A family of fibrinogen-related proteins that precipitates parasite-derived molecules is produced by an invertebrate after infection

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ABSTRACT After infection with the digenetic trematode Echinostoma paraensei, hemolymph of the snail Biomphalaria glabrata contains lectins comprised of 65-kDa subunits that precipitate polypeptides secreted by E. paraensei intramolluscan larvae. Comparable activity is lacking in hemolymph of uninfected snails. Three different cDNAs with sequence similarities to peptides derived from the 65-kDa lectins were obtained and unexpectedly found to encode fibrinogen-related proteins (FREPs). These FREPs also contained regions with sequence similarity to Ig superfamily members. B. glabrata has at least five FREP genes, three of which are expressed at increased levels after infection. Elucidation of components of the defense system of B. glabrata is relevant because this snail is an intermediate host for Schistosoma mansoni, the most widely distributed causative agent of human schistosomiasis. These results are novel in suggesting a role for invertebrate FREPs in recognition of parasite-derived molecules and also provide a model for investigating the diversity of molecules functioning in nonself-recognition in an invertebrate.

Nonself-recognition is a key component of internal defense, and one of the major goals of comparative immunobiology is to identify molecules functioning in this capacity in invertebrates. Although significant advances have been made in understanding insect defense molecules (1), among the molluscs, the second largest invertebrate phylum, little explicit sequence information has thus far been obtained for any molecules capable of binding nonself. Such molecules might be present on the surface of circulating, multifunctional defense cells called hemocytes or in the fluid portion of the hemolymph (2, 3).

In response to infection with the digenetic trematode Echinostoma paraensei, the freshwater gastropod Biomphalaria glabrata produces hemolymph proteins that bind to and precipitate secretory/excretory products (SEP) derived from cultured intramolluscan sporocyst stages of this parasite. Similar reactions do not occur in hemolymph from unexposed snails or in hemolymph derived from snails subjected to experimental wounding or that have been injected with bacteria (4, 5). This suggests that B. glabrata responds uniquely to digenean infection by initiating or increasing synthesis of humoral defense proteins.

Both snail hemolymph and SEP are complex polypeptide mixtures. SEP-binding proteins in hemolymph can be partitioned into two distinct classes. The first consists of two groups of previously defined lectins (5) that form a precipitate (designated as ppt 1) with particular components of SEP when small quantities of SEP are added to the hemolymph. If ppt 1 is removed by centrifugation and additional SEP is added to the hemolymph, a second precipitation event (ppt 2) is observed. Ppt 2 contains a second class of hemolymph proteins comprised of 65-kDa subunits that migrate as a broad band, suggestive of the presence of different polypeptides of slightly different mass (4). The major SEP component present in both ppt 1 and 2 is a 49-kDa polypeptide. From 4 to at least 14 days postinfection, hemolymph contains the lectins that participate in formation of ppt 1. The 65-kDa polypeptides involved in formation of ppt 2 are detectable from 2–8 days postinfection (4).

It was of interest to determine if formation of ppt 1 and 2 was comparable to arthropod coagulation or prophenoloxidase cascade reactions or to complement fixation or coagulation pathways of vertebrates, all of which rely on proteases (6, 7). Also, because little progress has been made in characterizing nonself-recognition molecules in molluscs, the molecular structure of the 65-kDa polypeptides of B. glabrata was further characterized.

METHODS AND MATERIALS

Parasite and Snails. E. paraensei (8) and two laboratory strains (M-line and 13–16-R1) and two Brazilian isolates (Salvador Bahia, Belo Horizonte) of B. glabrata were maintained in the laboratory. Several other pulmonate snails were examined: B. alexandrina (Planorbidae, Egypt); Bulinus truncatus (Planorbidae, Africa); Helisoma trivolvis (Planorbidae, New Mexico); Lymnaea stagnalis (Lymnaeidae, Holland); Stagnicola elodes (Lymnaeidae, Michigan); and Helix aspersa (Helicidae, New Mexico).

Precipitate Formation. SEP derived from 24-h cultures of ~10,000 E. paraensei sporocysts were concentrated 20-fold (4) before use in assays. Hemolymph was obtained from M-line snails infected for 4 days with E. paraensei (4). Protein content of SEP and cell-free hemolymph (plasma) was quantified using the DC Bio-Rad protein assay. SEP and plasma were mixed as described (4) to obtain ppt 1 and ppt 2. Precipitates were visualized by SDS/PAGE (8).

Protein Biochemistry. Potential modulatory factors were added to plasma samples 10 min before addition of SEP. The following factors were tested (final concentration indicated): (i) proteases: thrombin (5 units), transglutaminase (25 mU) [both blood clotting-activating proteases (7, 9)], and trypsin (5 μg); (ii) protease inhibitors: antipain, aprotinin, bestatin,

Abbreviations: FREP, fibrinogen-related protein; SEP, secretory/excretory products; RT-PCR, reverse transcriptase-PCR.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. BgMFREP1 (U82471), BgMFREP2 (U82479), BgMFREP3 (U82480), BgMFREP4 (U82478), BgRFREP1 (U82472), BgBFREP1 (U82477), BgSFREP1 (U82476), BalFRREP1 (U82473), HtrFREP1 (U82475), BtrFREP1 (U82474), BgMEST4 (U82753), Bgl1 (P80742), BgD4 (P80743), BgS5 (P80744), and BgO6 (P80745)].

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chymostatin, E 64, leupeptin, Pefablock (Pentapharm, Basel, Switzerland), pepstatin, phosphoramidon, EDTA, and EGTA (used at concentrations recommended by Boehringer Mannheim); and (iii) monosaccharides (each at 100 mM): α-methyl-D-mannoside, D-(-)arabinose, L-(-)fucose, D-(-)fructose, D(+)maltose, D(+)mannose, N-acetyl-galactosamine, N-acetyl-glucosamine, and rhamnose.

To determine if SEP and plasma formed precipitates in a manner analogous to antigen–antibody precipitin reactions, 0.1-, 1.0-, 2.5-, 5.0-, 10-, 25-, and 50-μl volumes of SEP (5 μg/μl of protein) were mixed with 25-μl aliquots derived from a pool of plasma (30 μg/μl of protein). After 1.5 h, precipitates were collected by centrifugation and visualized. Protein content of the resulting precipitates was determined by SDS-PAGE. To investigate the level of glycosylation, the 65-kDa polypeptides characteristic of ppt 2 were treated with N-glycosidase F for 24 h according to instructions provided by Boehringer Mannheim. The results were monitored by SDS-PAGE.

**Amino Acid Sequencing.** Precipitated 65-kDa polypeptides were gel-purified and subjected to internal trypsin digestion, and four of the resultant peptides (designated Bg01, Bg04, Bg05, and Bg06) were sequenced at the Microchemistry Facility, Memorial Sloan–Kettering Cancer Center, New York.

**PCR Experiments.** Peptide fragments obtained were used to design PCR primers. Genomic DNA (10 ng) extracted from snails or E. paraensei adult worms using a CTAB (hexadecyltrimethylammonium bromide)-based method (10) was used as template for PCR experiments (Perkin–Elmer, Foster City, CA). The temperature profile used was 4 min at 94°C; 30 cycles of 1 min at 92°C; 30 s at annealing temp; 1 min at 72°C (extension interval was increased by 4 s with each cycle); and 7 min at 72°C. For each primer combination used, the lower primer annealing temperature reported by the primer manufacturer (Ransom Hill, Ramona, CA) was selected.

**Cloning and Sequencing.** PCR fragments were cloned into pCRII (TA-cloning kit, Invitrogen). Sequencing reactions (Sequenase kit; Applied Biosystems) were initiated from vector and the manufacturer (Ransom Hill, Ramona, CA) was selected. Primer annealing temperature reported by the primer manufacturer (Ransom Hill, Ramona, CA) was selected.

**cDNA Library.** A A-ZAPII cDNA library (Stratagene) was produced using 1 mg of total RNA isolated from head–foot tissue of 40 B. glabrata snails harboring 4-day old E. paraensei infections (RNA kit, Stratagene). A ZAPII cDNA library (Stratagene) was produced.

**Nucleic Acid Hybridizations.** Restriction enzyme digests of genomic DNA were separated on agarose gels. Total RNA, isolated with an RNA kit (Stratagene), was separated on formaldehyde gels or used directly for slot blots. Total RNA from snails and E. paraensei was transferred to nitrocellulose filters, probed with [32P]-cTPP-labeled (T Prime-it kit, Stratagene), fibrinogen-related protein (FREP)-encoding, PCR-generated probes. The final wash was done at 60°C. Signal strength on Northern slot blots was quantified densitometrically (5) and expressed relative to housekeeping gene (BgMEST4)-specific signal strength. BgMEST4 was identified from the cDNA library by random expressed sequence tag sequencing and showed no similarities to known genes.

**Reverse Transcriptase (RT)-PCR.** RT-PCR was performed according to Perkin–Elmer kit instructions. The RT step involved antisense primers designed from previously obtained relevant sequences. These were then used in conjunction with matching sense primers for the PCR part of the procedure. Templates consisted of RNA (RNA kit, Stratagene) isolated from head–foot tissue or from hemocytes derived from individual E. paraensei-infected and uninfected B. glabrata. BgMEST4-specific primers designed to amplify a 204 bp product were used in control reactions.

**Sequence Analysis and Submission.** Sequence alignments were done using DNA STAR, ver. 2.01, software (Hitachi Software, San Bruno, CA) and were optimized by eye. Sequence analysis used BLAST and ENTREZ WEB services of the National Center for Biotechnology Information and the Prosite motif search facility of EXPASY. All sequences obtained were submitted to GenBank or SwissProt.

**RESULTS**

**Biochemical Results.** Addition of variable amounts of SEP to a constant amount of plasma resulted in formation of distinct precipitates (Fig. 1A). In the presence of low doses of SEP, precipitates contained snail-derived polypeptides of 200 and 80–120 kDa, previously designated ppt 1 (4). With increasing amounts of SEP, the quantity of ppt 1 diminished (Fig. 1A), suggestive of a condition comparable to “antigen-excess” noted with antigen–antibody precipitin reactions. Also, a second distinct precipitate appeared containing snail-derived polypeptides of 65 kDa (Fig. 1A), previously identified as ppt 2 (4). A state of antigen-excess was not achieved for ppt 2. However, both ppt 1 and 2 could be readily dissolved in the presence of excess SEP (Fig. 1B). SEP-free medium had no effect. These results indicate that SEP and plasma components form complexes in a manner analogous to precipitin formation after mixture of antigens and antibodies (11).

Neither precipitation reaction is influenced by the presence of protease inhibitors nor can comparable reactions be provoked in the absence of SEP by blood clotting-activating proteases (thrombin, transglutaminase) or trypsin (not shown). The plasma components of both precipitates behave as calcium-dependent lectins (12); they lack enzymatic activity and are inhibited by EDTA, EGTA, or 100 mM of L-fucose.

**Protein Sequencing.** Four peptides derived from gel-purified 65-kDa polypeptides were sequenced: VVTVLASEGLEMCDITTDDGGWTFIQ (Bg01); GKPDAF3XIDI-TWESVEXXI (Bg05); LEHDLAOGYVVVDLTA (Bg04); and FFTTFDKNDDQQONDN (Bg06). Alignments to known proteins revealed considerable similarity of Bg01 (bolded residues up to 73% identity) and Bg06 (whole length up to 56% identity) to the FREP domain of human serum lectin P35, tenascin, β-fibrinogen, and other known FREPs (13).

**Cloning and Sequencing.** Primers designed from peptides Bg01–06 failed to amplify products from genomic DNA of E. paraensei. One primer combination (sense Bg01s: 5′-GAYACIACIACIGAYGGNG-3′; antisense Bg06as2: 5′-GYGITCITITCTTYRTTCTRAA-3′ where I = inosine, N = A/T/C/G, R = A/G, and Y = C/T) was designed assuming that the relative orientations of the sequences used for primer design corresponded to homologous regions in the human fibrinogen gene sequence (13). Using these primers, a 354-bp product (designated BgMFREP1) was amplified from B. glabrata genomic DNA. This product was cloned and sequenced. Regions of this PCR fragment internal to the primers also were found to be similar to the fibrinogen domains of several FREPs, both at nucleotide level (e.g., 55% identity over 181 residues of tenascin from the pig Sus scrofa; GenBank accession no. X61599) and at deduced amino acid level (e.g., 58% identity over 106 amino acids relative to Homo sapiens serum lectin P35; GenBank accession D49353). Fur-
Presence of FREPs in Other Snail Species. Using the same primer combination, comparably sized FREP-encoding sequences were amplified from genomic DNA derived from other gastropod molluscs. All PCR fragments except those obtained from lymnaeid snails and *H. aspersa* were cloned and sequenced. The sequences obtained also displayed FREP characteristics. As an example, the *Bulinus truncatus*-derived FREP (BtrFREP1) is aligned with other FREPs and with a partial FREP sequence (14) from the pulmonate slug *Limax flavus* (*Limacidae*), which was obtained independently from our work (Fig. 2A).

*B. glabrata* Contains a Family of FREP-Encoding Genes. BgMFREP1 hybridized to at least five unique restriction fragments in *B. glabrata* genomic DNA. No hybridization was observed with *E. paraensei* genomic DNA (Fig. 2B), which, in conjunction with the PCR results previously described, confirms that the FREPs are produced by the snail host and not the parasite. The presence of multiple restriction fragments that hybridize BgMFREP1 suggests the occurrence of a FREP-encoding gene family in the *B. glabrata* genome.

**cDNA Analysis.** One clone from the cDNA library containing a BgMFREP1-reactive cDNA insert of 1411 bp contained a possible start site at the 5′ end of a 1116-nt ORF and a polyadenylation signal (aataaa) and a poly(A) tail at the 3′ end. Each of six independent cDNA inserts encoding this particular message had the same 5′ sequence. Surprisingly, none contained a predicted signal peptide, and the sequence obtained may not be full length. Nevertheless, the 1411-bp cDNA must be close to full length because it hybridized a ~1450-nt message on a Northern blot of RNA from an *E. paraensei*-infected *B. glabrata* snail (see below).

The ORF would encode a 372-amino acid peptide with a *M*<sub>r</sub> of 43.3 kDa and six possible N-linked glycosylation sites. After N-glycosidase F treatment, the *M*<sub>r</sub> of purified 65-kDa hemolymph polypeptides dropped by up to 28% to 46.7 kDa (Fig. 2C), indicating that the 65-kDa lectins are heavily glycosylated. The discrepancy of ~10% between the predicted and the actual deglycosylated *M*<sub>r</sub> of the polypeptides may then reflect a less than full length coding sequence represented in this cDNA, incomplete deglycosylation of the purified protein, nonlinear migration of the deglycosylated molecule on an SDS/PAGE gel, or any combination of these factors.

The 1411-bp cDNA was termed “BgMFREP2” because of evident FREP characteristics and its marked differences from the original internal peptide sequence data and BgMFREP1 (Fig. 2A). The deduced amino acid sequence of BgMFREP2 contains a FREP motif. The fibrinogen-related domain contains a FREP domain at the C terminus that are separated by a region that varies in length between the different BgMFREPs.

The N-terminal, nonfibrinogen-related portion of BgMFREP2 showed no clear overall similarity to any known sequence. However, predicted amino acid sequences flanking two cysteines spaced 80 residues apart have similarity with sequences containing the intra-chain loop-forming domains of Ig superfamily members. Eighteen of 20 residues conserved among invertebrate Ig superfamily members were present (16). Comparable results also were found in additional BgMFREP-encoding cDNAs (Fig. 2D).

Partial sequencing of cDNAs hybridizing with the original BgMFREP1 probe revealed two additional unique FREP-containing sequences (BgMFREP3 and 4). These partial sequences revealed the same basic structure as BgMFREP2; all contain a homologous sequence at the N-terminal end and a FREP domain at the C terminus that are separated by a region that varies in length between the different BgMFREPs. BgMFREP2 has the shortest such interceding region (Fig. 2E). Assuming that they have sequence similarity to BgMFREP2 at both 5′ and 3′ ends, BgMFREP3 and BgMFREP4 would have estimated *M*<sub>r</sub>s of 56 and 47 kDa, respectively, within the

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**Fig. 1.** (A) SDS/PAGE gradient (5–20%) gel of precipitates formed 1.5 h after mixing 0.1, 1.0, 2.5, 5, 10, 25, or 50 μl of SEP with different 25-μl aliquots of a common plasma pool. Shown beneath the gel is a graph of protein content (micrograms) of precipitates from each lane. The results are explained in the text. Note the presence of a 49-kDa band derived from SEP in lanes receiving higher doses of SEP. *M*<sub>r</sub> markers (kDa) are indicated. (B) SDS/PAGE gradient (5–20%) gel showing that, after exposure to SEP-free medium, both ppt 1 and ppt 2 remained in an insoluble form in a pellet (lane 1); they were not solubilized, and thus were absent from the supernatant (lane 2). In contrast, after exposure to SEP, components of ppt 1 and ppt 2 were solubilized and thus were not retained in the pellet (lane 3) but appeared in the supernatant (lane 4). Polypeptides smaller than 65 kDa are from SEP. *M*<sub>r</sub> markers (kDa) are indicated.

Moreover, signature amino acid residues found to be conserved among 15 FREPs (13) were present in the deduced amino acid sequence encoded by the snail-derived PCR product (Fig. 2A).
FIG. 2. (A) Alignment of gastropod FREP domains with HsaFibB: Homo sapiens fibrinogen B, M64983; HsaP35: H. sapiens plasma lectin P35, D49353; PpaFREP: FREP Panstichopus parvimensis (sea cucumber), M31326; and LfISASL: Limax flavus (slug) sialic acid-specific lectin, A48505. Note the fibrinogen characteristics of deduced amino acid sequences of the B. glabrata FREPs BgMFREP1–4 and the Bulinus truncatus FREP (BtrFREP1) provided as an example of the other gastropod FREPs obtained. Identical amino acid residues are bolded. Stars indicate amino acids conserved among fibrinogen-related sequences (13). In all available snail sequences, the tryptophan (W) in position 84 has undergone a conserved replacement with a tyrosine (Y). Similarly, a Y (position 212) was replaced by a W. The spacings between residues 68 and 80 as well as 106 and 108 are snail-specific. The two shaded boxes indicate areas that are homologous to the calcium-binding sites that are the likely carbohydrate-binding domains of the human serum lectin P35 (15). The Prosite motif (PS00514) for fibrinogen is underlined: BgMFREP3 has one additional residue at position 203. Residues have been renumbered to allow comparison of the FREP domains. (B) Genomic DNA from one B. glabrata snail (Bg) and from E. paraensei sporocysts (Ep) was digested with EcoR1 (E) or HaeIII (H) and probed at high stringency with radiolabeled BgMFREP1.

estimated $M_r$ range of the deglycosylated 65-kDa polypeptides (Fig. 2C). At the deduced amino acid level, BgMFREP4 contains two stretches of sequence (VVVTLSGLIMC-DHDTDGGMGWTFIQ and LEIADLAQYVVDLTAR) that correspond exactly to peptides Bg01 and Bg05 obtained from the purified 65-kDa polypeptides. This provides an additional independent verification of the direct sequence correspondence between 65-kDa hemolymph polypeptides and the BgMFREP family. Because BgMFREP4 has the same general organization (including the Ig-like sequences toward the N terminus) as other BgMFREPs, it also links these FREPs more securely to the peptide data. Such similarities also support the concept that the BgMFREPs identified to date comprise a family.

**Increased Production of FREPs After Infection.** BgMFREP1 hybridized with at least five mRNAs (ranging from 1450 to 2500 nt) on a Northern blot of RNA from individual infected snails. The same bands were only weakly detectable in mRNA from uninfected snails. BgMFREP2 hybridized strongly with a transcript from infected snails of the expected size (1450 nt) and less strongly with a 2500-nt message. No hybridization was observed with mRNA derived from uninfected snails (Fig. 3A).

Three primer pairs designed for sequencing of BgMFREP1–3 in RT-PCR experiments demonstrated the presence of FREP-encoding messages in both infected and control snails. FREP messages, especially those amplified by BgMFREP2 specific primers, occurred more frequently in infected snails (Fig. 3B). RT-PCR also showed the presence of FREP-specific signals in RNA samples from hemocytes from both infected and control *B. glabrata* snails (not shown). When RNA from *E. paraensei* sporocysts was used as template, no FREP-specific products were amplified (not shown).

Quantification of the levels of FREP gene expression in individual 3-day infected snails and unexposed control snails

The probe hybridized with at least five *B. glabrata* DNA fragments (arrowheads). No signal was detected from *E. paraensei* DNA. (C) Reduction in $M_r$ of the 65-kDa band (Left) to 46.7-kDa band (Right) after 24 h of deglycosylation with N-glycosidase F. (D) Partial sequences from the 5′ nonfibrinogen-related portion of *B. glabrata* FREPs have homologies to parts of Ig superfamily molecules. In particular, two cysteines that are situated 80 residues sequences apart are both flanked by residues that align with residues around intra-chain, Ig-type, loop-forming cysteines. Alignments are shown for the cysteine residues (indicated by stars) present at both positions 31 (Upper) and 111 (Lower) of the complete BgMFREP2 sequence. Relative to the BgMFREP2 sequence, identical residues are bolded, and conserved replacements are italicized. Abbreviations and GenBank accession numbers of the Ig superfamily sequences used for the alignments in this figure: CelCAM: *Caenorhabditis elegans*, similar to C2-type domains of N-CAM proteins, U29082; GigaCAM-GRASP: Gallus gallus (chicken) cellular adhesion molecule GRASP, U22889; HsaiIgEVR: Homo sapiens IgE variable region, Z22638; HsaiIgKCVR: Homo sapiens Ig k chain variable region, L03684; HsaiIgLCVR: Homo sapiens Ig light chain variable region, X13084; HsaiTCRβVR: Homo sapiens T cell receptor β variable region, D13086; RnoTCRβVR: Rattus norvegicus T cell receptor β variable region, U11793; MmIgHCVR: Mus musculus Ig heavy chain variable region, S36380; LstMDM, Lymnaea stagnalis (pond snail), molluscan defense molecule, U58769; and OoTCRβVR: Oryctolagus cuniculus (rabbit), T cell receptor β variable region, d17420. (E) Schematic alignment of (partial) BgMFREPs shows that FREP domains are located at the 3′ end of each sequence, immediately upstream from a stop codon. Gray boxes indicate the positions of two putative calcium-dependent carbohydrate-binding sequences. At the 5′ end, BgMFREPs show homologies to each other, but similarities with any other known genes are minimal. The length of the interceding sequence between these two regions varied between BgMFREPs (black boxes); BgMFREP2 is the shortest *B. glabrata* FREP obtained. Arrowheads indicate N-linked glycosylation sites (PS00001, Prosite). The position of the Prosite motif is also indicated.

Fig. 3. (A) Northern blot of total RNA from an individual infected (I) or uninfected (U) control snail. Radiolabeled BgMFREP1 hybridized with at least five transcripts in RNA from the infected snail (arrow heads). Fewer and lighter transcripts were detected in RNA from the uninfected snail. The probe BgMFREP2 hybridized strongly with a 1450-nt transcript. No BgMFREP2-specific signal was detected from the uninfected snail. (B) RNA samples from infected (I) and uninfected (U) snails (two examples of each shown) served as template for RT-PCR reactions using BgMFREP1–3-specific sense and antisense primers. For each primer pair, the graph depicts the number of positive signals from 10 infected and 10 uninfected snails. BgMEST4 is a housekeeping gene control. (**I**) and uninfected (U) snails were probed with either BgMFREP1+2 or BgMEST4, a housekeeping gene. Densitometric intensity of BgMFREP-specific signal [expressed as arbitrary units (AU)] relative to the BgMEST4-dependent signal was calculated for six infected and five control snails. The mean signal strength was significantly higher in infected snails (Student’s $t$ test, $P = 0.0025$).
revealed that the FREP-specific signal (probed for with BgM-FREP1 and BgMFREP2) was increased 3-fold on average (ranging from 2- to 20-fold) in infected snails relative to controls (Fig. 3C).

**DISCUSSION**

The combined biochemical and molecular results show that *B. glabrata* produces a family of FREPs with lectin activity that also has sequence similarities with members of the Ig superfamily. FREP abundance increases in response to digenean infection, and FREPs can precipitate parasite-derived molecules. FREP transcripts also are present in hemocytes, the circulating defense cells of gastropods. *Echinostoma paraensei* infections stimulate a 3-fold increase in hemocyte numbers by 4 days postinfection (17), which may account for the increased abundance of FREP transcripts noted in infected snails. Additional studies are needed to determine if cells or tissues other than hemocytes are involved in FREP production (2).

The increased abundance of FREPs in snails with successfully developing parasites could be interpreted to mean that FREPs lack relevance in internal defense. However, FREPs may function to bind soluble parasite-derived molecules and eliminate them from circulation. Such a function would be relevant in the case of *E. paraensei*, sporocysts of which have been shown to release soluble factors that impair host hemocyte functions (18).

FREPs are present in vertebrates (13, 15), echinoderms (19), mollusces (this study, ref. 14), and insects (20), so a fibrinogen-related domain must have existed before the divergence of protostomes and deuterostomes. In the vertebrates, fibrinogen is involved in platelet aggregation and fibrin clot formation and is also an acute phase protein (21). Invertebrate fibrinogen is involved in platelet aggregation and fibrin clot formation and is also an acute phase protein (21). Invertebrate blood coagulation pathways, when they exist, are not homologous to the fibrinogen-mediated coagulation pathway of vertebrates (6). Mollusces generally are considered to lack a hemolymph coagulation system (22). Both the present study and previous work with the *Limax flavus* lectin indicate that molluscan FREPs function in binding of nonself (14, 23). Human serum lectin P35 is also a FREP and binds to foreign molecules (15), suggesting that a primordial FREP function may have been in nonself-recognition. Another mammalian coagulation protein, von Willebrand factor, has similarity to silkworm hemocytin, a lectin with a presumed role in nonself-recognition (24). Other primordial FREP functions may have been involved in regulation of embryological development or in cell adhesion; some known FREPS function in these contexts as well (19, 25).

It is significant that the FREPs reported in this and some other studies are lectins. Lectins are involved in the innate defense responses of vertebrates (26) and are frequently implicated as mediators of nonself-recognition in invertebrates (2, 3, 12). Human P35 lectin and the *Limax* lectin are oposinins, a further indication of a role for FREPs in internal defense. The presence of a FREP family in *B. glabrata* then assumes additional relevance because collectively the members of this family could recognize a greater spectrum of nonself-entities.

The relative ease with which the FREPs identified in this study can be inhibited by L-fucose may belie a more sophisticated ability to recognize nonself when they are confronted with complex carbohydrates on naturally occurring macromolecules.

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