrypsin, elastase, cathepsin G, or thrombin (Table II). This is in contrast to Kunitz inhibitors, which do inhibit these proteases (22).

Therostasin Molecular Cloning—Based on the first 33 amino acids of the therostasin N-terminal sequence, two degenerated oligonucleotide primers, (5'-TCTAGACCGGGCA(A/G)GA/C/T/TTG/C/T/CT/GA/AG/AA/C/T/AC3') and (5'-GAATGGCAGTCTG/C/T/GT/GT/AC/GT/CC/GC/AG/TG/TC/A/GC/AG/C-A-3'), were used in PCR, with first-strand cDNA generated by reverse transcription. A PCR-amplified oligonucleotide of 132 bp was obtained. Its sequence analysis showed an open reading frame following oligonucleotide 1 that extends through the known therostasin sequence (data not shown). Two positive clones (T1$\alpha$ and T1$^2$) of approximately 2 × 10$^6$ phage lysates from the *T. tessulatum* agt11 library were identified using the 132-bp oligo probe. After EcoRI digestion of the purified recombinant phage T1$\alpha$ or T1$^2$, a single band (approximately 900 bp) with a similar size was identified. After subcloning in pBluescript II KS vector, the nucleotide sequence of T1$^2$ cDNA insert was determined. This finding revealed that this clone contains an uninterrupted open reading frame of 303 nucleotides preceded by a 180-nucleotide 5'-untranslated sequence and followed by a 342-nucleotide 3'-untranslated region (Fig. 4). The first hexanucleotide AATAAA consensus signal for polyadenylation was found at position 740 (Fig. 4). Translation of the open reading frame gives a 101-amino acid residue mass of 8890 Da, which is in perfect agreement with the molecular mass measured by MALDI-TOF MS (8991 Da). This Factor Xa inhibitor, designated therostasin, is a cysteine-rich peptide without post-translational modifications.

![Amino acid sequence of therostasin.](image)

**Fig. 2.** Amino acid sequence of therostasin. The sequence (82 amino acid residues) results from the analysis of *s*-pyridylethylated therostasin, which provided good yields for 48 residues on a pulse liquid automatic sequenator (Applied Biosystems, model 473). Three arginylendopeptidase fragments and an overlapping region with three peptides from a *Staphylococcus aureus* V8 protease digestion were isolated by fractionating digested peptides using reversed-phase HPLC on a Lichrosphere C$_8$ column. The sequences of these peptides were used to obtain the complete sequence.

![Sequence alignment of therostasin, domain I of antistasin and ghilanten.](image)

**Fig. 3.** Sequence alignment of therostasin, domain I of antistasin and ghilanten (fragments 1–60), hydra antistasin (internal repeats 4 and 5 corresponding to amino acids 119–182), guamerin, piguamerin, and hirustasin using Clustal W 1.8 software. Positions that are identical or conserved in at least four sequences are shaded with black or gray boxes, respectively. The solid bar indicates the position of the active site (P4′-P4) in antistasin and the arrow the P1 residue.
sequence that matches the sequence of the mature protein (Fig. 4). A 19-amino acid pre-peptide beginning with the Met residue precedes the N-terminal Asp residue of mature therostasin. This 19-amino acid sequence contains a hydrophobic core of 17 amino acids. cDNA sequence comparison revealed no homology with any cDNA known, demonstrating that the therostasin cDNA sequence is also completely new.

Therostasin Cellular Localization—In situ hybridization with DIG-labeled riboprobes was used to detect the therostasin messages in leech paraffin sections. A relatively intense signal was detected in the salivary glands (Fig. 5A). Not all cells were stained. No signal was observed in sections hybridized with the therostasin sense strand (Fig. 5B). Using an antibody performed against the N-terminal part of synthetic therostasin on serial sections of T. tessulatum, a relatively weak, but specific, immunolabeling was found in salivary glands (data not shown), confirming that the protein is stored in these cells.

**DISCUSSION**

We have purified a novel Factor Xa inhibitor from the rhynchobdellid leech T. tessulatum, designated therostasin. This consists of an 82-amino acid residue peptide preceded by 19 residues, representing a signal peptide sequence, localized in the salivary glands of the leech. Therostasin is a novel and highly specific Factor Xa inhibitor (Ki: 34 pm) isolated from leeches. For the Factor Xa "family" inhibitors isolated from leeches, two molecules consisting of 119 amino acid residues with anti-metastasic activity, antistasin and ghilianenate, have been isolated in the Glossiphoniidae leeches Hementeria officinalis (1) and Hementeria ghilianii, respectively (2). These native molecules are slow, tight-binding inhibitors with estimated dissociation constants ranging between 0.31 and 0.62 nm (2).

Factor Xa inhibitor has been isolated from other invertebrates. For example, in the hookworm Ancylostoma caninum, a small molecule with a molecular mass of 8.7 kDa, and a high intrinsic Ki (323.5 pm), has been found (23). In the insect Ornithodoros moubata, a tick anticoagulant peptide with a reversible and potent Factor Xa inhibition (Ki: 0.18–0.59 nm) has been also reported (24). However, the sequence of therostasin revealed no homology with the hookworm or the tick molecule. Factor Xa inhibitor has also been found in prokaryotes. Ecotin has been characterized as a periplasmatic protein in Escherichia coli (25). This 18-kDa protein inhibits trypsin, chymotrypsin, and rat mast cell chymase. Its activity toward Factor Xa is very high with a Ki around 54 pm. This molecule seems to play a role in protecting bacteria from exogenous proteases found in the mammalian gut. Ecotin was considered the most potent Factor Xa inhibitor described to date. We report here a novel molecule, therostasin, that is smaller than ecotin but as active, and it is even more specific in inhibiting Factor Xa.

Based on its primary sequence as well as its cDNA, therostasin is a novel molecule. The 25–39 amino acid sequence (CLICK-GCNDADCRYIC) of therostasin contains a consensus pattern C-X-C-X-D-X-C-X-(F/Y)-C found in proteins belonging to the endothelin family. This signature pattern detected in sarafo toxins and bibrotoxin, potent vasoconstrictors isolated from Atractaspis snakes (26, 27), has never been observed in protease inhibitors. In leeches, among the different anticoagulant molecules involved in the inhibition of the coagulation cascade, three substances have been investigated in detail. These are hirudin (thrombin inhibitor) (1), antistasin (Factor Xa inhibitor) (1), and decorin (antagonist of platelet membrane glycoprotein IIb-IIIa) (29). Although these molecules differ in their biological activities, they share a common theme: they inhibit Factor Xa. They are small, specific inhibitors with subnanomolar affinities.

**TABLE II**

| Enzymes     | Therostasin | Antistasin |
|-------------|-------------|------------|
| Chymotrypsin| 5 nm        | 5 nm       |
| Trypsin     | 7.0 nm      | N.D.       |
| Cathepsin G | N.D.        | N.D.       |
| Plasmin     | N.D.        | N.D.       |
| Elastase    | N.D.        | N.D.       |
| Urokinase   | N.D.        | N.D.       |
| Thrombin    | 34 pm       | 0.62 nm    |

**FIG. 4.** Nucleotide and deduced amino acid sequences of therostasin. Nucleotides and amino acid residues are numbered in the right column. Amino acids are numbered from the first methionine residue and identified with single-letter codes. The peptide signal is underlined, and an asterisk indicates the stop codon (TAA) of the open reading frame. Possible polyadenylation signals are double-underlined.
amino acid sequences and inhibitory activities, their three-dimensional structures share the same conformational motif with that of the leech antithrombotic protein (C-X₆₋₁₂-C-X-C-X₃₋₅-C-X₃₋₅-C-X₈₋₁₄) (30). Therostasin did not share this leech antithrombotic protein conformational motif. Nevertheless, some homology was observed with antistasin, particularly in the active site domain (P₄-P₉, VRCRVHCP). By similarity, we speculate that the active site of therostasin (P₄-P₉, AQCRIYCP) will be at position 33–40. In this context the P₁ residue, which most often reflects the specificity of the protease that is being inhibited, e.g., lysine or arginine residues for trypsin-like enzymes and phenylalanine or leucine for chymotrypsin-like enzymes, would be an arginine in therostasin the same as for the Factor Xa inhibitors antistasin and ghilanten. However, in addition to the P₁ residue, the P₃ residue in antistasin (Arg 32) is particularly important for the interaction of the inhibitor with Factor Xa (31). In therostasin, this residue is replaced by Glu as in the putative active site of Hydra antistasin. Furthermore, it must also be noted that a putative exosite binding region has been defined in the N-terminal domain of antistasin, which explains the specificity and inhibitory potency of antistasin toward Factor Xa (31). This exosite binding region in position 15–17 (EGS) observed in ghilanten is not conserved in therostasin (DED). Because the spacing of cysteine residues in therostasin is somewhat different than in antistasin, it may be that the overall structures of antistasin and therostasin differ, leading to differences in the inhibitory mechanism.

When therostasin is compared with the other serine protease inhibitors found in the same species of leech, T. tessulatum, the sequence comparison reveals a high degree of sequence similarity. Therostasin shows 70 and 47% sequence identity with theromin, a thrombin inhibitor (16), and tessulin (17), a trypsin-chymotrypsin inhibitor, respectively. More particularly, the N-terminal parts (Cys²-Lys²⁸) of these three inhibitors are highly conserved (16). These results are of particular interest, as this conserved amino acid sequence among protease inhibitors with different specificities has never been observed previously in leeches. These similarities could be the result of an evolutionary divergence from an ancestral gene, arising after gene duplication, able to generate several peptides acting toward the specific substrates Factor Xa, thrombin, trypsin, and chymotrypsin (16, 17). This provides this leech species a high diversity of molecules acting at different points in its life cycle, such as coagulation, modulation of inflammation, storage, and preservation of blood and host-parasite communication. Because of its localization in the salivary glands, therostasin, like other protease inhibitors, may prevent blood clotting when biting and during subsequent storage in its foregut over several months. Nevertheless, storing blood requires the inhibition of proteases present in host leukocytes because lysis of these cells could induce an untimely and uncontrolled
digestion (32). We have previously demonstrated that the other protease inhibitors isolated in *T. tessulatum* are implicated synergistically in the inhibition of immunocyte activity (16, 17). We have also shown that therostasin is capable of inhibiting human leukocyte activation, similar to aprotinin, another serine protease inhibitor isolated from sea anemone (data not shown).

In conclusion, leeches have developed a panoply of molecules that may interfere with the coagulation cascade and host communication (33–35). We speculate that when biting, leeches inject substances able to block pain and inflammation and to induce vasodilatation in the victim. Noxious stimuli from injury will normally lead to an inflammatory response with a great deal of leukocyte activation. We speculate that when biting, leeches may interfere with the coagulation cascade and host communication (33–35). We speculate that when biting, leeches try to avoid this scenario. We therefore hypothesize that the challenge for leeches is to block peripheral nociception and local inflammation during the bite. In this context, the production of therostasin and other serine protease inhibitors, in conjunction with endocannabinoids, opiates known to be anti-nociceptive and neuro-signaling molecules, is a successful survival strategy to escape host-immune defense systems (33–35).

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REFERENCES

1. Tuszynski, G. P., Gasie, T., and Gasie, G. J. (1987) *J. Biol. Chem.* 262, 9718–9723
2. Brankamp, R. G., Blankenship, D. T., Sunkara, P. S., and Cardin, A. D. (1990) *J. Lab. Clin. Med.* 115, 89–97
3. Nutt, E., Gasie, T., Rodkey, J., Gasie, G. J., Jacobs, J. W., Friedman, P. A., and Simpson, E. (1988) *J. Biol. Chem.* 263, 10162–10167
4. Theunissen, H. J., Dijkema, R., Swinkels, J. C., de Poorter, T. L., Vink, P. M., and Van Dinther, T. G. (1994) *Thromb. Haemost.* 72, 4917–4927
5. Han, J. G., Law, S. W., Keller, P. M., Kniskern, P. J., Silberklang, M., Tung, J. S., Gasie, G. J., Friedman, P. A., and Ellis, R. W. (1989) *Gene* 75, 47–57
6. Ohta, N., Brush, M., and Jacobs, J. W. (1994) *Thromb. Haemost.* 72, 825–830
7. Whitlow, M., Arnaiz, D. O., Buckman, B. O., Davey, D. D., Griedel, B., Guilford, W. J., Koovakkat, S. K., Liang, A., Mohan, R., Phillips, G. B., Sets, M., Shaw, K. J., Xu, W., Zhao, Z., Light, D. R., and Morrissey, M. M. (1999) *Acta Crystallogr. Sect. D Biol. Crystallogr.* 55, 1394–1404
8. Malecha, J., Verger-Bouquet, M., and Tramu, G. (1989) *Can. J. Zool.* 67, 636–640
9. Bevins, M. M. (1976) *Anal. Biochem.* 72, 248–254
10. Laemmli, U. K. (1970) *Nature* 227, 680–685
11. Maniatis, T., Frisch, E. F., and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Vol. 1, pp. 7.19–7.23, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
12. Henderson, P. (1972) *Biochem. J.* 127, 321–333
13. Bieth, J. G. (1980) *Bull. Eur. Physiopathol. Respir.* 16, 183–195
14. Deleted in press
15. Sawyer, R. T. (1986) *Leech Biology and Behavior*, Vol. I, Oxford Science Publications, Clarendon Press, Oxford
16. Salzet, M., Chopin, V., Baert, J., Matias, I., and Malecha, J. (2000) *J. Biol. Chem.* 275, 10774–10780
17. Chopin, V., Stefano, G. B., and Salzet, M. (1998) *Eur. J. Biochem.* 256, 662–668
18. Holstein, T. W., Mala, C., Kur, E., Bauer, K., Greber, M., and David, C. N. (1992) *FEBS Lett.* 309, 288–292
19. Jung, H. I., Kim, S. I., Ha, K.-S., Joe, C. O., and Kang, K. W. (1995) *J. Biol. Chem.* 270, 13879–13884
20. Sollner, C., Mentele, R., Eckerskorn, C., Fritz, H., and Sommerhoff, C. P. (1994) *Eur. J. Biochem.* 219, 237–243
21. Kim, D. R., and Kang, K. W. (1998) *Eur. J. Biochem.* 254, 692–697
22. Laskowski, M., and Kato, I. (1980) *Ann. Rev. Biochem.* 49, 593
23. Capello, M., Vlasuk, G. P., Bergum, P. W., Huang, S., and, Hotze, P (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 6152–6156
24. Waxman, L., Smith, D. E., Arcuri, K. E., and Vlasuk, G. P. (1990) *Science* 248, 593–596
25. Seymour, J. L., Lindquist, R. N., Dennis, M. S., Mofat, B., Yansura, D., Reilly, D., Wessinger, M. E., and Lazarus, R. A. (1994) *Biochemistry* 33, 3849–3958
26. Kloo, Y., and Sokolovsky, M. (1989) *Trends Pharmacol. Sci.* 10, 212–214
27. Sokolovsky, M. (1991) *Trends Biochem. Sci.* 16, 261–264
28. Markwardt, F. (1957) *Hoppe-Seyler Z. Physiol. Chem.* 308, 147–156
29. Seymour, J. L., Hensel, W. J., Nevins, B., Stuils, J. T., and Lazarus, R. A. (1990) *J. Biol. Chem.* 265, 10143–10147
30. Krenzel, A. M., Wagner, G., Seymour-Ulmer, J., and Lazarus, R. A. (1994) *Science* 264, 1944–1947
31. Lapatto, R., Krenzel, U., Schreuder, H. A., Arkema, A., de Boer, B., Kalk, K. H., Hol, W. G., Grootenhuis, P. D., Mulders, J. W., Dijkema, R., Theunissen, H. J., and Dijkstra, B. W. (1997) *EMBO J.* 17, 5151–5161
32. Roter, F., and Zebe, E. (1992) *Comp. Biochem. Physiol.* 102, 85–92
33. Stefano, G. B., and Salzet, M. (1999) *Int. Rev. Cytol.* 187, 261–286
34. Salzet, M., Bisogno, T., Breton, C., and Di Marzo, V. (2000) *Eur. J. Biochem.* 267, 4917–4927
35. Salzet, M., Capron, A., and Stefano, G. B. (2000) *Parasitol. Today*, in press