Molecular Cloning of Chick β-Tectorin, an Extracellular Matrix Molecule of the Inner Ear

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Abstract. The tectorial membrane is an extracellular matrix lying over the apical surface of the auditory epithelium. Immunofluorescence studies have suggested that some proteins of the avian tectorial membrane, the tectorins, may be unique to the inner ear (Killick, R., C. Malenczak, and G. P. Richardson. 1992. *Hearing Res.* 64:21–38). The cDNA and deduced amino acid sequences for chick β-tectorin are presented. The cDNA encodes a protein of 36,902.6 D with a putative signal sequence, four potential N-glycosylation sites, 13 cysteines, and a hydrophobic COOH terminus. Western blots of two-dimensional gels using antibodies to a synthetic peptide confirm the identity of the cDNA. Southern and Northern analysis suggests that β-tectorin is a single-copy gene only expressed in the inner ear. The predicted COOH terminus is similar to that of glycosylphosphatidylinositol-linked proteins, and antisera raised to this region react with in vitro translation products of the cDNA clone but not with mature β-tectorin. These data suggest β-tectorin is synthesized as a glycosylphosphatidylinositol-linked precursor, targeted to the apical surface of the sensory epithelium by the lipid moiety, and then further processed. Sequence analysis indicates the predicted protein possesses a zona pellucida domain, a sequence that is common to a limited number of other matrix-forming proteins and may be involved in the formation of filaments. In the cochlear duct, β-tectorin is expressed in the basilar papilla, in the clear cells and the cuboidal cells, as well as in the striolar region of the lagena macula. The expression of β-tectorin is associated with hair cells that have an apical cell surface specialization known as the 275-kD hair cell antigen restricted to the basal region of the hair bundle, suggesting that matrices containing β-tectorin are required to drive this hair cell type.

THE tectorial membrane is a sheet of extracellular matrix that contacts the stereocilia bundles of the mechanosensitive hair cells in the inner ear. Shear generated between the lower surface of the tectorial membrane and the apical surface of the auditory epithelium as a result of sound-induced basilar membrane motion is considered to be the primary stimulus for the hair cells. The way in which the tectorial membrane behaves in response to sound stimulation is not fully understood, but in certain models, it has been proposed that it undergoes compression and expansion along the radial axis of the cochlea, and that it is a resonator tuned half an octave below the resonant frequency of the basilar membrane (Zwislocki, 1979; Allen, 1980; Neely and Kim, 1986). Models that incorporate the interaction of two broadly tuned resonators, the tectorial and basilar membranes, as well as an active mechanical amplifier provided by the electromotile outer hair cells (Brownell et al., 1985; Kachar et al., 1986), can provide a realistic description of observed cochlear behavior (Pickles, 1991).

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The mammalian tectorial membrane contains three different collagens, polypeptides reacting with antibodies directed against types II, V, and IX collagens (Richardson et al., 1987, Thalmann et al., 1987, Slepecky et al., 1992), and at least three collagenase-insensitive glycoproteins that may account for up to 50% of the total protein (Richardson et al., 1987). These glycoproteins form a matrix structure composed of two types of 7–9 nm diameter fibril, a light and a dark fibril, that are linked to one another by staggered cross-bridges and are arranged as flat sheets that are stacked one upon the other and that wrap around the collagen fiber bundles (Hasko and Richardson, 1988).

At the ultrastructural level, the avian tectorial membrane appears as a dense, fibrillo-granular matrix (Tanaka and Smith, 1975; see also Fig. 1 *a*). The available evidence suggests that collagens are not present in the avian tectorial membrane (Thalmann et al., 1987; Killick et al., 1992). However, it does contain a set of collagenase-insensitive glycoproteins electrophoretically similar to those of the mammalian tectorial membrane (Killick et al., 1992). SDS-PAGE of avian tectorial membrane proteins under nonreducing conditions reveals the presence of two major polypeptides. These have M_r = 196,000 and 41,000, and they will

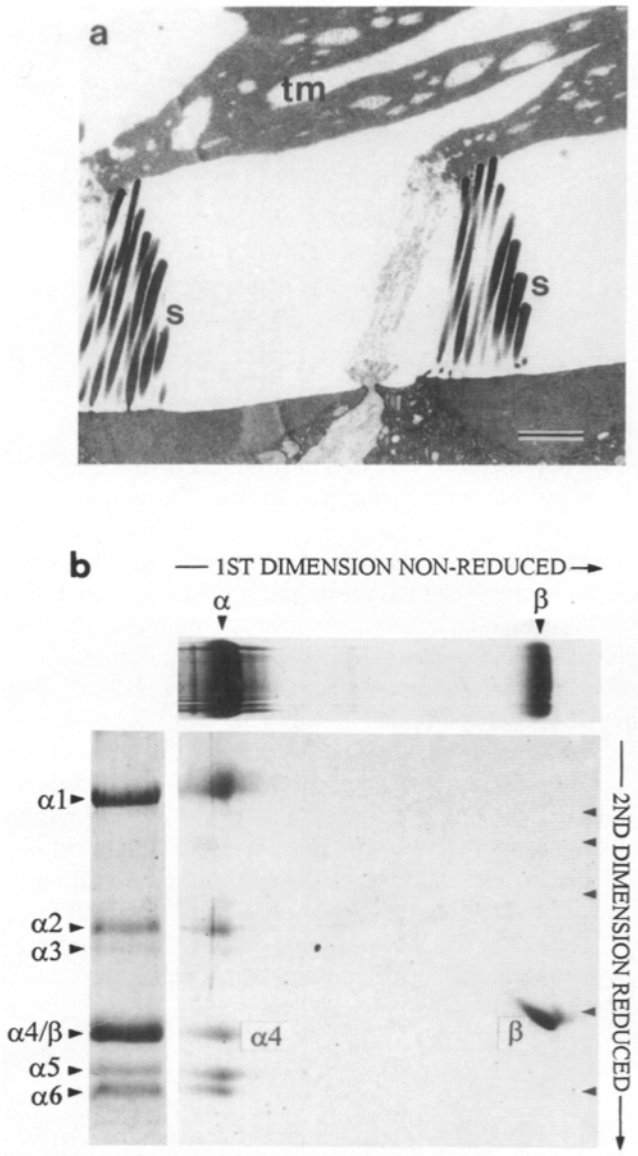


Figure 1. (*a*) Low power electron micrograph of the chicken basilar papilla showing the apical surface of the epithelium, the stereocilia bundles (*s*) of two hair cells, and the overlying tectorial membrane (*tm*). (*b*) One- and two-dimensional electrophoresis of chick tectorial membrane proteins. One dimensional gel electrophoresis under nonreducing condition (*top horizontal*) reveals two major bands, the α- and β-tectorins. One-dimensional electrophoresis under reducing conditions (*bottom left*) reveals the presence of six bands. Two-dimensional electrophoresis using nonreducing conditions in the first dimension and reducing conditions in the second (*bottom right*) reveals that α-tectorin is composed of six subunits, α1–α6, and that β-tectorin is not covalently associated with any other polypeptides. Arrowheads to the right indicate the positions of molecular mass markers, which are (*top to bottom*) 116, 96, 66, 45, and 29 kD.

be referred to as the α- and β-tectorins respectively (Fig. 1 *b* in this manuscript; see also Killick et al., 1992). SDS-PAGE under reducing conditions shows that α-tectorin is a disulphide cross-linked complex composed of six subunits, α1–α6, with M_r = 146,000 (α1), 60,000 (α2), 56,000 (α3), 43,000 (α4), 35,000 (α5), and 31,000 (α6) (Fig. 1 *b*). β-Tectorin shows a slight decrease in electrophoretic mobility under reducing conditions, and it migrates with an M_r similar to that of the α4 subunit of α-tectorin. These two low molecular mass components of the chick tectorial membrane, both of which have an M_r = 43,000 under reducing conditions, can be readily distinguished by two-dimensional gel electrophoresis. β-tectorin resolves as a set of discretely focused spots towards the basic end of the gel, while the α4-tectorin subunit forms a disperse smear towards the acidic end (Killick et al., 1992).

Immunofluorescence studies indicate that the chick tectorins have a restricted tissue distribution (Killick et al., 1992). Staining is not detected with anti-tectorin sera in a number of different avian tissues, including brain, liver, kidney, lung, heart, muscle, eye, tongue, skin, feather, and gizzard, although antibodies directed against the α1-tectorin subunit stain the glands supplying the mucus layer overlying the olfactory epithelium, and antibodies recognizing the α6 subunit stain granules in the cells lining the respiratory epithelium. These data suggest that some of the tectorins may be related to mucins and others may be novel matrix molecules. A full understanding of how the ear functions will ultimately require a knowledge of tectorial membrane structure and how this matrix interacts with the apical surface of the sensory epithelium. As a step towards this objective, we have used molecular cloning to deduce the primary sequence of β-tectorin.

Materials and Methods

Preparation and Screening of Cochlear cDNA Libraries

Cochlear ducts from the inner ears of 2–3-d-old chicks of the Isa Brown variety were dissected in cold PBS and snap frozen in liquid nitrogen. Total RNA was extracted with 4.0 M guanidinium isothiocyanate in 0.1 M Tris-HCl, pH 7.6, containing 1% β-mercaptoethanol (Chirgwin et al., 1979) and isolated by centrifugation through a 1.7 gm/ml caesium trifluoroacetic acid cushion (Okayama et al., 1987). Poly(A)$^+$ RNA was purified from total RNA by affinity chromatography using either oligo-dT cellulose (Pharmacia, Milton Keynes, UK), oligo-dT magnetic beads (Dynabeads; Dynal, New Ferry, UK), or poly-U paper (Amersham, Little Chalfont, UK). cDNA was synthesized from poly(A)$^+$ RNA using either murine or avian reverse transcriptases and oligo dT primers. Libraries were prepared in λ gt11 (Amersham) and λ UniZap XR (Stratagene, Cambridge, UK) bacteriophage vectors following the manufacturer's instructions. The λ gt11 library contained 4.1 × 10^5 recombinants and the λ Zap library 6.5 × 10^7 recombinants. Screening was done with unamplified libraries. A total of 6 × 10^4 clones from the λ gt11 library were immunoscreened (Mierendorf et al., 1987) with a mouse polyclonal anti-chick 43,000 tectorin serum that recognizes both β-tectorin and the α4-tectorin subunit (Killick et al., 1992) and peroxidase-conjugated rabbit anti-mouse antibodies, both of which had been preabsorbed with bacterial lysates and were used at a final dilution of 1:1,000. Peroxidase-conjugated antibodies were detected with 0.05% (wt/vol) diaminobenzidine-HCl, 0.01% (vol/vol) H_2O_2 in TBS (150 mM NaCl, 10 mM Tris-HCl, pH 7.2). A single immunopositive clone, clone 1, was identified and isolated through three successive rounds of screening. The cDNA insert from clone 1 was sequenced in both directions and found to encode the 3′ end of a poly(A)-tailed RNA. A ^{32}P-labeled DNA probe derived from clone 1 by PCR was used to screen 1 × 10^5 clones from the λ UniZap XR library, and nine clones were isolated. Two of these clones, clones 2 and 3, have been sequenced and are identical. The respective lengths and positional relationships of these three clones are illustrated in Fig. 2. Clones 2 and 3 from the λ Zap library were converted to pBluescript plasmids using Exassist helper phage rescue.

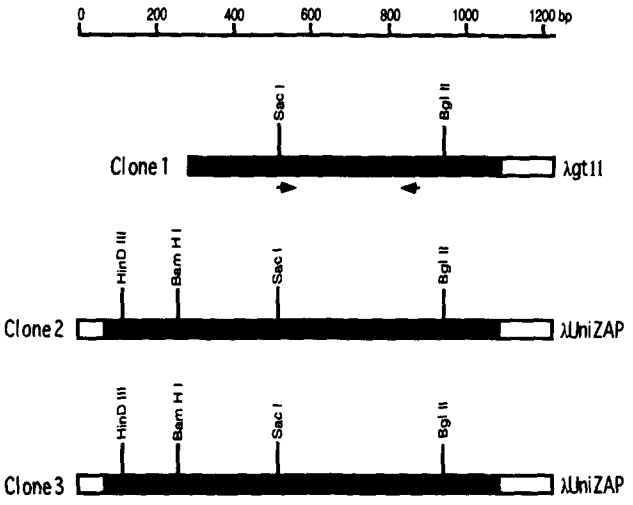


Figure 2. Cloning strategy for β-tectorin. Relationships between clone 1, from the λ gt11 library, and clones 2 and 3, from the λ UniZAP XR library, are shown. Filled boxes represent the open reading frame, and the open boxes represent the 5′ and 3′ untranslated regions. Positions of the PCR primers used to generate a ^{32}P-labeled probe from clone 1 for library screening and Northern blot analysis are marked by arrows.

Southern Blotting

Genomic DNA was isolated from chick heart and liver by a modification (Sambrook et al., 1989) of the method of Blin and Stafford (1976). Aliquots of DNA (17 μg) were digested to completion with EcoRI, PstI, SacI, KpnI, and XbaI, electrophoresed on a 0.7% agarose gel in 1× Tris-borate/EDTA buffer, and transferred to Hybond N$^+$ membrane (Amersham) (Southern, 1975). DNA was covalently linked to the membrane by treatment with 0.4 M NaOH for 20 min. Clone 2 was double digested with XbaI and XhoI, and the cDNA insert was isolated by gel electrophoresis. Insert cDNA was purified from the gel using Geneclean (Stratatech, Luton, UK) and random primer labeled (Feinberg and Vogelstein, 1983) with ^{32}P-dCTP using a MegaPrime labeling kit (Amersham). Unincorporated label was removed using Nick 50 columns (Pharmacia). Hybridization to ^{32}P-labeled β-tectorin cDNA probes and washing to high stringency were carried out according to the manufacturer's instructions.

Northern Blotting

Total RNA was prepared from muscle, cochlea, heart, kidney, liver, brain, eye, gizzard, and small intestine following the method described above. RNA was separated by denaturing electrophoresis in 1% agarose-formaldehyde gels, transferred by capillary blotting to Hybond-N (Amersham), and fixed by baking at 80°C for 2 h. An identical gel was run in parallel and stained with ethidium bromide to check the integrity of the RNA. Positions of the 28S and 18S ribosomal RNA bands were used as markers. A 386-bp DNA probe was prepared from clone 1 using PCR with sequencing primers, isolated by agarose gel electrophoresis, purified using Geneclean, and labeled with [^{32}P]dCTP using the Klenow fragment of DNA polymerase I and the PCR primers. The blot was hybridized to the probe overnight at 42°C in 5× SSC buffer containing 50% formamide, 10% dextran sulphate, 0.1% SDS. The blot was then washed three times for 20 min with 1× SSC, 0.1% SDS at room temperature, three times for 20 min with 0.2× SSC, 0.1% SDS at 65°C, and then exposed to Fuji RX film at −70°C for 5 d. The blot was subsequently stripped by immersion in boiling 0.1% SDS and reprobed with a ^{32}P-labeled mouse α-actin probe.

Reverse Transcription PCR

The following PCR primers containing internal EcoRI restriction sites (underlined) were used: βS1, CACTGGAATTCACACGTCTGTGACAGCG βAS1, GCATGAATTCGTGAAATGGTGCACAAGG. Poly(A)$^+$ RNA was isolated from cochlear, brain, kidney, colon, cartilage, heart, liver, and lung tissues of 1-2-d posthatch chicks using a Pharmacia Quick Prep Micro mRNA purification kit. Randomly primed first-strand cDNA was synthesized from 1 μg of poly(A)$^+$ RNA using a first-strand cDNA synthesis kit (Amersham). An aliquot (1 μl) of the first-strand cDNA reaction was added directly to a 50-μl PCR reaction containing 0.2 mM of each dNTP, 50 pmol of each of the above primers, and 2.5 U *Taq* DNA polymerase in 10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, and 0.1% gelatin. The reaction was heated to 94°C for 2 min followed by 30 cycles, each of 94°C for 45 s, 60°C for 45 s, and 72°C for 1 min. The reaction was completed with a 5-min incubation at 72°C. Reaction products were separated by electrophoresis in a 1.5% agarose gel and visualized with ethidium bromide (0.5 μg/ml).

Sequence Analysis

The cDNA insert from clone 1 was subcloned into M13, and single-stranded DNA was sequenced with universal and sequence specific primers using the dideoxychain termination method (Sanger et al., 1977) and Sequenase V.2.0 (Amersham). Double-stranded DNA from clones 2 and 3 was prepared from pBluescript plasmids using Magic Mini-preps (Promega, Southampton, UK) and denatured before sequencing. Sequence data was assembled and analyzed using DNA Star software (DNA Star, London, UK).

Lysogen Preparation

The *Escherichia coli* Y 1089 strain was infected with clone 1 phage, and a temperature-sensitive recombinant lysogen was selected. A cell lysate containing the recombinant fusion protein was prepared by freeze-thawing and sonication of the bacterial pellet. Lysate proteins were separated by SDS-PAGE and analyzed by Western blotting.

Preparation of Antisera

Polyclonal mouse antisera specific for β-tectorin and the α4-tectorin subunit were raised to proteins prepared by preparative SDS gel electrophoresis. Tectorial membranes were run on preparative 7.5% polyacrylamide gels under nonreducing conditions, regions of the gels containing the α- and β-tectorins were cut out, and the protein was eluted with 0.1% SDS in 50 mM ammonium bicarbonate. After dialysis against water, the samples were lyophilized. Protein from the α-tectorin band was subsequently dissolved in reducing SDS sample buffer, subjected to a second round of gel electrophoresis, and the region of the gel containing the α4-tectorin subunit was cut out and the protein extracted as described above. Mice were immunized intraperitoneally three times at three weekly intervals with protein obtained from a total of 200 tectorial membranes for each injection, and they were test bled from the tail veins 1 wk after the third injection.

Two 9-mer peptides, P1 (SEFKNPVYH) and P2 (RNKGLSRFY), based on the derived amino acid sequence of β-tectorin cDNA, were synthesized and coupled to bovine serum albumin using glutaraldehyde as described by Doolittle (1986). Rabbits were immunized subcutaneously three times at three weekly intervals with 500 μg of conjugate, and they were test bled from the ear veins 1 wk after the third injection. A mouse was also immunized subcutaneously with the P2-BSA conjugate using 50 μg of conjugate for each injection, the same immunization schedule, and was test bled from the tail vein.

Western Blot Analysis

One- (Laemmli, 1970) and two- (O'Farrell, 1975) dimensional gels were transferred to nitrocellulose using a dry blotting apparatus. Nonspecific protein binding sites were blocked with 3% (wt/vol) low-fat dried milk powder, 5% (vol/vol) heat-inactivated horse serum in TBS containing 0.05% (vol/vol) Tween-20 for 1 h. Antibodies were then added to a final dilution of 1:100 or 1:1,000, and the nitrocellulose transfers were incubated overnight. After washing in TBS, bound antibodies were labeled with peroxidase-conjugated anti-mouse Ig (1:200) or biotinylated swine anti-rabbit Ig (1:1,000) (Dako, High Wycombe, UK). Peroxidase anti-antibodies were visualized with 0.05% (wt/vol) diaminobenzidine-HCl in TBS containing 0.01% (vol/vol) H_2O_2. Biotinylated antibodies were labeled with alkaline phosphatase-conjugated streptavidin (1:1,000) (Vector Laboratories, Peterborough, UK), and they were visualized with 0.05 mg/ml bromochloroindolyl phosphate, 0.1 mg/ml nitro blue tetrazolium in alkaline phosphatase buffer (0.1 M NaCl, 0.1 M Tris-HCl, pH 9.5, and 5 mM $MgCl_2$).

Deglycosylation of β-Tectorin

Neuraminidase from *Arthrobacter ureafaciens*, *O*-glycosidase from *Diplo-*

coccus pneumoniae, and N-glycosidase F from *Flavobacterium meningosepticum* were obtained from Boehringer (Lewes, UK). β-Tectorin was prepared by preparative gel electrophoresis as described above. Lyophilized protein was dissolved in 10 μl H_2O, boiled for 1 min, allowed to cool, and CHAPS was added to a final concentration of 0.5% and the samples were boiled again for 1 min. Samples were then digested with either neuraminidase (20 mU/ml), *O*-glycosidase (25 mU/ml), neuraminidase plus *O*-glycosidase (20 and 25 mU/ml, respectively), *N*-glycosidase (4 U/ml), or a combination of all three enzymes overnight at 37°C. Reactions were terminated by the addition of 4× reducing SDS-PAGE sample buffer, and they were then analyzed on 10% polyacrylamide SDS gels.

Dot Blotting

Serial 10-fold dilutions of the synthetic peptides or gel-purified β-tectorin were spotted onto nitrocellulose sheets (Immobilon; Millipore, Watford, UK). The concentration of gel-purified β-tectorin was determined by densitometric scanning an aliquot of the same preparation that had been run on a Coomassie-stained gel. The nitrocellulose filters were baked at 90°C for 1 h, preblocked with 3% low fat milk powder in TBS containing 0.05% Tween-20, and then incubated in primary antibodies at a dilution of 1:100 overnight. After washing, bound antibodies were detected as described above using peroxidase-conjugated anti-antibodies.

In Vitro Transcription and Translation

Clone 2 plasmid DNA was digested with XhoI (Promega, Southampton, UK) in the supplied buffer, extracted twice with phenol/chloroform (1:1), once with chloroform, and ethanol precipitated. Linearized plasmid (5 μg) was transcribed in vitro in 50 μl of 40 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol, 10 mM NaCl, 6 mM $MgCl_2$, 2 mM spermidine, containing 500 μM each of ATP, CTP and UTP, 50 μM GTP, and 500 μM m^7 G(5')ppp(5')G (Boehringer), 50 U RNase inhibitor, and 20 U T3 RNA polymerase (Promega). After 1 h at 37°C, another 20 U of T3 polymerase was added, and the transcription was continued for an additional hour. RNA transcripts were recovered by phenol/chloroform extraction and ethanol precipitation. RNA transcripts were examined by formaldehyde agarose gel electrophoresis (Sambrook et al., 1989) before using in vitro translations. RNA transcripts from clone 2 (5 μg) were translated in vitro for 1 h at 30°C in a 250-μl reaction containing 160 μl rabbit reticulocyte lysate (Promega), 36 equivalents of canine pancreatic microsomes (Promega), 20 μM amino acids without cysteine, 100 μCi [^{35}S]cysteine (Amersham), 80 mM KCl, 12.5 mM DTT, and 100 U RNase inhibitor. Before immunoprecipitating the translates, PMSF was added to 1 mM, leupeptin to 10 μg/ml, benzamidine to 1 mM, and pepstatin to 10 μM, and the microsomes were then lysed by adding Triton X-100 to a final concentration of 1%. To deglycosylate the in vitro translation products, samples of the reaction were freeze-thawed, diluted with 9 vol of Tris-buffered saline (10 mM Tris-HCl, pH 7.4, and 150 mM NaCl), and treated with 0.2 U of *N*-glycosidase F for 2 h at 37°C.

Immunoprecipitations

Gel-purified β-tectorin was iodinated using Na^{125}I and chloramine T. Antipeptide sera were affinity purified on their respective peptides conjugated to cyanogen bromide–activated Sepharose 4B. Nonimmune rabbit IgG (Sigma) was used as a control for the affinity-purified antipeptide antibodies. Aliquots of [^{125}I]β-tectorin or the in vitro translation products of the clone 2 transcript were incubated overnight at 4°C with 25 μg of the affinity-purified antipeptide antibodies or nonimmune rabbit IgG, and with 2.5 μl of either mouse anti–β-tectorin or mouse nonimmune serum. Immunoprecipitates were collected with either protein A–Sepharose (rabbit antibodies) or a mixture of protein A– and protein G–Sepharoses (mouse sera), washed six times with PBS, eluted with gel electrophoresis sample buffer, and separated on 10% polyacrylamide gels. Gels were impregnated with fluor (NEN Enhance) and exposed to preflashed Fuji RX film for 24–48 h at −70°C.

In Situ Hybridization

Cartilaginous temporal bones were fixed at 4°C in 4% (wt/vol) paraformaldehyde in 0.1 M sodium acetate buffer, pH 6.5, for 1 h, and then for 3 h in 4% paraformaldehyde in 0.1 M borax buffer, pH 9.0. Tissue pieces were transferred to 4% paraformaldehyde in 0.1 M borax, pH 9.0, containing 30% (wt/vol) sucrose and stored overnight at 4°C before embedding in 1% low melting point agarose in 4% paraformaldehyde, 0.1 M borax, pH 9.0, containing 18% (wt/vol) sucrose. Cryosections were cut at a thickness of 20 μm, mounted on baked glass slides coated with 3-aminopropyltriethoxysilane, dried in a dessicator, and stored at -20°C until use. The cDNA insert from clone 1 was subcloned into the plasmid vectors pGEM3Z and pGEM4Z (Promega), and it was used to prepare ^{32}P-labeled RNA transcripts. In situ hybridization with ^{32}P-labeled probes was carried out following the method described by Simmons et al. (1989). RNA transcripts labeled with [^{35}S]UTP were prepared using pBluescript plasmid containing the β-tectorin cDNA insert from clone 2 as template and used as in situ hybridization probes as described by Wilkinson and Green (1990). After hybridization and washing, slides were dehydrated, air dried, and coated with Ilford K5 emulsion diluted 1:1 with distilled H_2O. Slides were stored for various time periods with desiccant at 4°C, developed in Ilford Phenisol, and counterstained with hematoxylin.

Immunofluorescence Microscopy

Cochlear ducts were fixed in 3.7% formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 1 h at room temperature, washed with PBS, cryoprotected with 30% sucrose in PBS, embedded in 1% low melting point agar in PBS containing 18% sucrose, and cryosectioned at a thickness of 10 μm. Sections were preblocked with 10% horse serum in TBS, incubated overnight in mouse anti–β-tectorin at 1:100 dilution, washed and labeled with FITC-

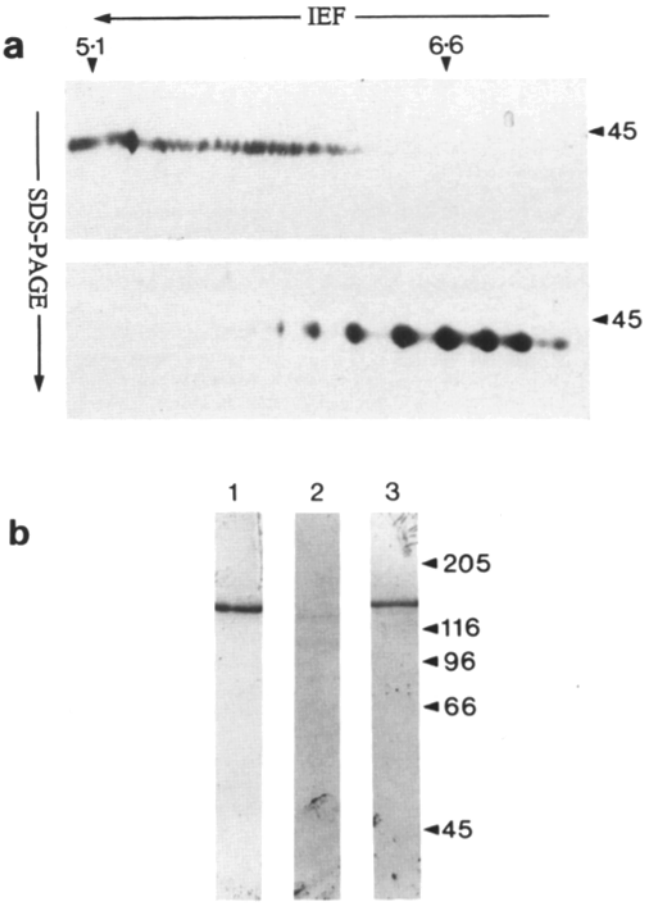


Figure 3. Western blot analysis of the λ gt11 clone, clone 1. (*a*) Specificity of the antisera. Tectorial membrane proteins were separated by 2-D electrophoresis and Western blotted with antisera specific for the α4-tectorin subunit (*upper panel*) and β-tectorin (*lower panel*). The two antisera can be seen to be specific for their respective antigens. (*b*) Reactivity of different antisera with the cloned fusion protein. A lysate from clone 1 was separated by 1-D SDS-PAGE, Western blotted, and stained with the polyspecific antiserum recognizing both the α4-tectorin subunit and β-tectorin (lane *1*), the antiserum specific for the α4-tectorin subunit (lane *2*), and the antiserum specific for β-tectorin (lane *3*).

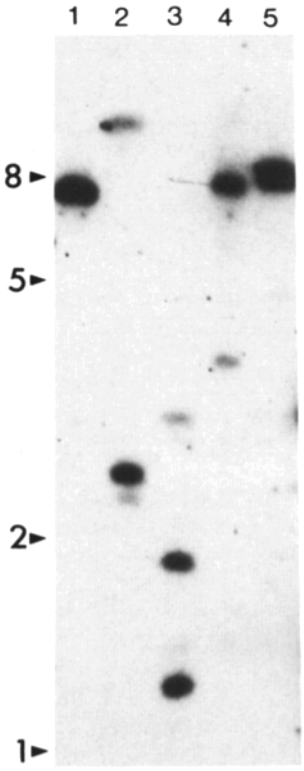


Figure 4. Southern blot analysis. A Southern blot of chick genomic DNA digested with EcoRI (lane *1*). PstI (lane *2*), SacI (lane *3*), KpnI (lane *4*), or XbaI (lane *5*) hybridized with a ^{32}P-labeled insert from clone 2. Positions of size markers in kilobases are indicated to the left.

conjugated rabbit anti-mouse Ig followed by FITC-conjugated swine anti-rabbit Ig, and then mounted in Tris-glycerol (1:9) containing 0.1% paraphenylenediamine. Mouse nonimmune serum was used as a control. Whole mounts of the lagenar macula were labeled with monoclonal anti-HCA as described by Goodyear and Richardson (1992).

Results

Identification of Clone 1 as a Chick β-Tectorin cDNA Clone

Clone 1 was isolated from the λgt11 expression library using a polyspecific antiserum recognizing both β-tectorin and the α4-tectorin subunit, and it was therefore necessary to determine which of these two polypeptides the clone encodes. Antibodies specific for either β-tectorin or the α4-tectorin subunit were raised, and their specificity was demonstrated on two-dimensional Western blots of tectorial membrane proteins (Fig. 3 *a*). Western blots of lysates prepared from a lysogen of clone 1 were tested with these antisera. The anti-chick 43,000 tectorin serum recognizes a β-galactosidase fusion protein with an M_r = 156,000. Only the anti-β-tectorin serum reacts with this band, the anti-α4-tectorin serum does not (Fig. 3b). The clones described therefore encode β-tectorin.

Southern Blotting, Northern Blotting, and Reverse Transcription PCR

Chicken genomic DNA was analyzed by Southern blotting using a DNA probe prepared from clone 2. A single band was obtained in the XbaI-digested lane (Fig. 4), suggesting that β-tectorin is derived from a single-copy gene. Total RNA was prepared from nine different tissues from the 2-d posthatch chicken and analyzed by Northern blotting using a DNA probe prepared from clone 1. A single band of 1.4 kb was detected in the inner ear tissue (Fig. 5 *a*). Bands were not detected in any of the other tissues. The blot was subsequently stripped and reprobed with an α-actin probe, confirming the integrity of the RNA (Fig. 5 *b*). Poly(A)$^+$ RNA was prepared from cochlea, brain, colon, kidney, cartilage, lung, liver, and heart, and used to prepare single-stranded cDNA. The cDNA was amplified using a primer pair based on the sequence of clones 2 and 3. A PCR product of the predicted size (334 bp) was observed with the cochlear cDNA, and also with the brain cDNA, but not with any of the other samples (Fig. 5 *c*). Poly(A)$^+$ RNA from the same tissues was also analyzed by Northern blotting using a probe prepared from clone 2. A single band of 1.4 kb was observed with the cochlear mRNA, but not with the mRNA from any of the other tissues, including brain (not shown).

Chick β-Tectorin cDNA Sequence

The sequence of the β-tectorin cDNA is presented in Fig. 6. The cDNA is 1,230 bp long, with the first ATG start codon at position 77. This codon has a purine (G) in the −3 position and a G at the +4 position, the minimum required by the Kozak consensus for initiation (Kozak, 1989). This initiation codon is followed by a 987-base open reading frame (ORF)[1] ending with a TAA stop codon at position 1,064. The ORF is followed by a 148-base 3′ untranslated sequence containing a potential polyadenylation signal and terminated by a poly(A) tail.

Predicted Amino Acid Sequence of β-Tectorin

The ORF encodes a protein of 329 amino acids with a molecular mass of 36,902.8 D. The predicted protein contains 13 cysteine residues and four potential N-glycosylation sites. The ATG start codon is followed immediately by a stretch of 17 hydrophobic amino acids that represent a putative signal sequence. Attempts to obtain direct amino acid sequence were not successful, hence the NH_2-terminal amino acid of the mature protein is not known. However, on the basis of the -1, -3 rule (von Heijne, 1986), cleavage may be between the alanine at position 17 and glycine at position 18. The protein terminates with a hydrophobic stretch of 23 amino acids that is preceded by several charged residues and is characteristic of proteins associated with the membrane via a glycosylphosphatidylinositol (GPI) tail (Cross, 1987; Ferguson and Williams, 1988; Cross, 1990). An extended basic sequence that may represent an endopeptidase recognition site is also present 21 amino acids upstream from the hydrophobic carboxy tail (Fig. 6) at positions 281–285.

Database Searches and Sequence Similarities

A search of the combined Swiss Prot, PIR, and Translated database (Release 80) with the β-tectorin sequence identified four proteins with a similarity index >15% when using a gap penalty of 4, a K-tuple of 2, and a window of 64. These were the pancreatic zymogen granule membrane glycoprotein GP2, the urinary protein uromodulin, and the zona pellucida (ZP) proteins ZP2 and ZP3. All these proteins belong to a

1. *Abbreviations used in this paper*: GPI, glycosylphosphatidylinositol; HCA, 275-kD hair cell antigen; ORF, open reading frame; ZP, zona pellucida.

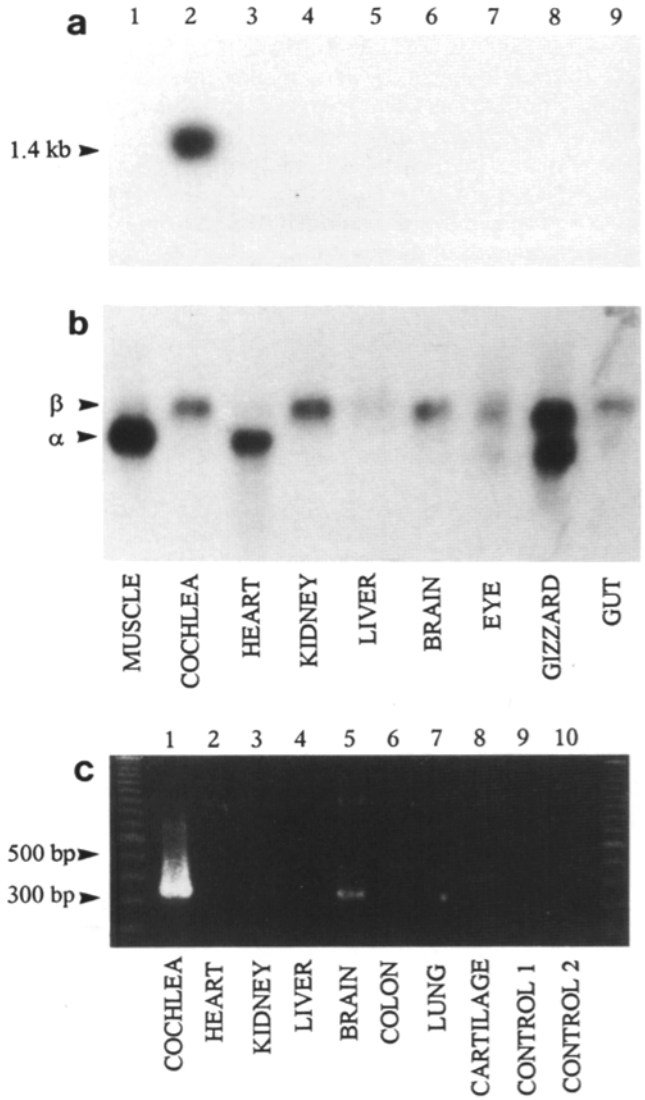


Figure 5. Northern blot and RT-PCR analysis of β-tectorin mRNA expression. (*a*) Total RNA from skeletal muscle (lane *1*), cochlea (lane *2*), heart (lane *3*), kidney (lane *4*), liver (lane *5*), brain (lane *6*), eye (lane *7*), gizzard (lane *8*), and gut (lane *9*), was hybridized with a ^{32}P-labeled probe prepared from clone 1. (*b*) The blot in *a* was stripped and reprobed with an α-actin probe that recognizes both α- and β-actins. (*c*) RT-PCR of single-strand cDNA synthesized from poly(A)$^+$ RNA prepared from cochlear (lane *1*), heart (lane *2*), kidney (lane *3*), liver (lane *4*), brain (lane *5*), colon (lane *6*), lung (lane *7*), and cartilage (lane *8*). Control 1 for the reverse transcriptase reaction (no poly(A)$^+$RNA) is shown in lane *9*, and control 2 for the PCR (primers alone without cDNA) is shown in lane *10*. Flanking the lanes are a 100-bp ladder, and the positions of the 500- and 300-bp markers are indicated on the left.

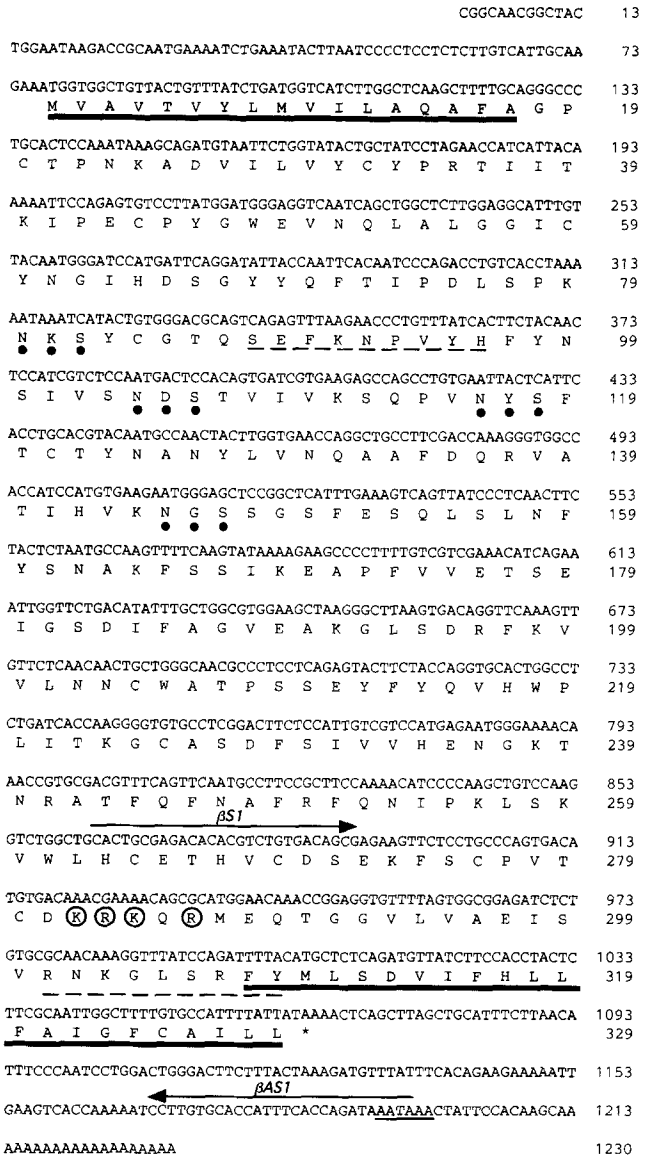


Figure 6. The cDNA and derived amino acid sequences of β-tectorin. The data shown is for clone 3. The putative NH_2-terminal signal peptide and the COOH-terminal hydrophobic sequence are heavily underlined. Consensus sites for N-glycosylation are indicated by dots. Charged residues representing a potential endopeptidase cleavage site are circled. Dashed underlining marks the sequences of the synthetic peptides used to raise antisera. The asterisk marks the in frame stop codon, and the polyadenylation signal is underlined. The positions of the RT-PCR primers are arrowed. These sequence data are available from EMBL/GenBank/DDJB under accession No. L38519.

recently defined family that shares a common 260–amino acid residue domain characterized by eight strictly conserved cysteine residues and a certain pattern of hydrophobic, polar and turn forming residues, known as the ZP domain (Bork and Sander, 1992). An alignment of the ZP domain of chick β-tectorin with the ZP domains of representative members of this family, dog GP2, human uromodulin, mouse ZP2, and human ZP3, is presented in Fig. 7. A comparison of the sequence similarity scores is provided in Table I.

Glycosidase Treatment of β-Tectorin

β-Tectorin was purified by preparative gel electrophoresis and digested with a number of different glycosidases, both alone and in combination. Neither neuraminidase nor *O*-glycosidase, nor a combination of these two enzymes cause any significant shift in the electrophoretic mobility of β-tectorin (Fig. 8, lanes *1–4*). After treatment with *N*-glycosidase, the mobility of β-tectorin increases significantly and a single band with an M_r = 30,000 is observed (Fig. 8, lane *5*). A

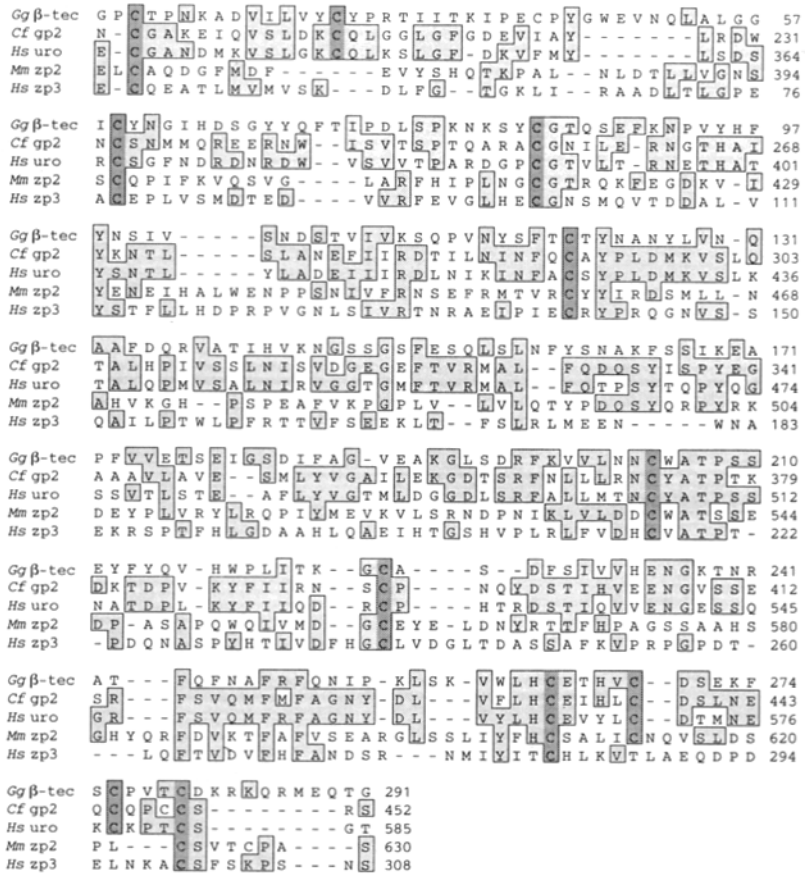


Figure 7. Comparison of β-tectorin with the ZP domains of GP2, uromodulin, ZP2, and ZP3. The ZP domains of dog GP2 (*Cf* gp2; Fukuoka et al., 1991), human uromodulin (*Hs* uro; Hession et al., 1987), mouse ZP2 (*Mm* zp2; Liang et al., 1990), and human ZP3 (*Hs* zp3; Chamberlain and Dean, 1990), as described by Bork and Sander (1992), were aligned with the predicted sequence of fully processed β-tectorin (*Gg* β-tec). Identical residues are boxed and lightly shaded. Conserved cysteines are heavily shaded.

single band with the same electrophoretic mobility is observed after treatment with a combination of all three enzymes (Fig. 8, lane *6*).

Blot Analysis with Antipeptide Sera

Examination of the derived protein sequence suggests that β-tectorin may be synthesized as a GPI-linked, membrane-associated precursor. Antibodies were therefore made to two synthetic peptides, P1 and P2, that are in the derived amino acid sequence. P1 is in the NH_2-terminal half of the predicted sequence, and P2 lies in a region of the COOH terminus that would be removed if the protein is lipid linked. Dot blots indicate that the rabbit anti-P1 serum, and both the rabbit and the mouse anti-P2 sera recognize their respective antigens. However, only the rabbit anti-P1 serum reacts with mature β-tectorin (Fig. 9). Furthermore, the polyclonal mouse anti-chick 43,000 tectorin serum that was originally used to isolate the β-tectorin clone recognizes the P1 and not the P2 peptide (Fig. 9). Western blots of tectorial membrane proteins separated by SDS-PAGE under reducing conditions show that a band with an M_r = 43,000 is labeled by the anti-P1 antibodies, but not by either of the two anti-P2 sera (Fig. 10 *a*). Blots of two-dimensional gels were also stained with the rabbit anti-P1 and the mouse anti-chick 43,000 tectorin sera. The anti-P1 serum only recognizes the eight isoelectric variants characteristic of β-tectorin, whereas the anti-chick 43,000 tectorin serum recognizes both the acidic smear formed by the α4-tectorin subunit, as well as β-tectorin (Fig. 10 *b*).

Reactivity of Antipeptide Sera with In Vitro Translation Products

Although the dot and Western blot analysis strongly suggests that mature β-tectorin does not contain the P2 sequence, the possibility that this epitope is masked or that the antibodies are of low affinity could not be excluded. Attempts were therefore made to immunoprecipitate the in vitro translation products of the β-tectorin cDNA clone. Translation of an RNA transcript prepared from clone 2 in the absence of microsomes produced bands of variable size, indicative of varying degrees of proteolysis (not shown). In the presence of microsomes, postranslational processing of β-tectorin gives two major bands of similar intensity with M_r = 47,000 and 43,500 (Fig. 11 *a*, lane *1*). Minor products with M_r = 40,500, 37,500, and 34,500 could also be observed in some gels. *N*-glycosidase treatment of the translation reac-

Table I. Comparison of the ZP Domains of β-Tectorin, GP2, Uromodulin, ZP2, and ZP3

Gg β-tec				
29.3	*Cf* gp2			
26.6	66.0	*Hs* uro		
14.1	18.2	17.8	*Mm* zp2	
13.1	19.5	20.2	21.2	*Hs* zp3

All scores are percentage sequence similarities between the full-length ZP domains of chick β-tectorin (*Gg* β-tec) and dog GP2 (*Cf* gp2), human uromodulin (*Hs* uro), mouse ZP2 (*Mm* zp2), and human ZP3 (*Hs* zp3), calculated from the multiple alignment shown in Fig. 7.

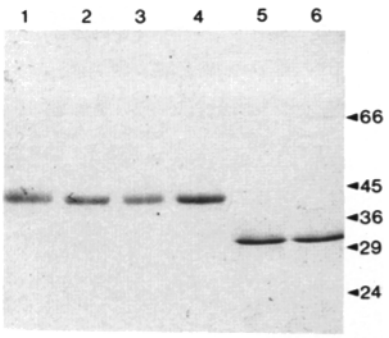


Figure 8. Glycosidase treatment of β-tectorin, Coomassie blue–stained gel. β-Tectorin was incubated overnight at 37°C in buffer (lane *1*), or buffer containing neuraminidase (lane *2*), *O*-glycosidase (lane *3*), neuraminidase and *O*-glycosidase (lane *4*), *N*-glycosidase (lane *5*), and a combination of all three enzymes: neuraminidase, *O*-glycosidase, and *N*-glycosidase (lane *6*). Positions of molecular mass markers are indicated to the right in kilodaltons.

tion generates a major deglycosylation product with an M_r = 34,500, and a minor component with an M_r = 37,500 (Fig. 11 *a*, lane *2*). These results suggest that all four potential N-glycosylation sites are being used. The bands with M_r = 37,500, 40,500, 43,500, and 47,000 represent, respectively, the addition of one, two, three, and four complex N-linked oligosaccharides to a core protein that has an M_r = 34,500. These in vitro translation products of clone 2 are immunoprecipitated by both the rabbit anti-P1 and the rabbit anti-P2 antibodies, as well as by the mouse anti–β-tectorin serum (Fig. 11 *a*, lanes *4–8*).

Immunoprecipitation of ^{125}I-labeled β-tectorin with the antipeptide antibodies confirms the results obtained by dot and Western blot analysis, with only the anti-P1 and not the anti-P2 antibodies precipitating mature β-tectorin (Fig. 11 *b*, lanes *1–5*). Together, the data from the blot and immunoprecipitation experiments indicate that mature β-tectorin does not contain the P2 peptide sequence.

In Situ Hybridization

The expression pattern of β-tectorin within the cochlea was investigated by in situ hybridization with ^{32}P- and ^{35}S-labeled RNA probes (Figs. 12 and 13). In the auditory region of the cochlear duct, antisense probes label the basilar papilla, the clear cells that lie adjacent to the papilla, and the cuboidal cells that lie over the inferior cartilaginous plate (Fig. 12, *a–c*). The homogene cells, the tegmentum vasculosum,

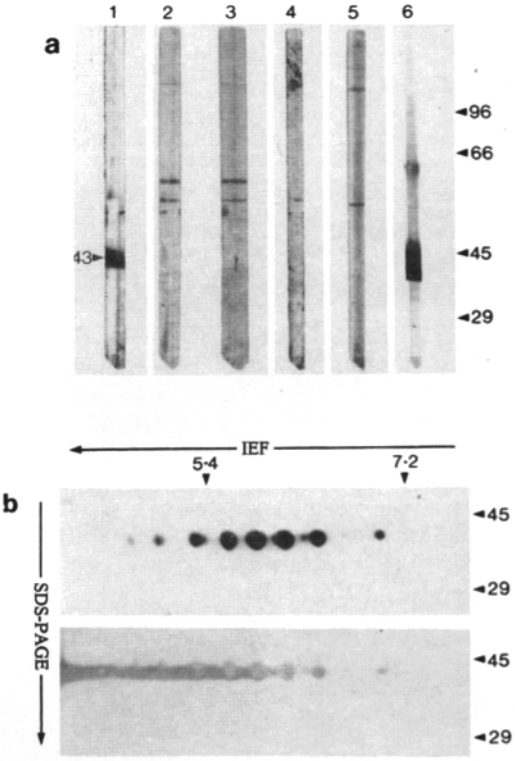


Figure 10. Western blot analysis of antipeptide sera reactivity. (*a*) Western blots of tectorial membrane proteins were stained with rabbit anti-P1 serum (lane *1*), rabbit preimmune serum (lane *2*), rabbit anti-P2 serum (lane *3*), mouse anti-P2 serum (lane *4*), mouse nonimmune serum (lane *5*), and mouse anti–chick 43,000 tectorin serum (lane *6*). (*b*) Two-dimensional Western blots of chick tectorial membrane proteins stained with rabbit anti-P1 serum (*top*) and mouse anti-43,000 tectorin serum (*bottom*). Positions of molecular mass markers are indicated to the right of the gels in kilodaltons.

the hyaline cells, the cochlear ganglion, and the cartilaginous plates are all unlabeled. No specific labeling was seen with the sense probe. Previous histological studies have not suggested that the tectorial membrane is attached to either the clear or the cuboidal cells, and expression of β-tectorin in these cell groups was therefore an unexpected finding. However, double labels using the anti–chick 43,000 tectorin serum to demarcate the tectorial membrane and rhodamine phalloidin to label the cuboidal cells clearly indicate that the tectorial membrane stretches across the duct and contacts both the clear and the cuboidal cells (Fig. 12, *d* and *e*).

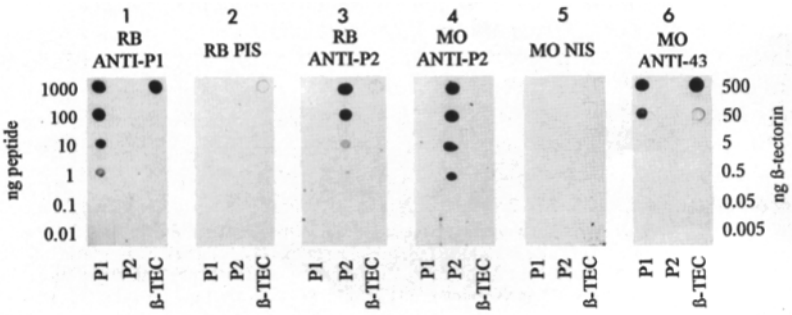


Figure 9. Dot blot analysis of antipeptide sera reactivity. Dot blots loaded with serial 10-fold dilutions of peptide 1 (*P1*), peptide 2 (*P2*), and gel-purified β-tectorin were reacted with rabbit anti-P1 serum (blot *1*), rabbit preimmune serum (blot *2*), rabbit anti-P2 serum (blot *3*), mouse anti-P2 serum (blot *4*), mouse nonimmune serum (blot *5*), and mouse anti–chick 43,000 tectorin serum (blot *6*).

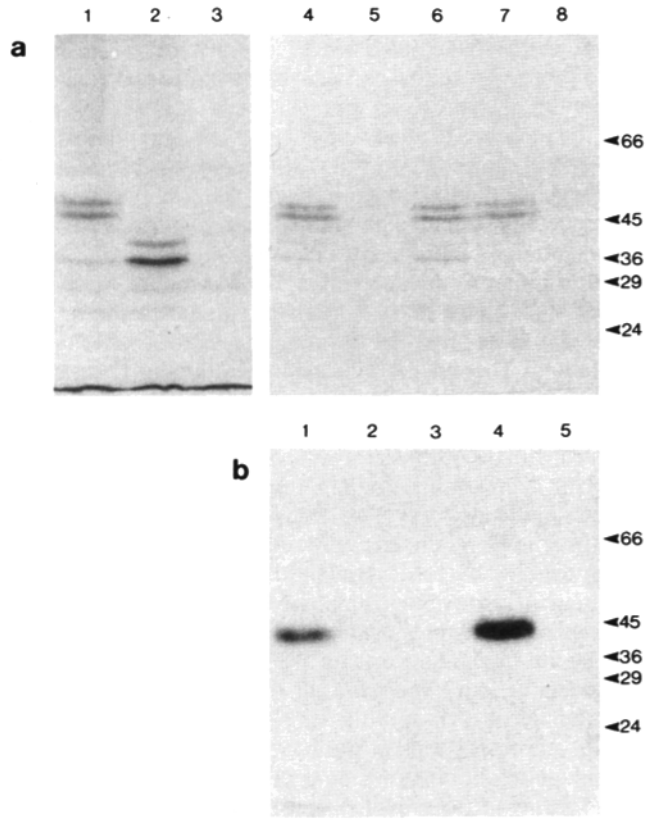


Figure 11. Immunoprecipitation analysis of anti-peptide sera reactivity. (*a*) ^{35}S-labeled in vitro translation products of a capped RNA transcript prepared from clone 2 after incubation in buffer (lane *1*), or buffer containing *N*-glycosidase F (lane *2*). Blank in vitro translation reaction (no RNA) treated with *N*-glycosidase (lane *3*). Aliquots of ^{35}S-labeled in vitro translation products of clone 2 RNA transcripts immunoprecipitated with rabbit anti-P1 antibodies (lane *4*), rabbit nonimmune IgG (lane *5*), rabbit anti-P2 antibodies (lane *6*), mouse anti-β tectorin serum (lane *7*), and mouse nonimmune serum (lane *8*). (*b*) Aliquots of ^{125}I-labeled β-tectorin immunoprecipitated with rabbit anti-P1 antibodies (lane *1*), rabbit nonimmune IgG (lane *2*), rabbit anti-P2 antibodies (lane *3*), mouse anti-β-tectorin serum (lane *4*), and mouse nonimmune serum (lane *5*). Positions of molecular mass markers are indicated to the side of the gels in kilodaltons.

The hair cells in the vestibular maculae are also covered by an extracellular matrix known as the otolithic membrane, and most antitectorin sera also stain this structure (Killick et al., 1992). In situ hybridization has also been used to study β-tectorin expression in one of these vestibular organs, the lagenar macula, which is situated at the end of the cochlear duct. In the macula of the lagena, expression of β-tectorin mRNA is restricted (Fig. 13, *a* and *b*) to a narrow zone in the sensory epithelium known as the striola. In addition, immunofluorescence microscopy with the anti-β-tectorin serum shows that β-tectorin protein is concentrated in the striolar region of the otolithic membrane in the lagenar macula (Fig. 13 *c*). This area can also be defined by the distribution of hair bundles that are only stained weakly by a monoclonal antibody recognizing a specialization of the apical surface of the hair cell surface known as the 275-kD hair cell antigen (HCA) (Richardson et al., 1990) (Fig. 13 *d*). The hair bundles in the striola stain weakly relative to those in the neighboring extrastriolar region because the HCA is restricted to the base of the bundles, whereas the extrastriolar hair cells that stain strongly have the HCA distributed over the entire surface of the hair bundle.

Discussion

The results describe the characterization of a cDNA for β-tectorin, one of the major glycoproteins of the avian tectorial membrane. The cDNA predicts a protein of 36,902.6 D with four potential N-glycosylation sites, 13 cysteine residues, a hydrophobic leader sequence, and a 23-amino acid, hydrophobic COOH-terminal tail. Mature β-tectorin has an M_r = 43,000 under reducing conditions (Killick et al., 1992), and treatment of β-tectorin with *N*-glycosidase F generates a core polypeptide with an M_r = 30,000, indicating that some 30% of the observed mass is accounted for by carbohydrate. Although the size of the protein predicted from the cDNA sequence is, as would be expected for a glycoprotein, smaller than that of the fully glycosylated product, it is actually significantly larger than that of the deglycosylated core polypeptide. Certain features of the NH_2 and COOH termini of the predicted protein suggest the core polypeptide is generated by posttranslational processing. Removal of the hydrophobic leader sequence from the deduced amino acid sequence at the position suggested by the von Heijne rule for signal peptidase cleavage would give a core protein with a mass of 35,109 D. This value is similar to that of the major band observed when the translation products of clone 2 are deglycosylated, suggesting the signal peptide is cleaved in the presence of microsomes. However, it is also nearly 5 kD greater than the mass observed for mature, deglycosylated β-tectorin, suggesting that the core polypeptide is normally processed further in vivo.

The predicted sequence of β-tectorin has a carboxy terminus with a high hydrophobicity index that terminates abruptly and is not followed by any stop-transfer animo acids or a hydrophilic cytoplasmic domain. Such sequences are characteristic of proteins that become membrane linked via a GPI tail, and are thought to be removed concomitantly with the attachment of the GPI moiety by a transamidase resident in the endoplasmic reticulum (Cross, 1987; Ferguson and Williams, 1988; Cross, 1990). The amino acid requirements adjacent to the cleavage site of the transamidase are not well defined, but the presence of small amino acids in the ω and ω+2 positions is considered necessary (Kodukula et al., 1993). The two glycines at positions 291 and 292, the alanine at position 296, or the serine at position 299 are all candidates for the terminal amino acid of a putative GPI-linked form of β-tectorin, according to the model of Kodukula et al. (1993). Antibodies recognizing peptide P2, lying just upstream of the hydrophobic COOH-terminal sequence in the predicted protein, react with the in vitro translation products of the β-tectorin cDNA, but not with mature β-tectorin, indicating that the hydrophobic COOH-terminal region is indeed lost during maturation of the protein. However, the results also indicate that the COOH-terminal tail is not processed in vitro, although the signal peptide is removed and the product is glycosylated.

If β-tectorin is synthesized as a GPI-linked, membrane-associated precursor, then it must be released from the mem-

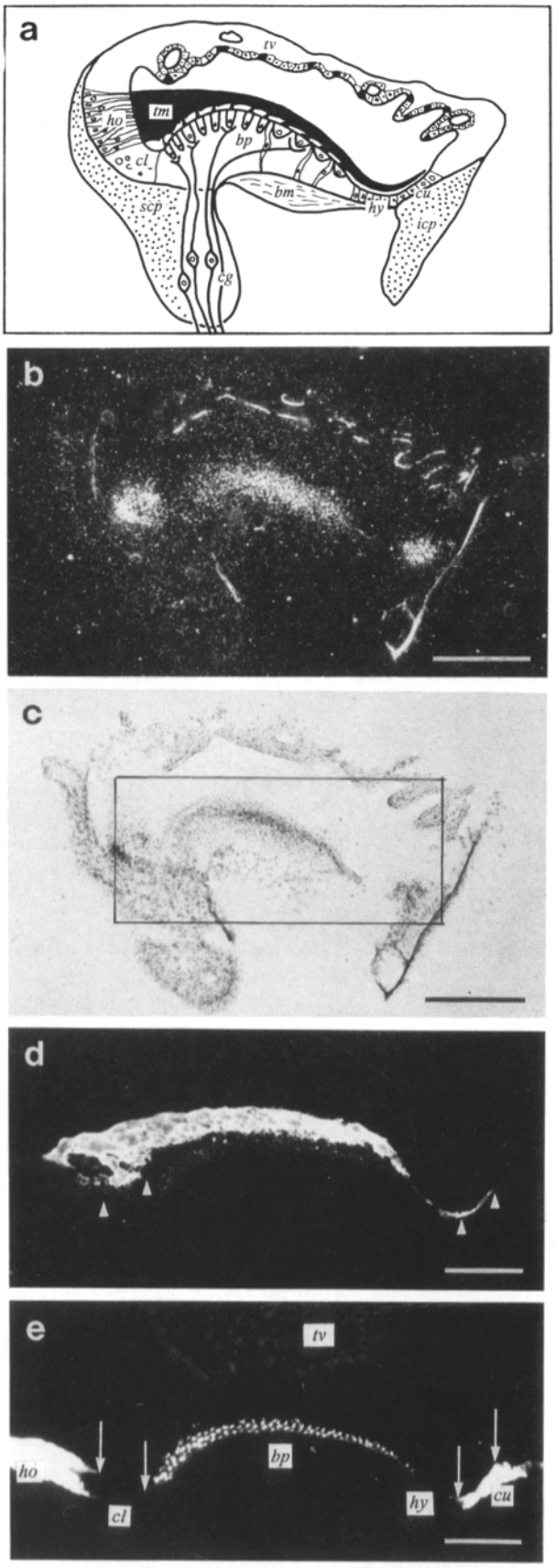
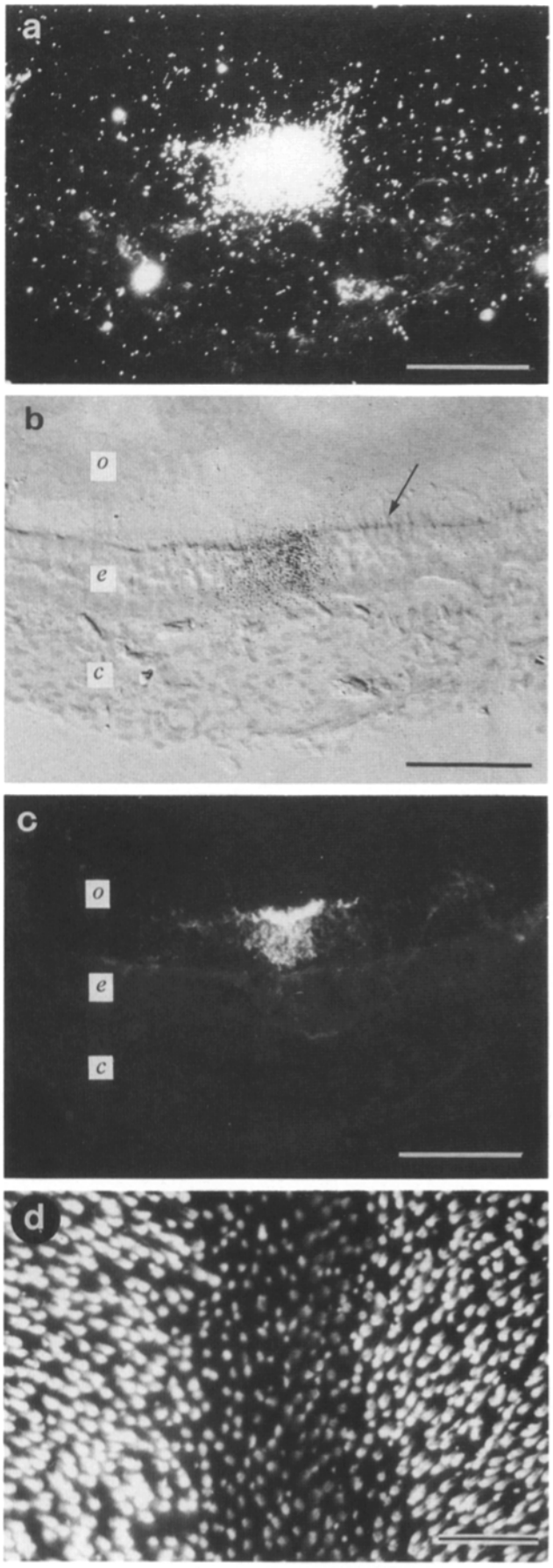


Figure 12. In situ hybridization analysis of β-tectorin expression in the cochlear duct. (*a*) Schematic diagram of a cross-section through the cochlear duct illustrating the different regions. *bp*, basilar papilla; *bm*, basilar membrane; *cg*, cochlear ganglion; *cl*, clear cells; *cu*, cuboidal cells; *hy*, hyaline cells; *ho*, homogene cells; *icp*, inferior cartilaginous plate; *scp*, superior cartilaginous plate; *tm*, tectorial membrane; *tv*, tegmentum vasculosum. (*b*) Dark-field micrograph of a section hybridized with a ^{32}P-labeled antisense β-tectorin probe. Grains are evident over the clear cells, the basilar papilla, and the cuboidal cells. (*c*) Bright-field micrograph of the field shown in *b*. An area similar to that boxed is shown below in *d* and *e*. (*d* and *e*) A cryosection of the cochlear duct double labeled with mouse anti-chick 43,000 tectorin serum (*d*) and rhodamine phalloidin (*e*) to illustrate the position of the tectorial membrane relative to the homogene cells, the clear cells, and the cuboidal cells. The boundaries of the clear and cuboidal cell regions are indicated by the vertical arrowheads in *d* and by the vertical arrows in *e*. The homogene and cuboidal cells are intensely stained by phalloidin. Bars, 200 μm.

brane, either by the action of an endogenous phospholipase or, as has been suggested for a GPI-anchored heparin sulphate proteoglycan that is shed into the medium (David et al., 1990), by proteolysis. In this respect, it is interesting to note the presence of an extended basic sequence at positions 282–286 in the predicted β-tectorin sequence that could act as an endopeptidase recognition site. Removal of the signal sequence after the alanine at position 17, and release from the membrane by proteolytic cleavage after the arginine at position 286 by an endopeptidase, would produce a polypeptide of 30,232 D, a mass that is very similar to the relative molecular mass observed for mature β-tectorin after deglycosylation. The final product would contain 12 cysteine residues that are probably all involved in intrachain disulphide bond formation since β-tectorin is not covalently associated with the other tectorins and it also undergoes a characteristic decrease in electrophoretic mobility on reduction.

The significance of synthesizing β-tectorin as a GPI-linked precursor is as yet unclear, although in many epithelial cell types, the GPI anchor is thought to be a signal for the apical targeting of proteins (Lisanti et al., 1988). β-Tectorin is secreted into the lumen of the cochlear duct, and the GPI tail may be the signal that sorts the protein to the apical surface of the epithelium. The tectorial membrane is attached to both the apical surface of the supporting cells in the basilar papilla and to the tops of the hair cell stereocilia bundles, and the possibility that this attachment is mediated via GPI linked forms of β-tectorin cannot be discounted. However, PCR experiments have not detected any alternatively spliced forms of the β-tectorin message that lack a potential COOH-terminal signal sequence for GPI addition (unpublished observations), suggesting that all β-tectorin, which is present throughout the entire tectorial membrane, is initially synthesized as a GPI-linked form.

A search of the current protein database with the β-tectorin sequence revealed homology with four other proteins, GP2, uromodulin, ZP2, and ZP3. GP2 was originally identified as the major component of the pancreatic zymogen granule membrane (MacDonald and Ronzio, 1972). It is a glycoprotein with a molecular mass that varies from 75–92 kD, depending on the species, it exists as both soluble and GPI-anchored, membrane-bound forms (LeBel and Beattie, 1988; Fukuoka et al., 1991), and it is also a major component of a recently identified, insoluble fibrillar network that



is present in the acinar ducts (Grondin et al., 1992). Uromodulin is an 85-kD glycoprotein isolated from the urine of pregnant women (Muchmore and Decker, 1985). The protein core of uromodulin is identical to the Tamm-Horsfall glycoprotein (Pennica et al., 1987; Hession et al., 1987), the major protein component of human urine, and the two proteins differ only in their patterns of glycosylation. The Tamm-Horsfall glycoprotein is localized to the thick ascending loop of Henle and the early distal convoluted tubule in the kidney (Hoyer and Seiler, 1979). It exists in urine as high molecular mass aggregates (Tamm and Horsfall, 1952), and it appears to form unbranched filaments of variable length (Porter and Tamm, 1955; Bayer, 1964; Fletcher et al., 1970). Like GP2, it can also exist as a GPI-anchored, membrane-linked form (Rindler et al., 1990). Although normally a "soluble" component of urine, solutions of uromodulin can readily be induced to form gels by high concentrations of calcium and sodium (Stevenson et al., 1971), and uromodulin is the major component of urinary casts (McQueen, 1966). ZP2 (M_r = 120,000) and ZP3 (M_r = 83,000), along with ZP1 (M_r = 200,000), are the major glycoproteins of the zona pellucida surrounding the mouse oocyte, and a similar set of glycoproteins probably exists in the zona pellucidae of oocytes from other mammalian species (Wassarman, 1988). The zona pellucida proteins are produced by the oocyte and form a dense fibrillogranular matrix composed of long, interconnected filaments which have a diameter of ~18 nm in rotary shadowed preparations. These filaments have a beaded appearance, and they are thought to be composed of alternately arranged molecules of ZP2 and ZP3. The filaments formed by these ZP2:ZP3 heterodimers are themselves cross-linked by ZP1 homodimers to form the observed, randomly interconnected network (Greve and Wassarman, 1985). ZP3 is the primary receptor for sperm and it alone can elicit the acrosomal reaction, with this property being associated with the O-linked sugar groups (Wassarman, 1988).

The common structural element shared by β-tectorin, GP2, uromodulin, ZP2, and ZP3 is the ZP domain. If the β-tectorin sequence is processed as described above, the mature protein would simply be a single zona pellucida domain. The function of this domain is not known, but it is also found in one other protein, the TGF-β type III receptor betaglycan, and experiments with bacterial fusion proteins have suggested that this domain may possess the TGF-β–binding activity (Fukushima et al., 1993). However, more recent experiments in which various betaglycan constructs have been expressed in COS cells indicate that the TGF-β–binding site

Figure 13. Expression of β-tectorin in the vestibular epithelium of the lagenar macula. (*a* and *b*) Dark-field micrograph (*a*) and corresponding bright-field micrograph (*b*) of the lagenar macula hybridized with a ^{35}S-labeled antisense β-tectorin probe. (*c*) Cryosection of the lagenar macula stained with mouse anti–β-tectorin. Staining is concentrated in the striolar region of the otolithic membrane. (*d*) Wholemount of the lagenar macula stained with a monoclonal antibody directed against the 275-kD hair cell antigen, a protein that is restricted to the apical surface of sensory hair cells. The central area where the cells are stained less brightly corresponds to the striola. *o*, otoliths; *e*, epithelium, *c*, cartilage. Bars, 100 μm.

is located towards the NH_2 terminus in the endoglin-related region, and it does not involve the zona pellucida domain (Lopez-Casillas et al., 1994). Soluble forms of betaglycan lacking the zona pellucida domain were found to be unstable, and it has been suggested that the function of this region may be to control the stability of the protein or enable it to interact with other components of the extracellular environment (Lopez-Casillas et al., 1994). Although the functions of GP2 and uromodulin are not yet clear, ZP2, ZP3, GP2, and uromodulin can, either alone or in combination with other components, form extracellular matrices or gels. The avian tectorial membrane is a dense, fibrillogranular matrix composed of 7–12-nm diameter filaments (Tanaka and Smith, 1975), and it superficially bears a resemblance to the zona pellucida. It is formed by a combination of α- and β-tectorins, but the way in which these two molecules interact to form the observed structure remains to be determined. The high density of fibril packing in the tectorial membrane has so far precluded detailed immunoelectron microscopic analysis of filament composition. However, it is possible that β-tectorin, like uromodulin, self associates to form homomeric filaments, and that these filaments are cross-linked by α-tectorin, in a way similar to that in which the heterodimeric ZP2:ZP3 fibrils of the zona pellucida are cross-linked by ZP1. Although circumstantial, the present evidence suggests the ZP domain may be the common element present in GP2, uromodulin, ZP2, ZP3, and β-tectorin that enables all five of these proteins to form filamentous extracellular structures.

Previous morphological studies have suggested that the tectorial membrane is secreted by both the supporting cells within the basilar papilla, and the homogene cells to which the tectorial membrane is attached along one of its edges (Cohen and Fermin, 1985; Shiel and Cotanche, 1990). The in situ hybridization data for the hatchling clearly indicate that β-tectorin mRNA is not expressed in the homogene cells, although it is expressed within the basilar papilla. In addition, expression is detected in two other groups of cells, the clear and the cuboidal cells, neither of which have been previously considered to be involved in tectorial membrane production, although both of which are in contact with the tectorial membrane. The homogene cells may well express β-tectorin during the early phases of development and further studies are underway to examine this possibility. However, during the regeneration of the tectorial membrane that occurs after its destruction by acoustic overstimulation, there is little evidence for the involvement of the homogene cells, and the tectorial membrane that forms de novo is probably produced entirely by cells within the papilla (Cotanche, 1987).

The results of this study also show that within the lagenar macula, the expression of β-tectorin RNA is restricted to a region of the sensory epithelium known as the striola, and that β-tectorin is concentrated in the overlying otolithic membrane. Several morphological studies have indicated that the structure of the otolithic membrane differs between the striolar and extrastriolar regions (Lindeman, 1969; Marco et al., 1971; Ross et al., 1987), and the selective expression of β-tectorin in the striola therefore correlates with this observed difference. The striola is a region of the vestibular maculae within which the hair bundles change their orientation (Hillman, 1976) and where there is a high density of type I vestibular hair cells relative to the surrounding extrastriolar regions (Wersall, 1956). In the chick inner ear, the striola can also be distinguished by the presence of hair cells that have the HCA restricted to the basal region of the hair bundle (Goodyear and Richardson, 1992). Similarly, the hair cells in the basilar papilla also have the HCA restricted to the basal region of their hair bundles, whereas in contrast, those in the extrastriolar regions of the maculae have the HCA distributed over their entire surface (Goodyear and Richardson, 1992). The HCA is a component of a type of interstereociliary cross-link called the shaft connector, and differences in shaft connector distribution may produce hair bundles with different mechanical properties (Goodyear and Richardson, 1992). The results of these studies therefore indicate that hair cells with the HCA restricted to the basal regions are associated with an overlying matrix enriched in β-tectorin, and they suggest that these matrices may be physically matched to the bundle type they drive by the presence of β-tectorin.

This study demonstrates a number of interesting points about one of the major glycoproteins of the avian tectorial membrane, an extracellular matrix that is involved in transmitting the motion of the cochlear fluids to the stereocilia bundles of the mechanosensory hair cells, and raises several possibilities. First, the data presented here indicate that β-tectorin is first synthesized as a GPI-linked, membrane-bound precursor that is then further processed. The GPI moiety may be responsible for targeting β-tectorin to the apical surface of the inner ear epithelium, and the protein may be released from the membrane by a phospholipase or an endopeptidase. Second, β-tectorin shares a common amino acid domain with a limited number of other proteins, all of which can form extracellular fibrils, either on their own or in combination with other components. This domain may therefore be involved in filament formation. Third, the expression patterns of β-tectorin in the inner ear show that it is expressed in association with matrices contacting hair cells that have a cell surface specialization known as the HCA restricted to the basal region of their hair bundles, raising the possibility that a matrix containing β-tectorin is required to generate the appropriate response from this type of hair cell. Molecular cloning of the α-tectorin subunits should further clarify how this extracellular matrix is formed and how it functions in the process of mechanotransduction.

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