The Mouse Tectorins

MODULAR MATRIX PROTEINS OF THE INNER EAR HOMOLOGOUS TO COMPONENTS OF THE SPERM-EGG ADHESION SYSTEM*

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The cDNA and derived amino acid sequences for the two major non-collagenous proteins of the mouse tectorial membrane, α- and β-tectorin, are presented. The cDNA for α-tectorin predicts a protein of 239,034 Da with 33 potential N-glycosylation sites, and that of β-tectorin a smaller protein of 36,074 Da with 4 consensus N-glycosylation sites. Southern and Northern blot analysis indicate α- and β-tectorin are single copy genes only expressed in the inner ear, and in situ hybridization shows they are expressed by cells both in and surrounding the mechanosensory epithelia. Both sequences terminate with a hydrophobic COOH terminus preceded by a potential endoproteinase cleavage site suggesting the tectorins are synthesized as glycosylphosphatidylinositol-linked, membrane bound precursors, targeted to the apical surface of the inner ear epithelia by the lipid and proteolytically released into the extracellular compartment. The mouse β-tectorin sequence contains a single zona pellucida domain, whereas α-tectorin is composed of three distinct modules: an NH₂-terminal region similar to part of the entactin G1 domain, a large central segment with three full and two partial von Willebrand factor type D repeats, and a carboxyl-terminal region which, like β-tectorin, contains a single zona pellucida domain. The central, high molecular mass region of α-tectorin containing the von Willebrand factor type D repeats has homology with zonadhesin, a sperm membrane protein that binds to the zona pellucida, whereas β-tectorin is considerably smaller (M₉ = 43,000) and not covalently associated with α-tectorin. The cDNA for chick β-tectorin has been recently cloned (3) and analysis of the sequence indicates β-tectorin is related to the pancreatic zymogen granule protein GP2, the urinary glycoprotein uromodulin (Tamm-Horsfall protein), and two components of the extracellular matrix that surrounds the unfertilized egg, the zona pellucida proteins ZP2 and ZP3.

The mammalian tectorial membrane is considerably more complex than that of the bird, containing polypeptides that react with antibodies to types II, V, and IX collagen (4, 5) and a number of collagenase-insensitive glycoproteins that account for up to 50% of the tectorial membrane protein (4). Under reducing conditions these non-collagenous proteins of the mouse tectorial membrane form three broad, diffuse bands on SDS gels with peak masses of 173, 60, and 45 kDa which have been referred to as the high, medium, and low molecular mass tectorins (HMM, 1 MMM, and LMM tectorin, Ref 6). The polydispersity of the mouse tectorins observed on SDS gels may result partially from glycosylation heterogeneity. For example, mouse HMM tectorin is sulfated and following treatment with endo-β-galactosidase it is converted from a diffuse smear to a sharp band with an M₉ of 160,000 (4, 6). Under nonreducing conditions most of the non-collagenous mouse tectorial membrane protein forms a large (M₉ > 240,000), disulfide cross-linked complex which may be homologous to chick α-tectorin. A proportion of the protein in the LMM tectorin fraction is not covalently associated with the other tectorins (4) and may represent the murine homologue of chick β-tectorin.

The studies of Kronester-Frei (7) originally described the presence of two major fibril systems in the mammalian tectorial membrane; the Type A protofibrils, straight unbranched filaments organized in bundles that run predominantly radially across the tectorial membrane, and the Type B protofibrils, described as branched, coiled, irregular diameter fibrils that...
could apparently exist in two states of hydration, with the highly hydrated form comprising the matrix within which the Type A protofibrils were found, and the weakly hydrated form forming the covernet fibrils, the marginal net, Hensen’s stripe, and the limbal undersurface of the tectorial membrane. The Type A protofibrils are entirely degraded by bacterial collagenase (8), and can be labeled by antibodies directed against Types II and IX collagen (9). The matrix within which the Type A protofibrils are embedded is resistant to degradation by bacterial collagenase and fixation in the presence of tunican acid reveals it is composed of two types of fine, 7–9-nm diameter filament; a light and a dark staining type that are linked to one another by staggered cross-bridges and arranged in sheets with the filaments lying within the plane of each sheet (8). The alternating arrangement of the two fibril types in these sheets gives the matrix a striated appearance, and these sheets roll up to form the thicker, hydrated Type B protofibrils described by Kroner-Frei (7).

To further our understanding of the way in which the tectorial membrane matrix is formed and functions in the process of mechanotransduction, we have now deduced the primary structure of the mouse tectorins. The data indicate that the HMM, MMM, and LMM tectorins observed on SDS gels are highly derived from a single large mouse α-tectorin sequence, with a smaller sequence, that of mouse β-tectorin, contributing partially to the LMM tectorin fraction. The ways in which these two proteins may interact via their different domains to form the observed structure of the non-collagenous matrix of the tectorial membrane are discussed.

**EXPERIMENTAL PROCEDURES**

**NH$_2$-terminal Protein Sequencing**—Mouse and chick tectorial membranes were collected by dissection in phosphate-buffered saline containing a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 10 μM pepstatin, and 1 μg/ml leupeptin), washed with the same buffer containing 0.1% Triton X-100 and stored at −70 °C until sufficient numbers had been collected. To prepare the mouse tectorins for sequencing, approximately 550 tectorial membranes were dissected from the cochleae of 100 2–3-day-old postnatal Swiss CD1 mice (Charles River, United Kingdom) in Hepes-buffered Hanks’ balanced salt solution, pH 7.0, collected separately, and snap frozen in liquid nitrogen. Poly(A)$^+$ RNA was isolated by a one-step oligo(dT)-cellulose selection method (Quick Prep Micro kit, Pharmacia, St. Albans, United Kingdom) and double stranded cDNA was synthesized from 2 μg of poly(A)$^+$ RNA in a final volume of 20 μl using a commercial kit (Boehringer Mannheim). Degenerate forward and reverse oligonucleotide primers based on the NH$_2$-terminal amino acid sequences of the chick α- and ε-tectorins (αε51, ATGGGCTI(TC)TNTA/TC/CCNT, αεR1, AANGG/GATAN-G(A)/GANGGCCAT; αε51, GTNACNGNAAG(A)/AATG/CAGAGGA; αεR1, TCT/CTT/CTG/TTT/TTGNGNTNAC) were used to isolate candidate α-tectorin RT-PCR products. A degenerate primer based on an ε-tectorin sequence of one of the mouse tectorin bands (mm4α1, GA/GACA/TC/ACNCCNAA/TC/CAAG/CG), and a degenerate antisense primer (βTDEGR, GGNGTNGCGCAAA/CA/CA/AG/TT) based on an amino acid sequence (CWATPS) conserved in chick β-tectorin and mouse α-tectorin were used to isolate a β-tectorin specific RT-PCR product. Separate PCR reactions using 1-μl aliquots of the two cDNA populations and 50 pmol of primer in a 50-μl reaction volume (as described in Ref. 3) were hot started and followed by 25 cycles of 50 °C for 15 s, 72 °C for 1 min, and 94 °C for 15 s. The reactions ended with a 10-min incubation at 72 °C. PCR products were analyzed by agarose gel electrophoresis in 1 × Tris borate-EDTA buffer and products specific to the GLER were isolated using GeneClean (Stratage Scientific, Luton, United Kingdom).

**Preparation and Screening of Mouse Cochlear cDNA Libraries**—GLER samples were collected from the cochleae of approximately 500 2–3-day-old postnatal Swiss CD1 mice as described above. Total RNA was isolated using guanidinium thiocyanate extraction (11) followed by cesium trifluoroacetate gradient centrifugation (12) and poly(A)$^+$ mRNA selected by oligo(dT)-cellulose chromatography (Quick Prep Micro kit, Pharmacia). A directionally cloned, oligo(dT)-primed cDNA library was constructed using commercial kits (Stratagene, Cambridge, United Kingdom). First strand cDNA synthesis was primed with an oligo(dT) primer containing a 5’ XhoI site, the cDNA was then double stranded, EcoRI adapters were added and the cDNA digested with XhoI. The cDNA was directionally ligated into λ UniZap XR arms and packaged in vitro. A randomly primed cDNA library was constructed in a similar manner, with the following modifications; poly(A)$^+$ RNA was isolated from cochlear tectorial membranes of 500 2–3-day-old postnatal mouse cochleae with a Quick Prep Micro kit, first strand cDNA synthesis was primed with a mixture of random hexamers and an antisense primer designed to the 5’ end of clone A2 (GAAATGGGCGGCTAGTGGTGC) and, in addition of EcoRI adapters, the double stranded cDNA was ligated into the EcoRI site of λ Zap II. Libraries were screened at high density with 32P-labeled DNA probes. Positive plaques were rescreened to purity and converted to plasmids by phage rescue.

**Southern and Northern Blotting**—Genomic DNA was isolated from neonatal mouse brain by a modification of the method of Blin and Stafford (13, 14). Aliquots of DNA (10 μg) were digested to completion with EcoRI, PstI, SacI, or KpnI in the supplied buffers (Promega Ltd., Southampton, United Kingdom), electrophoresed on a 0.7% agarose gel in 1 × Tris borate-EDTA buffer, transferred to nylon membrane (Hybond N, Amersham International, Little Chalfont, United Kingdom) (15), and covalently bound to the dried membrane by UV cross-linking. Hybridization to 32P-labeled DNA probes and washing to high stringency were carried out according to the manufacturer’s instructions.

Poly(A)$^+$ RNA was isolated from cochlear, brain, heart, liver, kidney, intestine, lung, and skin tissues of 2–3-day-old postnatal mice as described by RT-PCR. Aliquots of 3 (μg) were digested with 1 % agarose-formaldehyde gel (14) and transferred to nylon membrane as described above. Hybridization to 32P-labeled DNA probes and washing to high stringency were carried out as described by Angst et al. (16).

**Probe Preparation and Labeling**—Probes for Southern and Northern blotting were generated by PCR or restriction enzyme digestion of cDNA clones. Probe fragments were separated by gel electrophoresis, using standard procedures on a 47TA liquid-pulse instrument (PE Applied Biosystems) and a Beckman LF 3000 automated NH$_2$-terminal sequencer.

**Reverse Transcription PCR**—Differential analysis of reverse transcriptase (RT)-mediated PCR products obtained from a region of the cochlea that is known to be derived from tectorial cells but that is not involved in tectorial membrane production was used to identify products potentially derived from tectorin cDNAs. The combined greater and lesser epithelial ridges (GLER) which are known to produce the tectorial membrane, and the stria vascularis which are not involved in tectorial membrane synthesis were dissected from the cochleae of 100 2–3-day-old Swiss CD1 mice (Charles River, United Kingdom) in Hepes-buffered Hanks’ balanced salt solution, pH 7.0, collected separately, and snap frozen in liquid nitrogen. Poly(A)$^+$ RNA was isolated by a one-step oligo(dT)-cellulose selection method (Quick Prep Micro kit, Pharmacia, St. Albans, United Kingdom) and double stranded cDNA was synthesized from 2 μg of poly(A)$^+$ RNA in a final volume of 20 μl using a commercial kit (Boehringer Mannheim). Degenerate forward and reverse oligonucleotide primers based on the NH$_2$-terminal amino acid sequences of the chick α- and ε-tectorins (αε51, ATGGGCTI(TC)TNTA/TC/CCNT, αεR1, AANGG/GATAN-G(A)/GANGGCCAT; αε51, GTNACNGNAAG(A)/AATG/CAGAGGA; αεR1, TCT/CTT/CTG/TTT/TTGNGNTNAC) were used to isolate candidate α-tectorin RT-PCR products. A degenerate primer based on an ε-tectorin sequence of one of the mouse tectorin bands (mm4α1, GA/GACA/TC/ACNCCNAA/TC/CAAG/CG), and a degenerate antisense primer (βTDEGR, GGNGTNGCGCAAA/CA/CA/AG/TT) based on an amino acid sequence (CWATPS) conserved in chick β-tectorin and mouse α-tectorin were used to isolate a β-tectorin specific RT-PCR product. Separate PCR reactions using 1-μl aliquots of the two cDNA populations and 50 pmol of primer in a 50-μl reaction volume (as described in Ref. 3) were hot started and followed by 25 cycles of 50 °C for 15 s, 72 °C for 1 min, and 94 °C for 15 s. The reactions ended with a 10-min incubation at 72 °C. PCR products were analyzed by agarose gel electrophoresis in 1 × Tris borate-EDTA buffer and products specific to the GLER were isolated using GeneClean (Stratage Scientific, Luton, United Kingdom).
recovered with GeneClean or Gelex resin (Stratech Scientific), and random primer labeled (17) with [α-32P]dCTP using a MegaPrime labeling kit (Amersham). Unincorporated label was removed on Sephadex G-50 columns (Pharmacia) and the probes were denatured with alkali before use.

DNA Sequencing and Analysis—Double stranded plasmid DNA minipreps (Promega) of cDNA clones were sequenced by the method of Sanger et al. (18) using T7 DNA polymerase (Pharmacia) and a combination of sequencing templates from nested deletions (19) and templates primed with synthetic oligonucleotides. Analysis of DNA and derived amino acid sequences was performed using the DNA Star package (DNA Star, London, United Kingdom). DNA and protein sequence data bases (GenBank, EMBL, DDBJ, and PDB) were searched using the BLAST network service at the National Center for Biotechnology Information (20).

Preparation of Antipeptide Sera—Three synthetic peptides for α-tectorin [25–257] RELMYPFWQNITDRT; [325–335] TCPERPEYLEIDIN; and [342–346] KONTIGIEENGVSILT] and one for β-tectorin (mβ25–38) PTDETVLVHENGKD and one for β-tectorin (mβ25–38) PTDETVLVHENGKD] based on the derived amino acid sequences of α- and β-tectorin were synthesized and purified by high performance liquid chromatography. Peptides were coupled to bovine serum albumin using glutaraldehyde (21) and dialyzed against phosphate-buffered saline. Conjugates (500 μg) were suspended in Freund’s complete adjuvant and injected subcutaneously into rabbits. The immunization was repeated a further three times with Freund’s incomplete adjuvant at 4-week intervals. Antipeptide antibodies were affinity isolated using the respective peptide coupled to either cyanogen bromide-activated Sepharose 4B or Affi-Gel 15.

Western Blotting—Mucous tectorial membranes were solubilized in reducing SDS-PAGE sample buffer, separated on 7.5% polyacrylamide gels, and transferred to nitrocellulose membranes using semi-dry blotting. Nitrocellulose blots were preblocked for 1 h with 3% dried milk powder, 10% horse serum in Tris-HCl buffered salt solution containing 0.05% Tween 20 and incubated overnight in the same solution containing the affinity isolated antipeptide antibodies at concentrations of 5 or 50 μg/ml. After washing, bound antibodies were labeled with biotinylated anti-rabbit IgG (1:1000 dilution) (Dako, High Wycombe, United Kingdom) and detected using the respective conjugate. Bound antibody complexes were visualized using streptavidin–alkaline phosphatase activity was visualized with 0.05 mg/ml bromochloroindolyl phosphate, 0.1 mg/ml nitro blue tetrazolium in alkaline buffer; or 0.05 mg/ml nitro blue tetrazolium in alkaline buffer.

In Situ Hybridization—Inner ears from 2-day-old postnatal mice were immersion fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline for 1 h prior to overnight fixation in 4% paraformaldehyde in phosphate-buffered saline containing 30% sucrose. Tissues were embedded in agarose and cryosectioned at a thickness of 20 μm. Aliquots (10 μg) of plasmid minipreps (Promega) of the α- and β-tectorin cDNA clones A1 and B3 were linearized with either EcoRI or XhoI (Promega, United Kingdom). Antisense RNA was transcribed from EcoRI cut clones using T7 RNA polymerase and sense RNA was transcribed from XhoI cut clones using T3 RNA polymerase. In situ hybridization, washing and autoradiography were carried out as described by Goodyear et al. (22).

RESULTS

NH2-terminal Amino Acid Sequence of Chick and Mouse Tectorins—NH2-terminal amino acid sequence data for the six chick α-tectorins and four mouse tectorin bands are presented in Tables I and II. The sequences for the chick α2- and α3-tectorin bands are identical suggesting chick α3-tectorin is either a truncated or differentially glycosylated form of chick α2-tectorin. The sequences show no similarity to derived amino acid sequences or proteins in the current data bases (GenBank, EMBL, DDBJ, and PDB) except for the sequence of the mtm4 band which has 86% identity with the predicted NH2-terminal of mature chick β-tectorin (3). The mouse tectorin bands mtm2 and mtm3, which have Mr of 40,000 and 34,000, respectively, have almost identical NH2-terminal sequences, suggesting mtm2 is an incompletely deglycosylated form of mtm3 and that both tectorin bands are derived from the same protein. Furthermore, identity between the sequence YPFW in mtm3 and the α-tectorin suggests that the mtm3 sequence is derived from the mouse homologue of chick α4-tectorin. The first amino acids of the mtm1 band and chick α1-tectorin also share 67% identity suggesting mtm1 is the mouse homologue of chick α1-tectorin.

Isolation and Identification of Mouse α- and β-Tectorin cDNA Clones—Assuming that the different mouse α-tectorins were derived from a large precursor protein, RT-PCR was performed on poly(A)+ RNA from the GLER and stria vascularis with combinations of forward and reverse primers based on the NH2-terminal amino acid data. Using one primer pair based on the NH2-terminal amino acid sequences of the chick α4 and α5-tectorins (cα5S1 and cα4R1), a GLER-specific PCR product of 300 bp was amplified which, while smaller than expected, was not as amplified from the stria vascularis cDNA and hybridized to a cochlear-specific cDNA library. Thirty-two positive clones were identified, of which two, A1 and A2, were selected for further study. Clone A1 is encoded by a single gene as judged by

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**TABLE I**

<table>
<thead>
<tr>
<th>Tectorin</th>
<th>M&lt;sub&gt;r&lt;/sub&gt;</th>
<th>NH&lt;sub&gt;2&lt;/sub&gt;-terminal amino acid sequences of chick α-tectorins</th>
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<tbody>
<tr>
<td>α1</td>
<td>146,000</td>
<td>PHYHTTFDGFL</td>
</tr>
<tr>
<td>α2</td>
<td>60,000</td>
<td>MANELQXYQY</td>
</tr>
<tr>
<td>α3</td>
<td>56,000</td>
<td>MANELQXYQY</td>
</tr>
<tr>
<td>α4</td>
<td>43,000</td>
<td>MASLYPFW?</td>
</tr>
<tr>
<td>α5</td>
<td>35,000</td>
<td>SDFTPPTVTTAXNEDRT?</td>
</tr>
<tr>
<td>α6</td>
<td>31,000</td>
<td>SDFGTCHY/V/VEGXQX</td>
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</table>

**TABLE II**

<table>
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<tr>
<th>Polypeptide</th>
<th>M&lt;sub&gt;r&lt;/sub&gt;</th>
<th>NH&lt;sub&gt;2&lt;/sub&gt;-terminal amino acid sequences of mouse tectorins</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtm1</td>
<td>150,000</td>
<td>QEYGTFDGFL</td>
</tr>
<tr>
<td>mtm2</td>
<td>40,000</td>
<td>RELMYPPFAQ</td>
</tr>
<tr>
<td>mtm3</td>
<td>34,000</td>
<td>RELMYPPFWQN</td>
</tr>
<tr>
<td>mtm4</td>
<td>31,000</td>
<td>KXCTTPNK</td>
</tr>
</tbody>
</table>

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FIG. 1. Southern and Northern analysis. a and b, Southern blots of mouse genomic DNA digested with KpnI (lane 1), SacI (lane 2), PstI (lane 3), and EcoRI (lane 4), probed with (a) a PCR product from clone A1, and (b) the cDNA insert from clone B2. Positions of size markers (1-kbp ladder) are shown between the blots. c and d, Northern blot of mouse poly(A)+ RNA from cochlea (lane 1), heart (lane 2), lung (lane 3), brain (lane 4), liver (lane 5), kidney (lane 6), intestine (lane 7), and skin (lane 8). The blot was first hybridized with the cDNA insert of clone A2 (c), then stripped and reprobed with the cDNA insert of clone B2 (d). Positions of size markers (RNA ladder) in kilobases are located to the left of both blots.
Southern blotting and, like the GLER specific PCR product, hybridizes to a cochlear-specific mRNA of approximately 7.5 kb on a Northern blot (Fig. 1, a and c). Sequence analysis of A1 and A2 suggested that they were derived from the same mRNA, but both lacked the 5′ end. Clones extending further 5′ were obtained by screening 1.0 × 10⁵ plaque-forming units of the randomly primed mouse cochlear cDNA library with a PCR product from the 5′ end of clone A2. Twenty-four further positives were identified of which one, A3, was found to encode the 5′ end of the mRNA. Clone A1 was completely sequenced on both strands; A2 was sequenced on both strands where it extended 5′ of A1, and clone A3 where it extended 5′ of A2. Other regions were sequenced on one strand only (Fig. 2, a and c).

In a and c unshaded boxes indicate noncoding regions and shaded boxes indicate the open reading frames of the clones. In d the position of an alternatively spliced exon found in clone A1 is marked. In b the first in-frame start codon is underlined in the cDNA sequence of A1 and in both cDNA sequences (b and d) in-frame stop codons are marked with asterisks (·). These sequence data are available from the EMBL Nucleotide Sequence Data Base, accession numbers X99805 and X99806.
sequence of mtm1 only matches the derived sequence in 6 out of 9 positions.

RT-PCR on poly(A)
RNA from the GLER using degenerate primers for \( \beta \)-tectorin generated a 570-bp product (data not shown), which was of the size predicted from the chick \( \beta \)-tectorin sequence data. The product was used to screen 1.0 \( \times 10^5 \) plaque-forming units of the randomly primed mouse cochlear cDNA library and 9 positive clones were identified and re-screened to purity. Two of these, B1 and B2, were selected for further study. Clone B2 was completely sequenced on both strands, and B1 and B3 were sequenced on both strands where they did not overlap with B2 (Fig. 2c). Overlapping regions were sequenced on one strand only. The DNA sequences were assembled into a composite cDNA sequence of 2745 bp with a single open reading frame (Fig. 2d). The derived amino acid sequence matches the NH\(_2\) terminal sequence of mtm4 in 6 out of 7 positions and has 75.3% overall sequence identity with chick \( \beta \)-tectorin, confirming the cDNA sequence encodes the mouse homologue of chick \( \beta \)-tectorin.
Analysis of α- and β-Tectorin Sequences—The α-tectorin cDNA sequence (Fig. 2b) has a 5′-untranslated region of 267 bp, a single open reading frame of 6453 bp, and a 3′-untranslated region of 620 bp containing two consensus polyadenylation signals, the second of which is followed by a poly(A) tail. The open reading frame is initiated at base 268 by the second ATG codon in the cDNA sequence. This codon matches the consensus initiation codon (23) by the presence of a purine at -3 and a cytosine at -4, whereas the first ATG codon, at position 121, matches only at the -3 position and is also followed by three in-frame stop codons at positions 154, 184, and 227. The β-tectorin cDNA sequence (Fig. 2d) has a 5′-untranslated region of 130 bases followed by an open reading frame of 963 bp, which is initiated at base 131 by the first ATG codon in the sequence. This codon matches the consensus initiation codon (23) by the presence of a purine at -3 and a cytosine at -2. The 1652-bp 3′-untranslated region of β-tectorin is unusually long and contains two potential polyadenylation signals. The second of these is used and is followed 24 bases downstream by a poly(A) tail.

The open reading frame of α-tectorin encodes a polypeptide of 2150 amino acids which has a calculated molecular mass of 239,034 Da and contains 33 potential N-glycosylation sites (Fig. 2b), whereas that of β-tectorin encodes a much smaller polypeptide of only 320 amino acids with a calculated molecular mass of 36,074 Da and 4 potential N-glycosylation sites (Fig. 2d). A number of features are common to these two derived amino acid sequences. Both start with hydrophobic signal sequences, as expected for secreted molecules (24). Identity between the NH₂-terminal amino acid sequence of mtm2/3 residues 25–31 of the derived amino acid sequence of α-tectorin implies the signal peptide is cleaved between amino acids 24 and 25. The presence of an alanine at position 22 conforms to the -3 position of the signal peptide cleavage site (25), however, the proline at position 24 does not fit the consensus -1 position. Similarly, for β-tectorin, identity between the NH₂-terminal amino acid sequence for mtm4 and residues 18–24 of the derived amino acid sequence implies that the signal peptide is cleaved between amino acids 17 and 18 to generate the mature NH₂ terminus of β-tectorin. Alanine residues at positions 15 and 17 in the β-tectorin sequence conform completely to the -3, -1 rule of von Heijne (25) for signal peptide cleavage. The derived amino acid sequences of both α- and β-tectorin terminate with sequences of predominantly hydrophobic residues (Fig. 2, b and d) characteristic of proteins that are membrane bound via a glycosylphosphatidylinositol tail (26–28). The method of Kodukula et al. (29) predicts that the asparagine at position 2086 in α-tectorin is the most likely acceptor for the glycosylphosphatidylinositol anchor, but does not provide a clear candidate residue in the case of β-tectorin. These hydrophobic COOH termini are both preceded a short distance upstream by tetrasaccharide motifs (Fig. 2, b and d) that are characteristic of endoproteinase cleavage sites (30, 31) and it seems likely that, as previously suggested for chick β-tectorin (3), mouse α- and β-tectorin are both synthesized as lipid-linked, membrane-bound precursors, targeted to the apical surface of the cochlear epithelium by the lipid (32) and then released into the extracellular compartment by the action of an endoproteinase.

Alternative Splicing of α-Tectorin mRNA—Comparing the cDNA sequences of the three α-tectorin clones identified a 15-bp sequence present in clone A1, but absent in A2, which encodes five amino acids, PLAPS (Fig. 3a). PCR of mouse genomic DNA with primers immediately adjacent to this sequence amplified a product of 1.5 kbp (Fig. 3b), suggesting this sequence may be an alternatively spliced exon flanked by approximately 1.4 kbp of intron sequences. RT-PCR of mouse GLER cDNA amplified products of 77 and 62 bp corresponding to both possible splice variants and suggests the variant lacking the 15-bp exon is the more common form (Fig. 3c). Splicing this exon into the mRNA changes Ser1659 to an Arg residue, creating a new dibasic sequence, Lys-Arg

$\text{Ser}_{1659}^{\text{Arg}}$

Additional faint bands of 101 and 104 bp are also observed (Fig. 3c) suggesting there are additional splice variants in this region.

Sequence Similarities—Similarity searches of the current data bases suggest α-tectorin is composed of three distinct modules, each with homology to different proteins. The first 219 amino acids of the NH₂-terminal region of α-tectorin show 24.9% similarity to a part of the first globular domain (G1) of entactin/nidogen (Fig. 4a) (33, 34). A 39-amino acid stretch separates these NH₂-terminal domain from the central domain which is 1528 amino acids long and is composed of three full repeats and two partial repeats homologous to the D domains of prepro-von Willebrand factor (vWF), zonadhesin, and the intestinal mucin muc2 (Fig. 4b) (35–37). The D domains are rich in cysteine, and the positions of the cysteines within the individual domains of α-tectorin match well, with 28 out of 37 positions being fully conserved (Fig. 4b). At the carboxyl end of the α-tectorin sequence there is a stretch of 255 amino acids which exhibits similarity to the zona pellucida domains (38) of uromodulin (39) and GP2 (40) (Fig. 4c, Table III). This region of α-tectorin is also similar to other members of the zona pellucida domain family, and, most significantly, to chick and mouse β-tectorin (Fig. 4c, Table III). Mouse β-tectorin contains a single zona pellucida domain (Fig. 4c) and all the major features of chick β-tectorin are conserved in the mouse sequence, including the four consensus N-glycosylation sites, the 12 cysteine residues, and the extended basic sequence preceding the hydrophobic tail.

Western Blotting with Antipeptide Antibodies—Antibodies were raised to peptide sequences in the three different modules of the α-tectorin sequence and used to determine whether the HMM, MMM, and LMM tectorins observed on reducing SDS gels are derived from these different regions (Fig. 5). Antibod-
ies to a peptide sequence (m\textsuperscript{a}2742–755) in the second full vWF type D repeat of \( \alpha \)-tectorin detect a broad smear extending from the top of the separating gel down to the 170-kDa region of the gel corresponding to HMM tectorin (Fig. 5, lane 1). Antibodies to a sequence (m\textsuperscript{a}2000–2014) in the zona pellucida domain react with a band of 60 kDa corresponding to MMM tectorin, and additional polydisperse material in the 210–135-kDa region of the gel (Fig. 5, lane 2). Antibodies to a peptide sequence (m\textsuperscript{a}25–38) at the predicted NH\textsubscript{2} terminus of the module with similarity to the G1 domain of entactin, recognize a broad diffuse band in the 42–55-kDa region of the gel (LMM tectorin) and a faint band of 205 kDa (Fig. 5, lane 3). An antibody raised to a peptide in the derived amino acid sequence of \( \beta \)-tectorin recognizes a single sharp band of 45 kDa (Fig. 5, lane 4).

Expression Patterns of \( \alpha \) and \( \beta \)-Tectorin in the Inner Ear—In situ hybridization was used to study the distribution of \( \alpha \)- and \( \beta \)-tectorin mRNAs in the inner ear of the 2-day postnatal mouse. This stage of development was chosen as it is known to be a period when the tectorial membrane is being produced. In the cochleas, \( \alpha \)-tectorin mRNA is expressed on both sides of the organ of Corti. It is expressed in the pseudostratified cells of the greater epithelial ridge on the modiolar side of the inner hair cells, and in the immature Hensen’s cells that lie alongside the outermost row of outer hair cells (Fig. 6a). Expression of \( \beta \)-tectorin is also found in the immature Hensen’s cells and in the greater epithelial ridge. However, in comparison to \( \alpha \)-tectorin, \( \beta \)-tectorin is expressed within a more restricted zone of the greater epithelial ridge that lies adjacent to the inner hair cells of the organ of Corti (Fig. 6b). In addition, \( \beta \)-tectorin is expressed by the pillar cells that lie between the inner and outer hair cells within the organ of Corti, and the resultant pattern observed with \( \beta \)-tectorin antisense probes is one of three stripes arrayed across the organ (Fig. 6b). In the sacculus and utricule, \( \alpha \)-tectorin is expressed at low levels within the sensory macula, but strongly expressed in the transitional zone around the periphery of the macula and in a region that is producing the accessory membrane, a structure that connects the otolitic membrane to the roof of the organ (Fig. 6c).

In contrast, \( \beta \)-tectorin is expressed in a restricted region of the macula called the striola (Fig. 6d), a region where there is known to be a high density of vestibular Type I hair cells (41).

DISCUSSION

The results describe the isolation and characterization of cDNA clones for mouse \( \alpha \)- and \( \beta \)-tectorin. The cDNA for \( \alpha \)-tectorin encodes a large protein of 239 kDa and that of \( \beta \)-tectorin a smaller protein of 186 kDa, and together these two proteins can account for the HMM, MMM, and LMM tectorins observed with SDS-PAGE. The genes encoding these proteins are expressed at high levels in the developing inner ear, and not in a number of other tissues of the early postnatal mouse, indicating the tectorins may be matrix molecules unique to the inner ear. Sequence data show both proteins are probably synthesized as glycosylphosphatidylinositol-linked membrane bound precursors which may be targeted to the apical surface of the inner ear epithelia by the lipid and released by endoproteolytic activity. The two proteins share a common module, the zona pellucida domain, which may enable them to form filaments, and \( \alpha \)-tectorin contains additional modules which could allow it to interact with \( \beta \)-tectorin. Mouse and chick \( \beta \)-tectorin share 75% overall sequence identity, both the NH\textsubscript{2}-terminal sequence data for the chick \( \alpha \)-tectorins presented in this study and preliminary data from chick \( \alpha \)-tectorin cDNA clones\textsuperscript{2} indicate the chick and mouse \( \alpha \)-tectorins are conserved at a similar level, and the derived amino acid sequence of a human brain expressed sequence tag (accession number R84585) is 92% identical to mouse \( \alpha \)-tectorin. The tectorins are therefore highly conserved and may be fundamentally important in the process of mechanotransduction.

Matches between the NH\textsubscript{2}-terminal sequence data for the mouse and chick tectorins and the derived amino acid sequence of mouse \( \alpha \)-tectorin, together with data from Western blots with antipeptide antibodies, indicate that the HMM, MMM, and LMM tectorins are generated by proteolytic cleavage of \( \alpha \)-tectorin, with \( \beta \)-tectorin also contributing to the LMM tectorin band. The domain structure of \( \alpha \)-tectorin and way in which the sequence may be cleaved are shown in Fig. 7a. The NH\textsubscript{2}-terminal amino acid sequences of mtm1 and chick \( \alpha \)-1-tectorin match residues 328–338 of the derived \( \alpha \)-tectorin sequence in 6/9 and 10/10 positions, respectively, that of chick \( \alpha \)z3 tectorin partially matches residues 1659–1668 in 7/10 positions, and that of mtm4 is identical to the predicted mature NH\textsubscript{2} terminus of the \( \alpha \)-tectorin sequence. Polypeptides with these NH\textsubscript{2} termini would have predicted masses of 147,360, 47,020, and 34,092 Da, respectively (Fig. 7a), values which are close to those observed for mouse tectorins following enzymatic deglycosylation with a combination of endo-\( \beta \)-galactosidase and \( \beta \)-glycosidase F. Western blotting with antibodies to synthetic peptides based on different regions of the predicted \( \alpha \)-tectorin sequence provides further evidence for this scheme. Antibodies to a sequence in the second full vWF type D repeat react with HMM tectorin, antibodies to a sequence in the ZP domain stain MMM tectorin, and antibodies to the predicted mature NH\textsubscript{2} terminus of \( \alpha \)-tectorin stain LMM tectorin. Good partial matches for the NH\textsubscript{2}-terminal amino acid sequence data of the chick \( \alpha \)-5 and \( \alpha \)-6-tectorins (75 and 67%, respectively) are also found within the region of the predicted mouse sequence that gives rise to mouse HMM tectorin according to the scheme described above. Cleavage at these sites in chicken \( \alpha \)-tectorin may explain why the polypeptide core of the largest chick \( \alpha \)-polypeptide, chick \( \alpha \)-1-tectorin, is some 60 kDa smaller than that of the largest mouse \( \alpha \)-tectorin.\textsuperscript{3}

Although the predicted sequence of mouse \( \alpha \)-tectorin can account for the three major tectorins observed on SDS gels under reducing conditions, it is not clear whether the three \( \alpha \)-tectorins are genuine subunits generated by active processing or simply the result of proteolysis occurring between intra-chain disulfide bonds. Furthermore, the way in which this sequence appears to be processed does not clearly divide the protein into the three different modules defined by sequence analysis as the predicted NH\textsubscript{2} termini of HMM and MMM tectorin both occur within the vWF type D repeats. The presence of a splice variant introducing a dibasic, potential endoprotease cleavage site close to the theoretical start of the MMM tectorin polypeptide within the D4 repeat indicates this site may be of functional significance, but it should be noted that there are also many dibasic sites throughout the predicted sequence and none lie immediately upstream of the theoretical NH\textsubscript{2} terminus of HMM tectorin. However, the splice variant without the dibasic site upstream of the potential NH\textsubscript{2} terminus of MMM tectorin is the major species present and this may explain why the antibodies to the peptide sequence in the zona pellucida domain react with both MMM tectorin and higher molecular mass material.

While it is unclear whether \( \alpha \)-tectorin is processed or slowly subject to site-specific proteolytic damage \textit{in vivo}, the three distinct modules may play different roles in organizing the structure of the non-collagenous matrix of the mammalian

\textsuperscript{2} Coutinho, P., Legan, P. K., Goodyear, R., and Richardson, G. P., manuscript in preparation.

\textsuperscript{3} G. P. Richardson, unpublished observations.
Matrix Proteins of the Inner Ear

**a**

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**b**

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**c**

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<td>BFL, ALGV, LSE, VYF, TFP, YFP, YFP</td>
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**FIG. 4**

Matrix Proteins of the Inner Ear

Comparison of the ZP domains of α- and β-tectorin, GP2, uromodulin, ZP2 and ZP3

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<th>Gg β-tec</th>
<th>Mm β-tec</th>
<th>Mm α-tec</th>
<th>Cf GP2</th>
<th>Hs uro</th>
<th>Mm ZP2</th>
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<td>82.4</td>
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<td>27.9</td>
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<td></td>
<td>47.6</td>
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<td>18.8</td>
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Percentage sequence identities between the ZP domains of chick and mouse β-tectorin (Gg and Mm β-tec), mouse α-tectorin (Mm α-tec), dog GP2 (Cf GP2), human uromodulin (Hs uro), mouse ZP2 (Mm ZP2), and human ZP3 (Hs ZP3).

Table III

Fig. 5. Western blots of mouse tectorial membrane proteins stained with antibodies to peptides ma742–755 (lane 1), ma2000–2014 (lane 2), ma292–38 (lane 3), and ma282–240 (lane 4). Positions of molecular mass markers in kDa are indicated to the left of the blot.

Fig. 6. Expression of α- and β-tectorin in the inner ear of the 2-day postnatal mouse. Sections of the cochlea (a and b) and the sacule (c and d) hybridized with 35S-labeled antisense probes for α (a and c) and β (b and d) tectorin. Positions of the inner (i) and outer (o) hair cells are indicated in a and b. In c the region of the roof (r) and the transitional zone (t) expressing α-tectorin are indicated, and in d the striolar region (s) is labeled. Bar in d = 100 μm and applies to all panels.

Specific manner (49, 36). Zonadhesin is a transmembrane protein with 3 types of extracellular domain, an NH2-terminal domain with no homology to other proteins, a threonine-serine/proline-rich mucin type domain, and a membrane proximal domain with 1 partial and 4 full vWF type D repeats (36) (Fig. 7b). During sperm maturation the first two domains of zonadhesin are lost and the membrane proximal domain with the vWF repeats is cleaved into two, disulfide cross-linked polypeptides with Mr of 105,000 and 45,000 which as a complex have the ability to bind to the zona pellucida. Not only is the homology between the vWF type D repeats of zonadhesin and α-tectorin high (26%), but the order of these repeats is the same in both α-tectorin and zonadhesin, whereas it is different in vWF...
and the mucins. Homomeric filaments of α-tectorin and β-tectorin may therefore interact with one another via the HMM, zonadhesin-like module of α-tectorin, either alone or in conjunction with LMM tectorin. Although the LMM module shows similarity with nidogen, an organizer of the extracellular matrix assembly, although this may not be necessarily the oligomerization of α-tectorin to form filaments as there is no evidence for the presence of covalently linked α-tectorin multimers in the tectorial membrane. A third potential model for the organization of α- and β-tectorin in the matrix would be one in which the two proteins interact via their ZP domains to form heteromeric filaments like the zona pellucida proteins, ZP2 and ZP3 (47). These in turn could bind to one another via the HMM module of α-tectorin, again either with or without the participation of the LMM module.

In situ hybridization shows that there are groups of cells in the inner ear that express either α- or β-tectorin, or both molecules simultaneously. While this suggests α- and β-tectorin can probably form homomeric filaments, it does not rule out the possibility they form heteromeric filaments in those areas where they are co-expressed. The patterns of α- and β-tectorin expression observed in the inner ear are more complex than expected and do not obviously correlate with the regional variations in matrix structure that have been described in previous morphological studies (7, 55, 8). For example, the immature Hensen’s cells are thought to produce the marginal band, a region of densely packed matrix, and the cells in the greater epithelial ridge lying adjacent to the inner hair cells form the midbody of the tectorial membrane where the matrix is loosely packed (7), yet both of these cell groups co-express α- and β-tectorin. Likewise the limbal undersurface of the tectorial membrane and Hensen’s stripe are regions of densely packed matrix, and yet the former is most likely to be produced by the cells of the greater epithelial ridge lying next to the limbus that are expressing only α-tectorin, and the latter could be produced by the pillar cells that are expressing only β-tectorin. Regional differences in matrix structure may be important for eliciting the correct response from different types of hair cells. For example, as in the chick, β-tectorin expression in the otolithic maculae is restricted to the striolar region, an area in the mammal where a high density of type I hair cells is found (41). While differences in the ratio of α- and β-tectorin expressed in any one region may be one factor that influences matrix structure, variations in their glycosylation patterns, or the α-tectorin splice forms that are used could also be important determinants. Modulation of the relative expression of α- and β-tectorin with respect to time during development could also play a role in shaping the structure of the tectorial membrane.

The results of this present study provide a complete molecular characterization of the two major non-collagenous glycoproteins of the mammalian tectorial membrane, an extracellular matrix essential for the perception of sound. These data should now allow the structure and properties of this matrix to be manipulated both selectively and non-invasively and reveal how the tectorial membrane influences frequency tuning in the cochlea.

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Matrix Proteins of the Inner Ear

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The Mouse Tectorins: MODULAR MATRIX PROTEINS OF THE INNER EAR HOMOLOGOUS TO COMPONENTS OF THE SPERM-EGG ADHESION SYSTEM
P. Kevin Legan, Angela Rau, Jeff N. Keen and Guy P. Richardson

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