Cloning and Characterization of Physarum polycephalum Tectonins

HOMOLOGUES OF LIMULUS LECTIN L-6*

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Previous investigators have reported the presence of two dominant proteins, tectonin I (25 kDa) and tectonin II (39 kDa), in nuclei and nuclear matrix from plasmodia of Physarum polycephalum. We demonstrate, by a modification of the nuclear isolation protocol and by protease sensitivity, that the tectonins are not nuclear proteins but rather are located on the exterior surface of the plasma membrane.

We report the sequences of cDNAs of tectonins I and II, which encode 217 and 353 amino acids, respectively. Tectonin I is homologous to the C-terminal two-thirds of tectonin II. Both proteins contain six tandem repeats that are each 33–37 amino acids in length and define a new consensus sequence. Homologous repeats are found in L-6, a bacterial lipopolysaccharide-binding lectin from horseshoe crab hemocytes. The repetitive sequences of the tectonins and L-6 are reminiscent of the WD repeats of the β-subunit of G proteins, suggesting that they form β-propeller domains. Tectonin II has an additional N-terminal domain that includes a 47-residue sequence highly similar to the galactoside-binding sequence of the B-chain of ricin. The tectonins may be lectins that function as part of a transmembrane signaling complex during phagocytosis.

In its plasmodial form, the myxomycete Physarum polycephalum exists as a multinucleated syncytium that feeds on bacteria and organic detritus by phagocytosis. The many nuclei within a single plasmodium progress through the cell cycle synchronously, and at the end of the G2 phase undergo closed mitosis. Because of these characteristics, several investigators have examined the P. polycephalum nuclear matrix (1–4) and reported that, as with mammalian nuclear matrix, the P. polycephalum matrix contained a number of proteins ranging from approximately 40 kDa to more than 100 kDa but that it differed from mammalian nuclear matrix by having two dominant proteins with reported molecular masses of 23–28 and 35–38 kDa as determined by SDS-PAGE.1 The same proteins have also been found associated with purified rDNA chromatin (5). We have termed these proteins tectonins I and II, respectively.

In the present study we report the cloning and sequencing of the cDNAs for the tectonins, and we use trypsin digestion of cell fractions to assess their localization in the plasmodium. We find that the tectonins are not nuclear proteins but instead are located on the plasmodial surface, and we report a method for purifying P. polycephalum nuclei not contaminated with the tectonins.

The tectonins share with lectin L-6 of horseshoe crab hemocytes (6) six repeats of a novel consensus sequence that may form a β-propeller structure. Additionally, tectonin II contains in its N-terminal region a sequence similar to the galactose-binding domain of the B-chain of the plant toxin ricin (7). The tectonins may share with lectin L-6 the ability to recognize the outer membrane lipopolysaccharide of Gram-negative bacteria for phagocytosis and utilize an additional affinity for galactose to expand the number of ligands that they recognize.

MATERIALS AND METHODS

Strain and Culture of P. polycephalum—P. polycephalum M3, a diploid subline of the natural isolate Wisconsin I (8), was used. Microplasmodia were cultured in the semi-defined liquid medium of Daniel and Baldwin (9) at 27 °C in the dark with shaking.

Preparation of Nuclei, Nucleoli, Matrices, and Mitochondria—Initially, nuclei were isolated from exponentially growing microplasmodia by the method of Mohberg and Rusch (10) which uses 250 mM sucrose, 10 mM Tris-HCl, pH 7.6, 10 mM CaCl₂, and 0.1% Triton X-100 as the homogenization buffer (ST buffer). These nuclei were further purified by sedimentation (1000 × g, 30 min, 4 °C) through 50% Percoll (Pharmacia Biotech Inc.) in ST buffer.

Later, nuclei were isolated by the following procedure that was developed to remove contaminating tectonins from nuclei. Microplasmodia were collected by centrifugation (200 × g; 2 min, 23 °C), washed briefly with deionized water at 23 °C, and immediately homogenized in 5–10 volumes of 4 °C isolation buffer (200 mM glycerol, 20 mM Tris-HCl, pH 7.5, 40 mM KCl, 10 mM NaCl, 7 mM MgCl₂, and 0.1% Triton X-100) in a Waring blender at low speed using three 15-s pulses. Large debris were removed by passing the homogenate through a milk filter. The nuclei were sedimented from the homogenate (700 × g, 10 min, 4 °C) and suspended in 4 volumes of isolation buffer. The suspension was made 14% in Percoll and centrifuged (700 × g, 10 min, 4 °C), yielding a pellet of tectonin-free nuclei and a tectonin-rich pellicle which formed on top of the buffered Percoll. Entrapped nuclei were recovered from the pellicle by centrifugation along with the Percoll supernatant to a new centrifuge tube, mixing well, and centrifuging. After two to three repetitions, nearly all the nuclei were collected in the pellet.

Nucleoli were obtained by suspending microplasmodia 1.5 (v/v) in 25 mM sucrose, 3 mM EGTA, 10 mM Tris-HCl, pH 7.2, and disrupting them with a French pressure cell (10,000 p.s.i., 4 °C). As the lysate was collected, aliquots of 1 mM CaCl₂ were added to bring the Ca²⁺ concentration to 10 mM. Nucleoli were sedimented from the lysate (1000 × g, 15 min, 4 °C), washed 3–4 times in ST buffer before being sedimented through 50% Percoll in ST buffer (1200 × g, 30 min, 4 °C), and finally washed and resuspended in ST buffer.

Nuclear matrix was prepared by mixing equal volumes of the resuspended nucleoli and 200 mM NaCl, 20 mM MgCl₂, 74 mM Tris-HCl, pH 7.4, and digesting with 50 µg/ml RNase A, 150 µg/ml DNase I, and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) AF041455 and AF041456 for the tectonin I and II cDNA sequences, respectively.

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The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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50 μg/ml micrococcal nuclease (3–5 h, 37 °C). After being sedimented (800 × g, 15 min), washed, and resuspended in ST buffer, the nuclei were dialyzed against 2.5 mM NaCl, 25 mM β-mercaptoethanol in ST buffer (1–2 h, 4 °C). The nuclei were collected by sedimentation and washed with ST buffer, and the nucleosome digestion and NaCl dialysis were repeated two to three times (dialysis was redone by centrifugation (10,000 × g) to yield nucleolar matrix. Nuclear matrix was prepared by the same procedure except that the salt extraction solution was added directly to the nucleate-treated nuclei at a 20:1 ratio, and each extraction was for 30 min.

Mitochondria were prepared from microplasmodia that were washed with buffered water and then homogenized in 10 volumes of ST buffer without Triton X-100. After removing nuclei and unbroken cells by centrifugation (1000 × g, 5 min, 4 °C), the supernatant was collected followed by recentrifuging several times until microscopic examination confirmed that virtually all nuclei had been removed. A crude preparation of mitochondrial nucleoli was then collected by centrifugation (10,000 × g, 10 min, 4 °C).

Tectonin Purification—Plasmoidal tectonins I and II were purified from urea-solubilized nuclear matrices by isoelectric focusing followed by preparative SDS-PAGE (11).

Additionally, purified tectonins were obtained after cloning the cDNAs of both tectonins into expression vectors in Escherichia coli as follows. The coding sequences and 5′- untranslated regions of cDNAs encoding tectonins I and II were inserted between the BamHI and EcoRI sites of pET-3a (12) to create pHKI and pHKII, respectively. The constructions involved reverse transcriptase-polymerase chain reaction (PCR) with total plasmoidal RNA as template for tectonin I and PCR with a gt11 recombinant DNA template for tectonin II. The primer complementary to the 5′ end of the coding body carried an NdeI site to permit insertion into the vector so that translation would begin at the initiation codons of the tectonins. Between the 3′ end of each tectonin cDNA insert and the BamHI site of pET-3a, pHKI and pHKII carry a 21-base pair EcoRI-BamHI fragment from the polylinker region of pHBI24 (International Biotechnologies Inc.), left from cloning the amplified tectonin cDNAs in pHBI24 before moving them to pET-3a. The inserts of pHKI and pHKII were verified by DNA sequencing.

When exponentially growing cultures of E. coli BL21(DE3)(pLysE), harboring pHKI or pHKII were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside, tectonins I and II accumulated in 2–3 h to between 20 and 30% of the cell protein mass. These cells were collected by centrifugation and resuspended in 100 μg/ml lysozyme, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 10 mM Tris-HCl, pH 8.0. After being held at room temperature for 5 min, the cells were lysed by sonication (5–8 bursts on ice), followed by centrifugation (10,000 × g, 10 min). Approximately 80% of the tectonins remained in the soluble fraction and were purified by chromatography on DEAE-cellulose (DE52, Whatman Laboratory Products) using 5 mM Tris acetic acid, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol as buffer and eluting with a 20, 50, 80, and 100 mM NaCl gradient. The tectonins, which eluted with the 50 mM NaCl step, were then chromatographed on CM-cellulose (CM52, Whatman Laboratory Products) using 5 mM sodium acetate, pH 4.5, 1 mM EDTA, 1 mM dithiothreitol as buffer and eluting with a 100, 200, and 400 mM NaCl gradient. Both tectonins eluted with the 200 mM NaCl step.

Preparation of Antibodies—New Zealand White rabbits were immunized against either plasmoidal tectonin II or tectonins I and II expressed in E. coli. Freund’s complete adjuvant and 20–50 μg of purified tectonin were used for the first injection. Booster doses contained Freund’s incomplete adjuvant and 20–50 μg of tectonin. All antibody titers were greater than 1:1000.

IgG antibodies were separated from the anti-plasmoidal tectonin II serum by diluting the serum 1:10 with 15 mM sodium phosphate, pH 6.3, and passing it through a DEAE 250 column (Cuno Laboratory Products). IgG in the flow-through was precipitated by 40% (NH₄)₂SO₄, and then dissolved in 10 mM sodium phosphate, pH 6.8. Antiserum against the bacterially expressed tectonins were used without fractionation of the immunoglobulins.

Immunoblots—For Western blots, proteins were separated by 12% SDS-PAGE, transferred to nitrocellulose membranes by semi-dry electrophoresis (13), blocked for 30 min with 3% bovine serum albumin, and incubated with tectonin antibodies diluted in phosphate-buffered saline, and then incubated in 2% milk/0.05% Tween 20 and 30% of the cell protein mass. These cells were collected by centrifugation (10,000 × g, 10 min, 4 °C).

Selection of Tectonin cDNAs—A gt11 expression library carrying P. polycephalum plasmoidal cDNAs inserted at the EcoRI site of the vector (gift of Volker Vogt) was plated at 100 plaques per cm² and immunohybridization-screened by standard methodology (14). Anti-tectonin II IgG and [35S]-labeled protein A were used to detect clones expressing tectonins. Tectonin I clone 1 was identified by hybridization of the tectonin II cDNA labeled with [35S] by nick translation (Life Technologies, Inc., kit). Hybridization was for 24–48 h at 37 °C in 6X SSC, 50% formamide, 0.05% sodium pyrophosphate, 0.1% SDS, 0.02% Ficol, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, and 100 μg/ml sheared E. coli DNA. After hybridization, the filters were subjected to three 15-min washes in 2× SSC, 0.1% SDS at room temperature, followed by two 30-min washes in 0.1× SSC, 0.1% SDS at 37 °C.

To clone the complete 5′ ends of the tectonin cDNAs, first strand cDNA synthesis was performed (Riboclone, Promega) with 5 μg of total plasmoidal RNA and primer RM6, complementary to both tectonin I (nucleotides 91–111) and II (nucleotides 499–519). The cDNA was separated from the primer by electrophoresis in a 1.5% low melting point agarose gel (Life Technologies, Inc.), recovered by phenol extraction, and poly(A)-tailed. For the tailing reaction, an aliquot of the first strand cDNA in 50 μl of TE buffer was mixed with 26.5 μl of H₂O, heated for 5 min at 95 °C, and immediately cooled on ice. Twenty μl of 5X DNA tailing buffer (0.5 mM potassium cacodylate, pH 7.2, 10 mM CaCl₂, 1 mM dithiothreitol), 1.5 μl of 14 mM dATP, and 2 μl of terminal deoxynucleotidyltransferase (30 units, Life Technologies, Inc.) were added, the tube was incubated at 37 °C for 10 min and was then dissolved by one phenol/chloroform extraction and three ethanol precipitations. The final pellet was dissolved in 80 μl of H₂O. The poly(dA)-tailed first strand cDNA of tectonin II was amplified by PCR using RM6 and an XhoI-oligo(dT) primer, 5′-d(GTCGACTCTAGATTTTTTTTTTTTTTTTTTTTTTTTTTTTTT)-3′, and Taq polymerase (2.5 units, Perkin-Elmer). After amplification, the cDNA was purified by agarose gel electrophoresis, digested with XhoI and SalI, and cloned into the corresponding restriction sites of pBI24 and pBI25 using standard methodology (14).

Primer Extension—Primer extensions to determine the 5′ ends of tectonin mRNAs were performed by the procedure described for first strand cDNA synthesis, using primers that had been 5′-32P-labeled with polynucleotide kinase (Promega). The primers were complementary to nucleotides 74–94 of the tectonin I coding sequence and nucleotides 121–140 of the tectonin II coding sequence.

RNA Preparation and Blots—Total RNA was isolated from microplasmodia solubilized with guanidinium thiocyanate using a commercial kit (CLONTECH). For Northern blots, total RNA (15 μg per lane) was electrophoresed in a 1% agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized to 32P-labeled cDNA under the conditions described for cDNA library screening. The final washes were at 37 °C for low stringency and at 65 °C for normal stringency.

Circular Dichroism—Circular dichroism spectra were obtained for each tectonin chromatomgraphy were diaлизated against 2 mM phosphate buffer, pH 7.2, 200 mM NaCl, and circular dichroism (CD) spectra were measured at 20 °C using a Jasco J-500A spectropolarimeter and a 0.2-mm cell. SDS-PAGE with Coomassie Blue stain demonstrated that the preparations used for CD analysis contained less than 2% contaminating proteins.

Protein Digestions—Digestion with V8 protease (Miles Scientific) at 5 μg/ml was in 60 mM Tris-HCl, pH 7.5, 10 mM NaCl, 37 °C. Free trypsin and trypsin attached to acrylic beads were purchased from Sigma. Digestions with trypsin were in ST buffer without Triton X-100. At intervals, aliquots were removed and heated with SDS-containing Laemmli sample buffer to stop the digestion. The products were separated by SDS-PAGE and analyzed by protein staining and by Western blotting.

DNA Sequencing and Analysis—Single-stranded copies of pBI24 and pBI25 carrying the tectonin cDNAs were prepared using M13K07 helper phage (14). Sequence analysis was performed by the dyeoxy chain termination method using the single-stranded phagemid DNA as template, deoxyadenosine 5′-α-[32P]thio-triphosphate for labeling, and a Sequene kit from U. S. Biochemical Corp. Products were analyzed in 5% polyacrylamide/8 M urea gels.

An NCBI BLAST search (15) of protein data bases was used to identify proteins with sequences similar to the tectonins. Sequences were aligned using Lipman and Pearson’s Align program on the Southamton Bioinformatics Data Server at the University of South Hampton, UK, and ClustalW at EMBL, Heidelberg, Germany. The pls were calculated with the Compute p/MW Tool of ExPASy at the University of Geneva, Switzerland.
immunoscreening the
obtain the complete 5′ poly(A)-tailing of the first strand cDNA were performed to
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the major mRNA species with a minor species two nucleotides
reading frame of the cDNA was equivalent in size to tectonin II,
gene of the vector. Although the protein predicted from the
leader being present in half the clones. Primer extension from
leader represented 9–25 nucleotides long, with the shortest
leader being present in half the clones. Primer extension from
nucleotide 121 of the coding sequence using total plasmodial RNA confirmed that the 19-nucleotide 5′ leader represented the
major mRNA species with a minor species two nucleotides longer (Fig. 3A). Among the six sequenced cDNAs, there were only three nucleotide variances, and these were all in the
noncoding 5′ and 3′ ends (Fig. 2A), suggesting that they were transcripts of different alleles in the diploid organism.

To verify the sequence as encoding tectonin II, the open reading frame was moved to pET-3a, bacterially expressed, and the encoded protein purified. The bacterially expressed protein co-migrated with plasmodial tectonin II upon SDS-PAGE. Partial digestion of the two proteins with V8 protease produced identical sets of peptides as follows: 34, 30, 28, 26.5, 22, 19, 16 kDa apparent molecular mass. Trypsin rapidly degraded both proteins to a 25-kDa core peptide. Furthermore, antisera raised against the bacterially produced protein bound strongly to plasmodial tectonin II and weakly to a 25-kDa protein (presumably tectonin I) in Western blots of total P. polycephalum plasmodial proteins.

**RESULTS**

**Cloning Tectonin II**—Tectonin II cDNAs were identified by immunoscreening the λgt11 expression library carrying *P. polycephalum* microplasmodial cDNAs, using antibodies raised against tectonin II that had been purified from *P. polycephalum*. In Western blots, this antibody reacted strongly with the 39-kDa tectonin II and weakly with a 25-kDa protein, indicating the presence of a shared epitope in the smaller protein (Fig. 1). Among 32,000 recombinant phage screened, four immunopositive clones were found; all contained the same 1.13-kilobase pair insert, based on restriction enzyme digestion patterns. Sequencing revealed a single open reading frame of 1059 nucleotides that was fused in frame at its 5′ end to the lacZ gene of the vector. Although the protein predicted from the reading frame of the cDNA was equivalent in size to tectonin II, the reading frame did not contain an initiating methionine codon. Therefore, to establish the N terminus of the cDNA-encoded protein, anchored reverse transcriptase-PCR using total plasmodial RNA, a primer complementary to nucleotides 498–518 downstream from the lacZ junction, and poly(A)-tailing of the first strand cDNA were performed to obtain the complete 5′ end of the cDNA. Cloning and sequencing of six of these cDNAs showed that the only nucleotide missing from the reading frame of the original cDNA was A of the AUG initiation codon (Fig. 2A). Preceding the initiation codon was a leader 19–25 nucleotides long, with the shortest leader being present in half the clones. Primer extension from nucleotide 121 of the coding sequence using total plasmodial RNA confirmed that the 19-nucleotide 5′ leader represented the major mRNA species with a minor species two nucleotides longer (Fig. 3A). Among the six sequenced cDNAs, there were only three nucleotide variiances, and these were all in the noncoding 5′ and 3′ ends (Fig. 2A), suggesting that they were transcripts of different alleles in the diploid organism.

**Tectonin II mRNA and Gene**—To ascertain that the major species of tectonin II mRNA was represented by the cloned cDNA, a Northern blot of plasmodial RNA was probed with the tectonin II cDNA (Fig. 4). A single mRNA species of 1,200 nucleotides corresponding in size to the full-length tectonin II cDNA was observed. With low stringency hybridization conditions, weak labeling of a band below the tectonin II mRNA was observed. This will be shown to correspond to tectonin I mRNA.

The number of genomic loci encoding tectonin II was examined by hybridizing tectonin II cDNA to Southern blots of restriction digests of *P. polycephalum* DNA. All probes identified a single 14.5-kilobase pair BamHI-XbaI fragment indicating a single tectonin II gene. Restriction digests mapped at least five exons that encoded tectonin II that were distributed over a 4-kilobase pair segment of the *P. polycephalum* genome.

**Cloning Tectonin I**—Because tectonin I appeared to be related to tectonin II on the basis of the Northern blot of total plasmodial RNA, the λgt11 cDNA expression library was screened for tectonin I clones by low stringency hybridization to the tectonin II cDNA. Of four selected clones, three were incomplete tectonin II cDNAs, but the fourth carried a 582-nucleotide open reading frame 73% identical to the C-terminal portion of tectonin II. Anchored reverse transcriptase-PCR with the same primer used to obtain the 5′ end of the tectonin II cDNA and cloning in pIBI24 yielded two identical independent cDNAs that overlapped the putative tectonin I reading frame by 21 nucleotides and extended it for an additional 102 nucleotides. Combined, the cDNA sequences encode a tectonin I-sized 25-kDa protein (Fig. 2B). There is a single methionine codon 33 nucleotides from the 5′ end. As was the case with the tectonin II sequence, the AUG initiation codon is preceded by an A at −3 and has a G at +4 nucleotides in accordance with the initiation codon rule of Kozak (16). A poly(A) signal sequence AATAAA is located 11 nucleotides upstream from the 3′ poly(A) tail. Primer extension from nucleotide 74 within the coding body using total cellular RNA confirmed that the 5′ leader of the complete cDNA corresponds in length to that of the tectonin I mRNA (Fig. 3B).

To confirm the cDNA sequence as tectonin I-encoding, the deduced amino acid sequence was compared with the N-terminal sequence of purified *P. polycephalum* tectonin I. Unfortunately the majority of N termini were blocked, so that an apparent identification could be made for only 17 of the initial 20 amino acids, VTVWKEWEGLRVVGXAXLN. Nevertheless, 14 of the 17 amino acids were identical to the cDNA-predicted residues with the N-terminal methionine removed. Further evidence was obtained by joining the cDNA sequences to yield a complete reading frame, which was cloned in pET-3a for bacteriological expression, and the resulting protein was used to raise antiserum. This antiserum recognized tectonin I strongly and tectonin II less well and, when adsorbed against tectonin II, recognized only tectonin I in a Western blot of total *P. polycephalum* plasmodial proteins (Fig. 1). The bacterially expressed tectonin I and the plasmidial tectonin I co-migrated upon SDS-PAGE.

**Tectonin I mRNA**—A Northern blot of plasmodial RNA was probed with tectonin I cDNA (Fig. 4) to determine if the major species of tectonin I mRNA was represented by the cloned cDNA. A single mRNA species of 900 nucleotides corresponding in size to the full-length tectonin I cDNA was observed.

**Tectonins I and II Contain a Repeated Sequence**—The deduced amino acid sequences for the tectonins show that tectonin I and the C-terminal two-thirds of tectonin II are 73% identical and are comprised of six similar repeats that vary from 33 to 37 residues in length. An alignment of the repeats is shown in Fig. 5A. The tectonins appear to have diverged from
each other after the set of six repeats was established because the sequences are more conserved between corresponding repeats of the two tectonins than between repeats within the individual proteins.

Both proteins are unusual in their high content of histidine and tryptophan. Of 217 amino acid residues in tectonin I, 26 of them are histidine and 13 are tryptophan, whereas in tectonin II, 23 of the 353 amino acid residues are histidine and 15 are tryptophan.

A search of protein data banks showed that lectin L-6 from horseshoe crab hemocytes (6) is a homologue of tectonin I and the repeat domain of tectonin II. It is comprised entirely of six copies of the same repeat element as the tectonins (Fig. 5A). The amino acid sequence of lectin L-6 is 33% identical to both tectonin I and the repeat domain of tectonin II, and an additional 40% of lectin L-6 residues are conservative substitutions. The consensus sequence defined by combining all 18 repeats of the tectonins and lectin L-6 residues is 30–32 amino acids long and contains two conserved blocks separated by 3 to 5 amino acids as shown in Sequence 1.

Polar residues are represented by p and nonpolar by n; Q represents positively charged residues. The repeats are joined by linkers of 4–7 amino acids. Fig. 6 shows composite secondary structure predictions for the tectonin and L-6 repeats. Collectively, the repeats have four similarly sized regions predicted as β-strand separated by regions predicted to have ei-

### A Tectonin I cDNA

![Image of a table showing the nucleotide sequence of tectonin I cDNA.](image)

![Image of a table showing the nucleotide sequence of tectonin II cDNA.](image)

![Image of a table showing the deduced amino acid sequences of tectonin I and II cDNAs.](image)
ther turn or random coil secondary structure. Thus, each of the repeats appears to form a four-stranded β-sheet. This pattern of multiple repeats 23-40 amino acids long, containing four short β-strands, is thematic of β-propeller proteins (17). If indeed the tectonins and lectin L-6 are β-propeller proteins, they would seem most closely related to the family of WD proteins, which have the consensus structure and sequence shown at the bottom of Fig. 5A. In some cases the WD residues at the ends of the repeats are replaced with YQ (18) as would be the case with the tectonins and L-6.

Within the N-terminal, non-repetitive domain of tectonin II, there is a 47-amino acid segment homologous to the N-terminal galactose-binding site 1 of the ricin B-chain (Fig. 5B). There is 31% identity and an additional 20% conservative substitution of amino acids within the segment. The Asp-22 and Asn-46 residues that were shown by x-ray crystallography to be directly involved in galactoside binding by ricin (7, 20) are present in tectonin II at positions 63 and 87, respectively. Other important binding residues in ricin, Trp-37 and Lys-40, are present in tectonin II as Tyr-79 and Lys-82. Only Gln-35 of the ricin-binding site is missing in tectonin II, which has Leu at the corresponding position, and this Gln residue is also missing from the homologous second galactose-binding site 2 that is found in the C-terminal half of the ricin B-chain.

**Circular Dichroism of the Tectonins—** The circular dichroism (CD) spectra of pure solutions of bacterially expressed tectonins I and II were compared with reference spectra (21) to determine the relative contents of α-helix and β-sheet (Fig. 7). However, both tectonins contain such large amounts of aromatic amino acids, which absorb strongly below 200 nm, that the spectra could not be measured below 200 nm where the signature spectrum of β-sheet is found. Thus no direct estimation of the amount of β-sheet secondary structure was possible. The CD spectrum expected for an α-helical protein should have two large negative peaks of similar magnitude at 208 and 222 nm (21). Although both tectonins I and II have negative CD values near 208 nm, their molar ellipticities at that wavelength are only 6 and 9% of the values for a pure α-helical protein, respectively, implying that less than 10% of the total secondary structure of either tectonin is likely to be α-helix. Furthermore, the negative CD peak at 222 nm is lacking in the spectra of both tectonins. Thus, neither the magnitudes nor the shapes of the CD spectra support the presence of a significant amount of α-helix secondary structure in either tectonin I or II.

When reference spectra for α-helix, β-sheet, β-turn, and random coil (21) as well as the CD spectra of tryptophan and tyrosine (22) and histidine (23) were used, a CD spectrum for each tectonin protein could be calculated that closely matches the experimental CD spectrum (Fig. 7). The contributions of β-sheet could not be reliably quantitated for the reasons noted above; however, the amounts of α-helix structure for these spectra were calculated as zero.

Because of the large amount of histidine present in the tectonins, and the fact that histidine ionizes near cytoplasmic pH,
CD spectra were also measured for the tectonins at pH values that bracket the pH of histidine. However, no alterations in the CD spectra were found between pH 4 and 7.5 for tectonin I and between pH 6 and 7.5 for tectonin II.

Distribution of Tectonins among Cellular Compartments—To assess the reported presence of the tectonins as major components of the nuclear/nucleolar matrix, nuclei were isolated from microplasmodia by the procedure of Mohberg and Rusch (10), which has been used by most investigators of the *P. polycephalum* matrix. Nucleoli were isolated after disrupting the nuclei in a French press, and nucleolar matrix was obtained by repeated nuclease digestion and extraction with 2.5 M NaCl. SDS-PAGE showed that tectonins I and II were enriched in the nuclei and nucleoli, and they dominated the nucleolar matrix profile (Fig. 8). We also found that mitochondria prepared by differential centrifugation of a plasmodial homogenate contained tectonins, and the tectonins dominated the proteins present in mitochondrial nucleoids obtained by sucrose gradient centrifugation of Nonidet P-40 lysed mitochondria.

If tectonins are components of mitochondrial nucleoids, digestion of intact mitochondria with trypsin should leave the tectonins undegraded, and disruption of the mitochondrial membrane with Nonidet P-40 should expose them to degradation. Likewise, if tectonins are found in the nuclear interior as
part of the nucleolar matrix, they should not be digested when intact nuclei are treated with trypsin immobilized on acrylic beads to prevent its entry into nuclei. We found, however, that the tectonins were digested in all these cases. As shown in Fig. 9, the tectonin II present in mitochondrial preparations was about 80% degraded by trypsin within 5 min and nearly totally degraded by 20 min regardless of the absence or presence of Nonidet P-40. The kinetics of tectonin I degradation could not be precisely determined since it co-migrated with a relatively trypsin-resistant 25-kDa core of tectonin II on SDS-PAGE. However, by 40 min both the tectonin II core and tectonin I were undetectable on a Western blot. As a control, the activity of the mitochondrial interior enzyme succinate dehydrogenase was monitored in the same digestion reactions. It responded as expected, being eliminated by the trypsin digestion only when the mitochondria were lysed (Fig. 9). Similarly, immobilized trypsin degraded the tectonin II in nuclear preparations to a 25-kDa core. Thus we concluded that the tectonins were associated with the exterior of mitochondria and nuclei, perhaps present as contaminants.

Evidence for the tectonins actually being components of the plasmodial surface came from trypsin digestion of intact microplasmodia. As shown by Western blot (Fig. 10), the tectonins...
could be degraded at a similar rate regardless of whether the microplasmodia had been permeabilized by a 30-min incubation with 0.1% Triton X-100. However, the tectonins were not components of the slime that layers above plasmidia after brief centrifugation, as determined by an immunoblot of this extracellular material (not shown).

Removal of Tectonin Contaminants from Nuclei—Because the tectonins were apparently contaminants in preparations of nuclei, we sought to devise a method to remove them. We succeeded by modifying the isolation procedure of Mohberg and Rusch (10). Some changes were made in the homogenization buffer, such as substituting 7 mM Mg\(^2+\) for Ca\(^2+\) and 0.2 M glycerol for the 0.25 M sucrose, and including 40 mM KCl, but the critical difference was to eliminate sedimentation of the nuclei through 1 M sucrose. Instead, a crude nuclear pellet was collected by centrifuging the homogenate at 700 \(g\), followed by suspension and sedimentation from a 14% Percoll solution at 700 \(g\). A viscous, tectonin-rich fraction collected at the top of the tube while the nuclei sedimented to the bottom forming a pellet. SDS-PAGE and Western blot of the proteins in the homogenate, the supernatant (S700), and crude nuclear pellet (P700) after the first centrifugation and the tectonin fraction and purified nuclei after the Percoll centrifugation are shown in Fig. 11. Although a significant portion of the tectonins remained in the S700 supernatant, they were still the dominant components in the P700 nuclei. However, nearly all of both tectonin I and II was removed by Percoll centrifugation of the P700 pellet, confirming that the tectonins are not nuclear proteins.

DISCUSSION

The amino acid sequences of tectonins I and II deduced from cloned cDNAs show that the two proteins are closely related, with 73% of the amino acid residues in tectonin I and in the C-terminal two-thirds of tectonin II being identical. The tectonin genes are transcribed to yield poly(A)-tailed mRNAs of 870 and 1200 nucleotides for tectonin I and II, respectively. The only variations within either mRNA were extension of a few nucleotides at the 5' end for minor species of the tectonin II mRNA and isolated base changes in the untranslated leaders and 3' regions of both mRNAs, which indicate transcription of both alleles of the genes in the diploid plasmodium that was used as the source of mRNA in constructing the cDNA library.

The calculated pI for tectonin I is 6.49 and for tectonin II is 6.27. By using two-dimensional electrophoresis, we have determined pI values of 7.2 for tectonin I and 6.7 for tectonin II, whereas Denovan-Wright and Wright (3) reported 7.4 for tectonin I and 6.70–6.85 for isoforms of tectonin II which they isolated from nuclear matrix preparations. These values all show reasonable agreement, with tectonin II being somewhat more acidic than tectonin I.

The tectonin homologue lectin L-6 has been shown to have affinity for bacterial lipopolysaccharide, and it appears to participate in the anti-bacterial defense system of the horseshoe

FIG. 9. Accessibility of tectonin II in mitochondrial preparations to trypsin digestion. Isolated mitochondria that were intact (closed symbols) or lysed with Nonidet P-40 (open symbols) were digested with trypsin for different times and then assayed for relative amounts of intact tectonin II (circles) and succinate dehydrogenase activity (squares), an intraorganellar enzyme.

FIG. 10. Sensitivity of tectonin in microplasmodia to trypsin digestion. Microplasmodia that were intact or permeabilized by treatment with Triton X-100 were digested with trypsin for different times and then subjected to SDS-PAGE. A, Coomassie Blue-stained gel. B, Western blot probed with a mixture of antisera against tectonins I and II. The arrow indicates the position of tectonin II.

FIG. 11. SDS-PAGE of cellular fractions during separation of nuclei from tectonin-containing membranes. A, Coomassie Blue-stained gel. B, Western blot probed with a mixture of antisera against tectonins I and II. Lane 1, whole plasmodia; lane 2, homogenized microplasmodia; lane 3, S700; lane 4, P700; lane 5, tectonin-rich pellicle that floats on buffered 14% Percoll; lanes 6 and 7, purified nuclei. Equal proportions of each fraction were loaded except in lanes 6 and 7 that contain 5- and 10-fold greater amounts, respectively. M indicates protein mass standards.
crab (6). By virtue of the similarity between lectin L-6 and the tectonins, we suggest that the tectonins may perform a similar bacterial recognition function and thus aid *P. polycephalum* in identifying and phagocytosing its food.

The N-terminal domain of tectonin II seems likely to have an additional lectin activity, β-galactoside binding. A segment of 47 amino acids (residues 47–94) shares 31% identity with part of the B-chain of ricin and an additional 20% conservative substitution of amino acids. The B-chain of ricin contains two similar galactoside-binding sites created by gene duplication. The two halves of the B-chain have a 32% identity in amino acids (7). It is the N-terminal half of ricin that is most similar to the tectonin II segment, but the putative galacto-side-binding site in tectonin II shares features of each of the ricin B-chain sites. Specifically, x-ray crystallography of a ricin-lactose complex (20) has shown that the N-terminal site uses Asp, Gln, Trp, Lys, and Asn to bind galactose, whereas at the second site there is no Gln and Tyr replaces Trp. Tectonin II has Asp, Leu, Tyr, Lys, and Asn at the equivalent positions in the sequence. Lectin activity resident in this segment of tectonin II could augment its breadth of specificity or its affinity for potential foodstuff. The surface slime of *P. polycephalum*, which is a galactan (25), does not appear to be a ligand of the tectonins, as we have not detected either tectonin in slime recovered from culture medium.

Denovan-Wright and Wright (3) have reported that tectonin II is a glycoprotein, with up to four isomers of the protein having been detected by concanavalin A. Perhaps the apparent isomers were the result of tight binding of the tectonins to oligosaccharides. If there are covalently linked saccharide residues, their number must be low since we find that plasmodial tectonin II co-migrates with tectonin II expressed in *E. coli*, which should not be glycosylated.

Originally described as possible nuclear matrix proteins, our results indicate that the tectonins are instead principally cell-surface proteins that are accessible to digestion by trypsin in unpermeabilized plasmodia. In other experiments using antitectorin antibodies for immuno-electron and fluorescence microscopy,2 we have confirmed the surface location of the tectonins and found that they also are bound to the inner surface of certain cytoplasmic vesicles. On the external membrane surface, the tectonins are concentrated in crowns similar to the structures believed to be involved in phagocytosis and macropinocytosis in *Dictyostelium discoideum* (27, 28). At the crowns of *D. discoideum*, coronin accumulates on the cytoplasmic side of the plasma membrane and interacts with actin. The crowns subsequently enclose bacteria or fluid in a vesicle for digestion. The aggregation of tectonins on the external surface of similar crowns supports a role for tectonins in such endocytoses by binding food and perhaps triggering vesicle formation.

How newly synthesized tectonins are transported and bound to the plasmoidal surface is not clear. Their cDNAs do not encode precursor forms with N-terminal signal sequences, and neither protein has recognizable sequences for membrane transport or attachment of lipids.

We are able to isolate *P. polycephalum* nuclei lacking the tectonins with mild conditions, which confirms that they are not nuclear proteins. By sedimentation through buffered 14% Percoll, tectonin-free nuclei are separated from contaminating membranes, and both tectonins are found in a viscous, membranous pellicle at the top of the Percoll solution as would be expected for fragments of the plasmoidal surface. Unless the tectonin-rich membranes are separated from the nuclei or nucleoli that are used to prepare matrix, we confirm that they appear as dominant components as reported by other investigators (1–3). We have also observed that the tectonins can contaminate mitochondrial preparations, and Amero et al. (5) have reported that they were present as possible contaminants of *P. polycephalum* rDNA chromatin. Why are the tectonins such ubiquitous contaminants? A likely answer is that they are part of complexes on the surface of plasmodia, which are associated with the viscous polysaccharide slime that coats the exterior surface. When fragmented by homogenization, surface fragments enriched in tectonins could entrap organelles and large molecular complexes via the associated polysaccharide. The purification method of Mohberg and Rusch (10), which relies on sedimentation through 1 M sucrose, may actually have driven the entrapment due to the sucrose concentration. The fact that tectonins can be separated from nuclei under low ionic strength and with 0.1% Triton X-100 in the buffer shows that their apparent association with nuclei is not a matter of strong interactions that must be disrupted. The buffered 14% Percoll that we used for purification of nuclei provides the density differential and low solute concentration needed to separate nuclei from tectonin-containing fragments of the cell membrane.

Together with limulus lectin L-6, the tectonins establish a new family of proteins based on the repeated consensus sequence: \(\text{pWpXIVIPgLpVpX}_{14}\text{pVWGVNpXpYP1Yp}\). Including the additional 4–7 residues that connect the six tandem repeats of this sequence in the tectonins and L-6, the repeat units are 33–38 amino acids long. Conserved residues in the consensus sequence define two blocks of 13–14 amino acids that are connected by 3–5 residues in the middle of the consensus sequence. The second or third residue at the beginning of each block is tryptophan or another aromatic amino acid, and the penultimate residue at the end of the second block is tyrosine. Valine or isoleucine is found at four rather evenly spaced intervals across the consensus sequence. In lectin L-6, a tight three-residue turn is locked in place between every other repeat of the consensus by a disulfide bond between cysteines that bracket the turn (6); this feature is absent from the sequences that link the tectonin repeats.

Collectively, the tectonin and L-6 repeat sequences predict that each repeat is comprised of four similarly sized short β-strands connected by turns or random coils. Thus, each of the repeats appears to form a four-stranded β-sheet. This pattern of multiple repeats 23–40 amino acids long, each containing 4 short β-strands typifies β-propeller proteins (17). Furthermore, the tectonin consensus sequence is similar, particularly in its latter half, to the repeated WD sequences of the β-subunits of G proteins, where YQ sometimes replaces the WD residues (18). Much is known regarding the position of G\(\beta\) on the inner surface of plasma membranes and its function in transmembrane signaling (26). The apparent similarity of tertiary structure of the tectonins may reflect their proposed function as external components of a signal system for response to food.

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P. polycephalum Tectonins: Homologues of Lectin L-6

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