

# Identification of a 275-kD Protein Associated with the Apical Surfaces of Sensory Hair Cells in the Avian Inner Ear

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**Abstract.** Immunological techniques have been used to generate both polyclonal and monoclonal antibodies specific for the apical ends of sensory hair cells in the avian inner ear. The hair cell antigen recognized by these antibodies is soluble in nonionic detergent, behaves on sucrose gradients primarily as a 16S particle, and, after immunoprecipitation, migrates as a polypeptide with a relative molecular mass of 275 kD on 5% SDS gels under reducing conditions. The antigen can be detected with scanning immunoelectron microscopy on the apical surface of the cell and on the stereocilia bundle but not on the kinocilium. Double label studies indicate that the entire stereocilia bundle is stained in the lagena macula (a vestibular organ), whereas in the

basilar papilla (an auditory organ) only the proximal region of the stereocilia bundle nearest to the apical surface is stained. The monoclonal anti-hair cell antibodies do not stain brain, tongue, lung, liver, heart, crop, gizzard, small intestine, skeletal muscle, feather, skin, or eye tissues but do specifically stain renal corpuscles in the kidney. Experiments using organotypic cultures of the embryonic lagena macula indicate that the antibodies cause a significant increase in the steady-state stiffness of the stereocilia bundle but do not inhibit mechanotransduction. The antibodies should provide a suitable marker and/or tool for the purification of the apical sensory membrane of the hair cell.

**T**HE mechanosensory hair cells of the acousticolateralis sense organs of vertebrates are epithelial cells capable of responding to displacements of less than a few nanometers. Hair cells are both morphologically and functionally polarized, with an apical end specialized for the detection of stimuli and a basolateral surface specialized for the release of neurotransmitter. A highly organized bundle of stereocilia is located at the apical end of the hair cell, and displacement of this stereocilia bundle results in the gating of nonspecific cation channels located in the apical membrane. Depolarization of the hair cell resulting from channel opening causes neurotransmitter to be released around the basolateral surface, and information about mechanical events at the periphery is thereby transmitted to contacting afferent nerve fibers and relayed to the central nervous system. Although much is known about both the physiology and morphology of hair cells and the biophysics of mechanotransduction, the molecular properties of the apical surface membrane of the hair cell remain completely uncharacterized.

To understand more about the molecular events underlying mechanotransduction, the proteins involved in this process will have to be identified. Purification and characterization of the apical plasma membrane of the hair cell will, however, be difficult since no specific markers or ligands are known for this surface of the cell. We have, therefore, used an immunological approach to generate such a ligand. This study describes the production of antibodies that are specific, within the inner ear, for the apical end of the hair cell and the

identification of a 275-kD protein that is associated with the sensory membrane.

## Materials and Methods

### Immunization

Lagena maculae were obtained from inner ears of 1–3-d-old domestic fowl, and a crude membrane fraction was prepared by differential centrifugation. Anti-lagena macula sera were produced by immunizing BALB/c mice (Charles River Breeding Laboratories, Ltd., Manston, UK) intraperitoneally three times at 1-mo intervals. Material from 200 ears was used for each injection. Animals were test bled at times ranging from 1 to 12 mo after the last injection.

### Absorbing Polyclonal Sera

Anti-lagena macula sera were absorbed sequentially with (a) homogenates prepared from cochlear tissues that do not contain hair cells (i.e., the tegmentum vasculosum and the cochlear ganglion; see Fig. 1); (b) a homogenate from the small intestine; and (c) a mixture of tectorial membranes and otoliths. Tectorial membranes and otoliths were extracted twice with 1% (vol/vol) Triton X-100 (TX-100)<sup>1</sup> in PBS before use as absorbents. Amounts of tissue required were determined empirically for each test bleed, and the efficacy of the absorption procedure was monitored by immunofluorescence microscopy. Immune sera from two different mice (IS1 and IS2) were used in this study.

1. *Abbreviations used in this paper:* TBS/HS, TBS containing 10% heat-inactivated horse serum; TX-100, Triton X-100.

## Immunofluorescence Microscopy

Tissues were fixed, sectioned, and stained as described previously (Richardson et al., 1987a) except that two layers of FITC-conjugated secondary antibodies (FITC-conjugated rabbit anti-mouse Ig followed by FITC-conjugated swine anti-rabbit Ig) were used to detect the primary antibodies. For double label studies rhodamine-conjugated phalloidin (1  $\mu\text{g}/\text{ml}$ ) was added to one of the FITC-conjugated antisera.

## Immunoelectron Microscopy

Cochlear ducts were dissected in PBS, and the distal ends were cut off and opened with a longitudinal slit along the ventral surface. After removal of the otoliths, otolithic membrane and tectorial membrane, the ducts were fixed in 3.7% (vol/vol) formaldehyde, 0.025% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 1 h. The pieces were washed three times in PBS and then incubated for 1 h in TBS (150 mM NaCl, 10 mM Tris-HCl, pH 7.4) containing 10% heat-inactivated horse serum (TBS/HS). Pieces were then incubated overnight in either absorbed mouse anti-lagena macula serum or absorbed mouse nonimmune serum (both diluted 1:100 in TBS/HS), washed five times with TBS, incubated in unconjugated rabbit anti-mouse immunoglobulins (1:50 dilution in TBS/HS) for 2 h, washed five times with TBS, and then incubated for 2 h in 20-nm-diameter colloidal gold particles coated with goat anti-rabbit immunoglobulins (Biochemical Services, Ltd., Cardiff, UK). Samples were then washed with TBS five times, fixed with 1% (wt/vol) osmium tetroxide in 0.12 M sodium cacodylate buffer, pH 7.2, for 1 h, dehydrated with acetone, critical point dried from liquid  $\text{CO}_2$ , mounted on stubs, and finally sputter coated with a thin ( $\sim 10$ -nm) layer of gold.

## Preparation of Monoclonal Antibodies

Monoclonal antibodies were prepared by the method of Kohler and Milstein (1975). Culture supernatants from hybridomas were screened and selected using immunofluorescence microscopy. The hybridoma used in this study was cloned out three times by limiting dilution on two separate occasions. Immunoglobulin type was determined using Ouchterlony double diffusion typing plates (ICN Biomedicals, High Wycombe, UK). Antibody titer was determined using a sandwich-type ELISA and mouse IgG (Sigma Chemical Co., Poole, UK) as a standard. Antibodies were purified using ammonium sulphate precipitation, DEAE ion exchange chromatography, and affinity chromatography on agarose-linked goat anti-mouse IgG antibodies.

## Preparation of Fractions from Lagena Macula

Lagena maculae were sequentially extracted with TBS, followed by 1 M NaCl in 5 mM Tris-HCl, pH 7.2, followed by 1% (vol/vol) TX-100 in 5 mM Tris-HCl, pH 7.2. All solutions contained 1 mM PMSF and 1 mM EDTA. Operationally defined soluble, high-salt soluble, detergent soluble, and insoluble (extracted) fractions were produced using 10-min 16,000  $g_{av}$  centrifugation steps.

## ELISA for Detergent Solubilized Antigen

Samples (50  $\mu\text{l}$  volume) were incubated in poly-L-lysine-coated microtiter plates overnight, then an equal volume of 2 $\times$  concentrated fixative (7.4% formaldehyde, 0.05% glutaraldehyde in 0.1 M sodium phosphate buffer) was added to each well, and the plates were left for 1 h. The wells were then emptied, washed once with TBS, washed twice with TBS containing 0.1 M glycine over a 30-min period, and preblocked with TBS/HS for a period of 1 h. Wells were then incubated with primary antisera diluted in TBS/HS for 3 h, washed three times with TBS, incubated for 1 h in peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, UK) diluted 1:200 in TBS/HS, and finally washed five times in TBS containing 0.05% (vol/vol) Tween 20. Bound antibodies were detected with *o*-phenylenediamine (20 mg/ml) in 0.1 M citrate phosphate buffer, pH 5.0, containing 0.001% (vol/vol)  $\text{H}_2\text{O}_2$  and 0.05% (vol/vol) Tween 20.

## Velocity Sedimentation

Detergent-soluble fractions of the lagena macula were prepared by sequential extraction as described above and loaded onto 5–20% sucrose gradients prepared in 150 mM NaCl, 5 mM Tris-HCl, pH 7.2, and 0.1% (vol/vol) TX-100. Gradients were centrifuged at 35,000 rpm (81,500  $g_{av}$ ) in a TLS-55 rotor for 6 h at 4°C and then split into 20 100- $\mu\text{l}$  fractions. Gradient markers

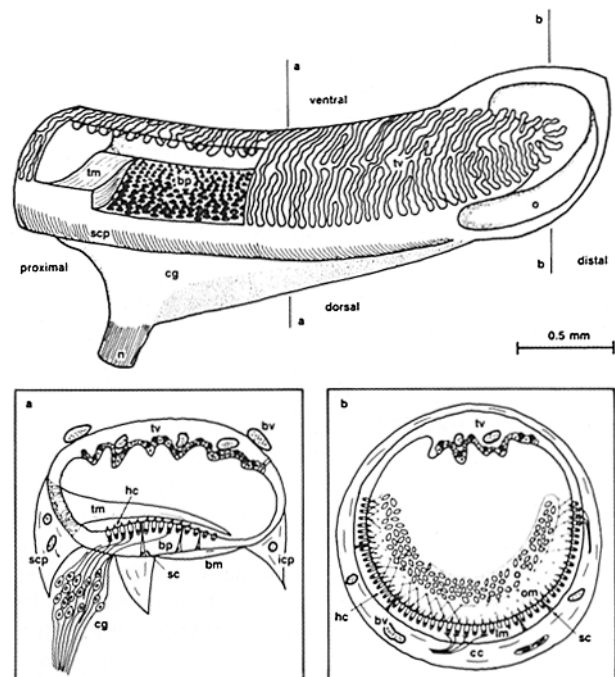
(catalase from bovine liver, ovalbumin, mouse IgG and IgM; 200  $\mu\text{g}/\text{tube}$ ) were run in separate tubes under identical conditions, and their positions were determined by gel electrophoresis. The relative amounts of antigen in each fraction were measured using ELISA. Proteins from pooled gradient fractions were concentrated and then separated on 7.5% polyacrylamide gels under nonreducing conditions. Immunoblotting procedures were as described previously (Richardson et al., 1987b).

## Immunoprecipitation Procedure

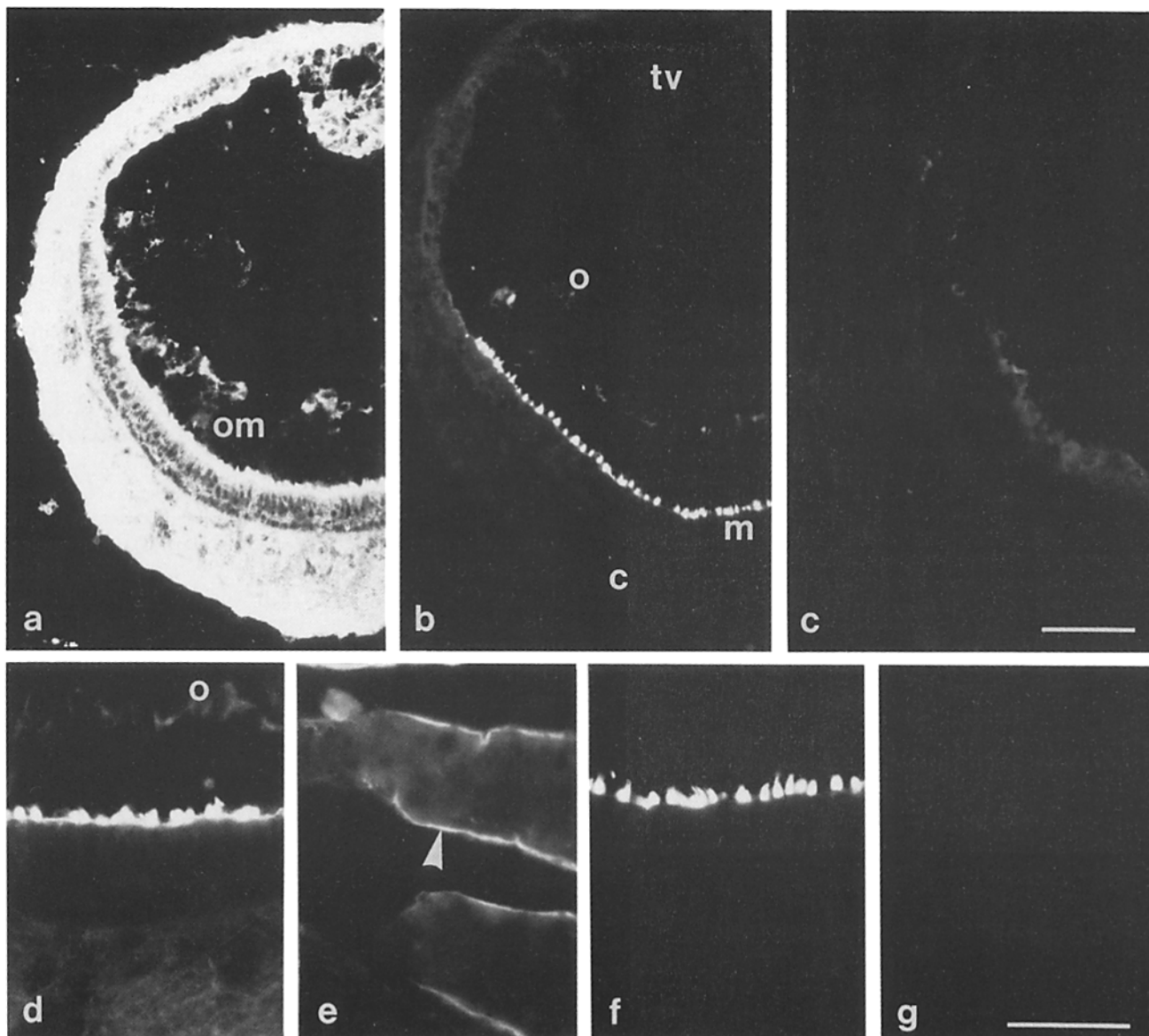
Lagena maculae were sequentially extracted with PBS, with 1 M NaCl in 10 mM sodium phosphate, pH 7.2, and finally with 1% (vol/vol) TX-100 in 0.1 M borate buffer, pH 9.0. All solutions contained 1 mM PMSF and 1 mM EDTA. The detergent soluble proteins were radiolabeled by reductive methylation (Rice and Means, 1971) using [ $^{14}\text{C}$ ]formaldehyde (New England Nuclear, Dupont Ltd., Stevenage, UK) or [ $^3\text{H}$ ]sodium borohydride (Amersham International, Aylesburg, UK) and dialyzed against TBS containing 1 mM EDTA, 0.1 mM PMSF, and 0.1% (vol/vol) TX-100. Procedures for immunoprecipitation were as described previously (Walker et al., 1982) except that a rabbit anti-mouse Ig (Dako) was used to precipitate the primary antibodies. Precipitates were run on 5 and 12.5% polyacrylamide gels under reducing conditions and analyzed by fluorography.

## In Vitro Studies

Organotypic cultures of the lagena macula were prepared from the cochlear ducts of embryonic day 13 chicks using a method previously described for



**Figure 1.** The upper half of the figure illustrates a cochlear duct dissected from a 1-d-old chicken. Only the distal two thirds of the duct are drawn, and the region proximal to the genu is not shown. Portions of the ventral wall and of the tectorial membrane overlying the basilar papilla have been cut away to reveal the auditory hair cells. The structure of the duct in sections cut transverse to its long axis at the levels indicated by the vertical lines (*a*—*a* and *b*—*b*) is illustrated below in *a* and *b*, respectively. The bar indicates the approximate size of the duct in the upper half of the figure. Cellular components are not drawn to scale. *bm*, basilar membrane; *bp*, basilar papilla; *bv*, blood vessel; *cc*, cartilaginous capsule; *cg*, cochlear ganglion; *hc*, hair cell; *icp*, inferior cartilaginous plate; *lm*, lagena macula; *n*, cochlear nerve; *o*, otolith; *om*, otolithic membrane; *sc*, support cell; *scp*, superior cartilaginous plate; *tm*, tectorial membrane; *tv*, tegmentum vasculosum.



**Figure 2.** (a–c) Sections of the lagena macula stained with unabsorbed polyclonal anti-lagena macula serum (a), polyclonal anti-lagena macula serum absorbed with cochlear ganglion and tegmentum vasculosum (b), mouse nonimmune serum absorbed with cochlear ganglion and tegmentum vasculosum (c). *om*, otolithic membrane; *o*, otoliths; *tv*, tegmentum vasculosum; *c*, cartilaginous capsule; *m*, lagena macula. (d–g) Sections of the lagena macula (d and f) and small intestine (e and g) stained with polyclonal anti-lagena macula serum absorbed with either (d and e) cochlear ganglion and tegmentum vasculosum or (f and g) cochlear ganglion, tegmentum vasculosum, and small intestine. Photographic exposure times for the negatives and the prints in d–g were the same. *o*, otoliths. Arrowhead in e points to the brush border. Bars: (a–c) 100  $\mu\text{m}$ ; (d–g) 50  $\mu\text{m}$ .

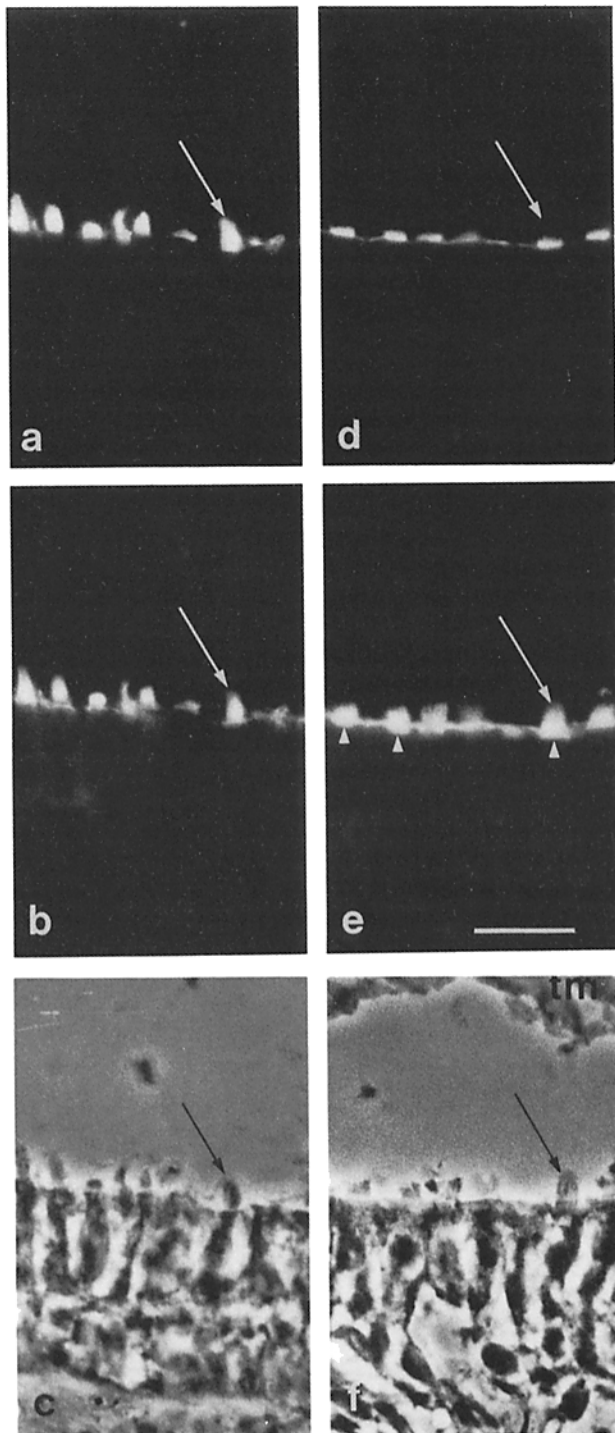
preparing mouse cochlear cultures (Russell and Richardson, 1987) except that the tissue pieces were incubated in trypsin (0.08% wt/vol) for 15 min at room temperature and washed three times with complete medium (90% MEM in Earle's salts, 10% FCS) and the otolithic membranes overlying the hair cells were scraped off with fine needles before explantation. Methods for intracellular recording and measurement of stereocilia bundle stiffness have been described previously (Russell and Richardson, 1987; Richardson et al., 1989; Kössl et al., 1990). The stiffness of the stereocilia bundle was calculated from the product of the stiffness of the probe ( $K_f$ ) and the difference between the displacements of the probe when just above and not in contact with the bundle ( $y_0$ ) and when attached to the bundle ( $y_1$ ). The stereocilia bundle stiffness ( $K_b$ ) is given by the equation  $K_b = K_f(y_0 - y_1)/y_1$ . Displacement measurements were made from the averages of 10–32 records of the differential voltage output of the displacement sensor for bundle displacements of  $\sim 40$  nm made 55–75 ms after the onset of the stimulus.

Results are expressed as a percentage of the mean stiffness measurement made for 10 cells in Hepes-buffered (10 mM, pH 7.2) Hanks' balanced salt solution at the start of each experiment.

## Results

### Structure of the Avian Cochlea

The structure of the avian cochlear duct is illustrated schematically in Fig. 1 as an introduction to the following results and as a guide for those unfamiliar with the anatomy of the chicken inner ear. The duct is shown as obtained from early postnatal chicks. It is a hollow, blind-ending tube  $\sim 4$  mm



**Figure 3.** Lagena macular (*a-c*) and basilar papillar (*d-f*) sections double labeled with phalloidin and polyclonal anti-lagena serum absorbed with cochlear ganglion, tegmentum vasculosum, small intestine, tectorial membrane, and otoliths. *a* and *d* reveal antibody binding sites through the fluorescein channel, *b* and *e* show F-actin distribution through the rhodamine channel, and *c* and *f* provide the corresponding phase-contrast images. The three arrows in *a-c* all point to the same lagena macular hair cell, and the three arrows in *d-f* all point to the same basilar papillar hair cell. Note the prominent cuticular plates (*arrowheads*) lying beneath the hair cell stereocilia bundles in the basilar papilla. *tm*, tectorial membrane. Bar, 20  $\mu$ m.

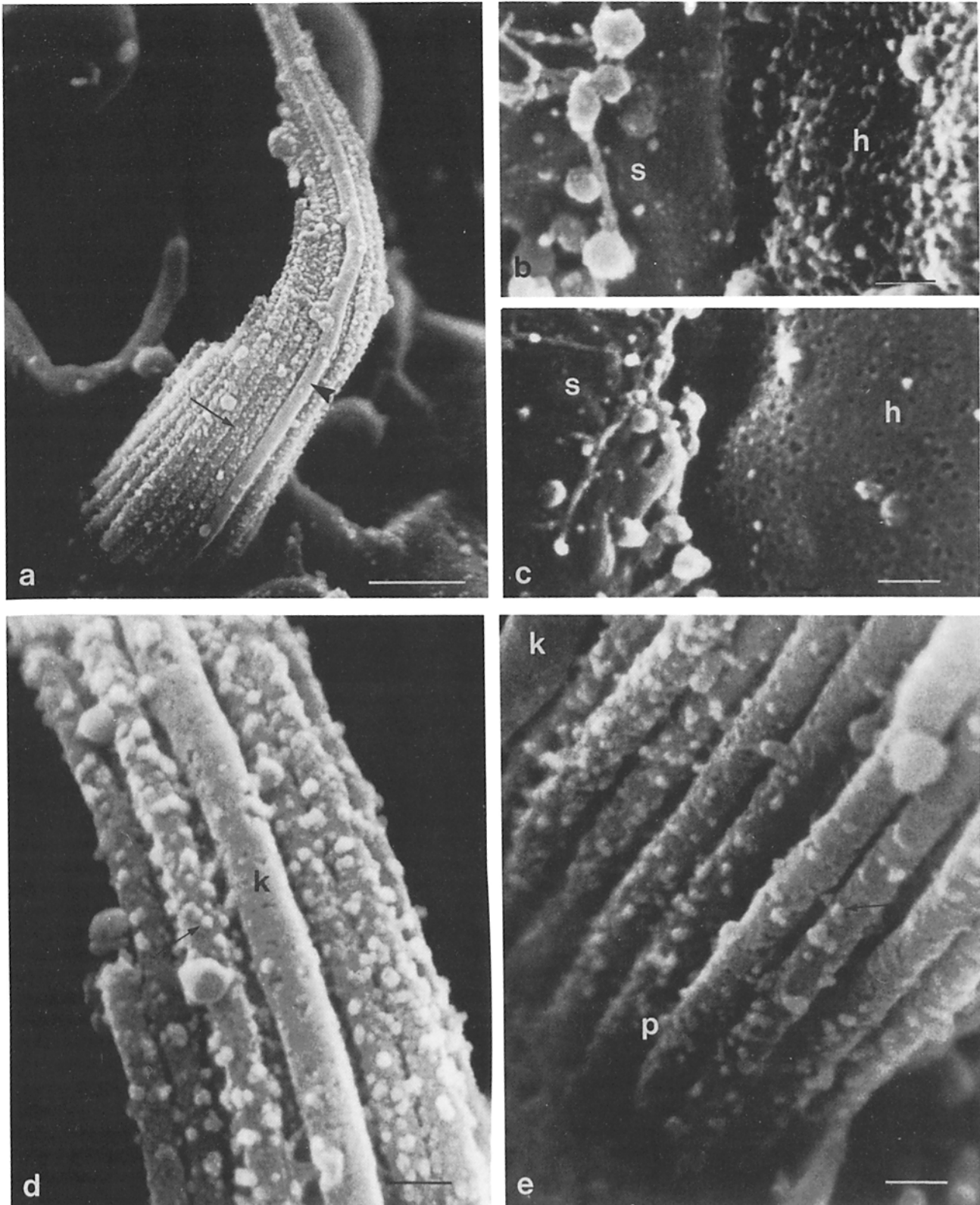
long. The blind, distal end houses the lagena macula, a gravity detector containing vestibular hair cells, which are covered by a pad of extracellular matrix, the otolithic membrane, on top of which calcium carbonate crystals, the otoliths, are embedded. The dorsal surface of the duct is formed by the basilar membrane on which the basilar papilla, an auditory receptor, sits. The basilar papilla contains auditory hair cells and is covered by a strip of extracellular matrix known as the tectorial membrane. The epithelial wall of the lower, ventral surface is highly pleated and forms the tegmentum vasculosum, a structure responsible for maintaining the unique ionic composition of the extracellular fluid that fills the duct. The hair cells in both the basilar papilla and the lagena macula are innervated by neurons located in the cochlear ganglion.

#### **Preparation of Polyclonal Anti-Hair Cell Sera**

Absorbing anti-lagena macula sera with tegmentum vasculosum and cochlear ganglion tissue provides antibodies that show considerable specificity for the apical ends of hair cells within the inner ear (Fig. 2, *a* and *b*). Nonimmune serum absorbed in a similar manner only gives faint, diffuse staining (Fig. 2 *c*). Although reasonably specific for hair cells, these absorbed anti-lagena macula sera still lightly stain support cells in the epithelium, surrounding cartilaginous tissue, otoliths, and tectorial membrane (Fig. 2 *d*). Such sera also stain structures in other tissues, including the edges of skeletal muscle fibers and brush borders in the small intestine (Fig. 2 *e*). Absorbing the sera further with intestinal tissue provides antibodies that still intensely stain the apical ends of hair cells but do not stain either skeletal muscle, small intestine, or any other cells apart from hair cells in the inner ear (Fig. 2, *f* and *g*). Some staining of the tectorial membrane and the otoliths is still detected after these absorption steps but this can be entirely eliminated without reducing hair cell staining by finally absorbing the sera with a mixture of tectorial membranes and otoliths (e.g., as in Fig. 3, *a* and *d*).

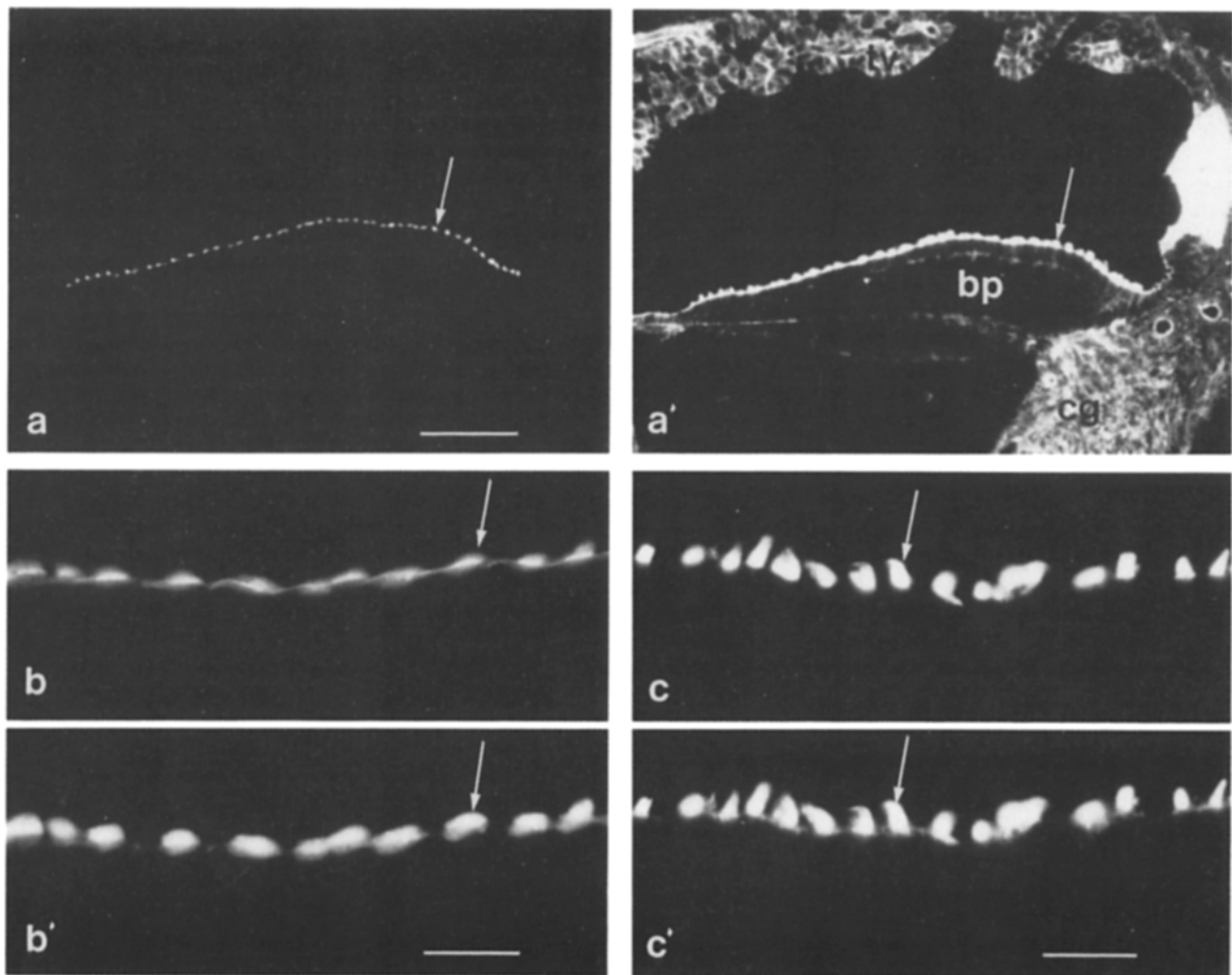
#### **Double Label Studies**

The distribution of staining observed with the absorbed antibodies and the distribution of F-actin revealed by rhodamine phalloidin have been compared in both the lagena macula and the basilar papilla (Fig. 3). In addition to the stereocilia bundles, the basolateral membranes of hair cells, the membranes of supporting cells, and the synaptic layer are all clearly depicted by rhodamine phalloidin staining (Fig. 3, *b* and *e*). In contrast, only the apical ends of the hair cells are stained by the absorbed antibodies. At the apical ends of hair cells in the lagena macula, the entire stereocilia bundle delineated by rhodamine phalloidin is stained by the absorbed antibodies (Fig. 3, *a* and *b*). However, in the distal, low frequency end of the basilar papilla (where the tallest stereocilia in the papilla are found), phalloidin stains the entire stereocilia bundle, but only the proximal region of the bundle nearest its insertion site and the surrounding apical membrane are stained by the antibodies (Fig. 3, *d* and *e*). Cuticular plates lying in the cytoplasm beneath the stereocilia bundles are prominent in the basilar papilla, and this region of the cell is unstained by the antibodies (Fig. 3 *d*). In more proximal, high frequency regions of the basilar papilla where



**Figure 4.** Scanning electron micrographs of inner ear tissues labeled with either absorbed anti-lagena macula serum (*a*, *b*, *d*, and *e*) or mouse nonimmune serum (*c*). (*a*) Apical end of a hair cell in the lagena macula. Arrowhead indicates the kinocilium, and the small arrow points to a group of colloidal gold particles. (*b* and *c*) A comparison between the apical surfaces of hair cells (*h*) and support cells (*s*) after labeling with either (*b*) absorbed immune serum or (*c*) nonimmune serum. Note how the texture of the hair cell surface differs considerably from that of the support cell in the unlabeled control (*c*). (*d* and *e*) A comparison between labeling observed on the stereocilia bundles of (*d*) a lagena macular hair cell and (*e*) a hair cell in the distal region of the basilar papilla. *k*, kinocilium, *p*, proximal region of the stereocilia bundle. The small arrows point to individual colloidal gold particles. In *e* note how the gold particle density decreases with distance from the apical surface of the hair cell. Bars: (*a*) 1  $\mu\text{m}$ ; (*b*–*e*) 0.1  $\mu\text{m}$ .





**Figure 5.** (a and a') Section through cochlear duct of an embryonic day 16 chick embryo double stained with monoclonal anti-hair cell antibodies (a) and rhodamine-conjugated phalloidin (a'). The arrows point to the same cell in each panel. *bp*, basilar papilla; *cg*, cochlear ganglion; *tv*, tegmentum vasculosum. (b and c). Hair cells in the proximal region of the basilar papilla (b and b') and the lagena macula (c and c') of the hatchling double stained with monoclonal anti-hair cell antibodies (b and c) and phalloidin (b' and c'). Arrows in b and b' and c and c' point to the same cells. Bars: (a and a') 100  $\mu$ m; (b and b') 20  $\mu$ m; (c and c') 20  $\mu$ m.

the stereocilia are considerably shorter, the difference between the antibody and F-actin staining patterns still can be detected (see Fig. 5 b), although it is not as marked as that seen toward the distal, low frequency end of the papilla.

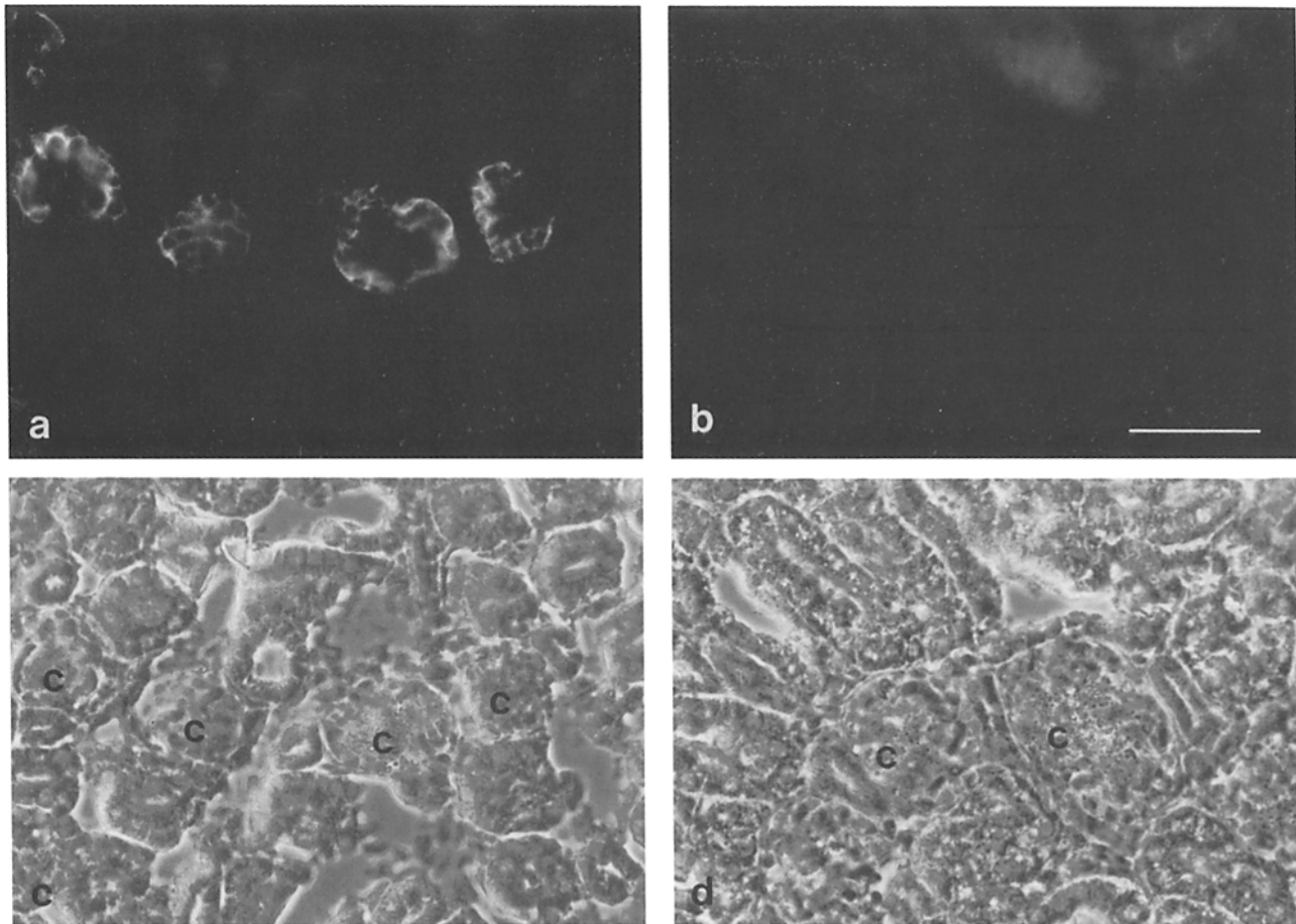
### Immunoelectron Microscopy

The distribution of absorbed antibody binding sites on the apical surfaces of hair cells has been examined with the scanning electron microscope using gold-labeled secondary antibodies. With the absorbed antibodies in the lagena macula, gold particles can be clearly resolved distributed up the entire length of the stereocilia bundle (Fig. 4 a). The apical membrane of the hair cell surrounding the insertion site of the stereocilia bundle is also labeled by the absorbed antibodies but not by nonimmune serum (Fig. 4, b and c). The kinocilium (Fig. 4, a, d, and e) appears not to be labeled. A comparison of gold particle distribution on hair cells stained with absorbed antibodies in the lagena macula (Fig. 4 d) with those in the distal region of the basilar papilla (Fig.

4 e) confirms the results obtained using light microscopy. Particles appear to be evenly distributed up the length of the lagena macular hair cell bundle but, in the distal region of the basilar papilla, decrease in density with distance from the insertion point of the stereocilia on the apical surface.

### Monoclonal Antibody

One of the mice that was producing a polyclonal anti-lagena macula serum that could be made hair cell specific by absorption was used to prepare monoclonal antibodies. Staining patterns observed within the inner ear with the monoclonal antibody at the light microscope level were identical to those described above for the absorbed, polyclonal serum (Fig. 5, a-c). The difference with respect to bundle staining observed between the basilar papilla and the lagena macula with the absorbed antibodies is also seen with the monoclonal antibody (Fig. 5, b and c). Evidence is presented below showing that the absorbed polyclonal sera and the monoclonal antibody all recognize the same antigen. The clone se-



**Figure 6.** Sections of hatchling kidney stained with (a) monoclonal anti-hair cell antibodies and (b) nonimmune mouse IgG. Photographic exposure times for the negatives and the prints in a and b were the same. Micrographs in c and d are phase-contrast images of a and b, respectively. c, renal corpuscle of *Malpighia*. Bar, 50  $\mu\text{m}$ .

cretes an IgG<sub>1</sub> type immunoglobulin, and the culture supernatants average from 40 to 50  $\mu\text{g/ml}$  immunoglobulin and can be used in immunofluorescence experiments at dilutions of up to 1:10,000.

#### **Staining of Other Tissues**

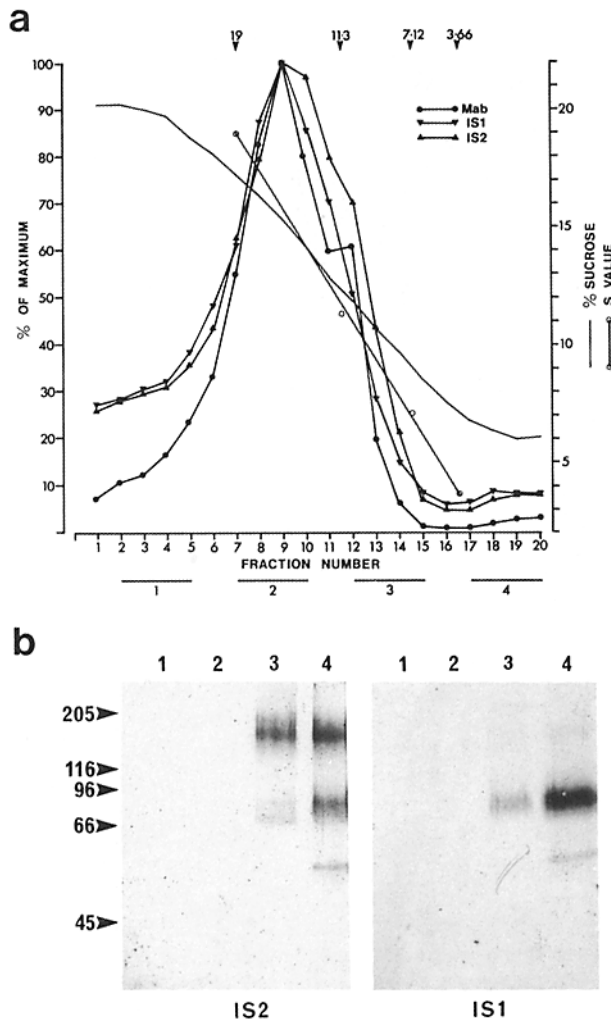
Sections of brain, liver, lung, gizzard, small intestine, skeletal muscle, skin, kidney, and heart of 1–2-d-old chicks were screened with absorbed antibodies. With eight of these tissues, no staining was detected in any cell type. Some very faint staining is observed in the kidney and this is specifically restricted to the renal corpuscles of *Malpighia* (not shown). Using the monoclonal antibody, a total of 12 different tissues, tongue, retina, and crop in addition to those listed above, have been screened. As with the polyclonal sera, the only organ in which staining can be detected is the kidney. The staining observed in the kidney is of considerably higher intensity than that observed with the absorbed polyclonal antibodies, and, as with these sera, staining with the monoclonal antibody is restricted to the renal corpuscles (Fig. 6). The staining in the renal corpuscles delineates cell boundaries although it can also be slightly granular. The antibodies do not stain the central core of the renal corpuscle, the pole where

the glomerular capillaries enter, or the region adjacent to the distal tubule of the nephron.

#### **Characterization and Identification of the Antigen**

**Neutralization of Staining.** To determine the solubility properties of the antigen(s) recognized by the different antibodies, various fractions prepared from the lagena macula were tested to identify those which neutralize the ability of the antibodies to stain hair cells. The staining properties of the absorbed polyclonal antibodies and the monoclonal antibody are only inhibited by preincubation with the nonionic detergent soluble fraction (not shown). Incubation of aldehyde-fixed sections in either 1.0 or 0.1% (vol/vol) TX-100 alone does not inhibit staining.

**Velocity Sedimentation of the Detergent Soluble Antigen.** The sedimentation behavior of the detergent solubilized antigen on 5–20% sucrose gradients is presented in Fig. 7 a. A major peak of activity is found with a sedimentation coefficient of  $\sim 16\text{S}$  with the two absorbed polyclonals and the monoclonal antibody. The peak is, however, not symmetrical, and a distinct shoulder with a sedimentation coefficient of  $\sim 12\text{S}$  is always observed on the trailing edge of the major peak. Treatment of the detergent soluble fraction with trypsin, followed by velocity sedimentation and subsequent ELISA



**Figure 7.** (a) Analysis of the sedimentation behavior of detergent soluble antigens on 5–20% sucrose gradients. Antigen concentration was determined by ELISA using monoclonal anti-hair cell antibodies (*Mab*) and the two absorbed polyclonal sera (*IS1* and *IS2*). Results are expressed as a percentage of the maximum absorbance reading obtained with each antibody. The remainders of each fraction were subsequently pooled into four main fractions (pools 1–4, as indicated by the horizontal bars below the ordinate) and analyzed by immunoblotting. (b) Nitrocellulose transfers of proteins from the pools 1–4 recovered from the sucrose gradients shown above, separated on 7.5% gels under nonreducing conditions, and stained with absorbed polyclonal sera *IS1* and *IS2*. Arrowheads indicate position of standards, with molecular masses given in kilodaltons.

analysis in the presence of trypsin inhibitor, results in a complete loss of the antigen (not shown). Fractions from the sucrose gradients were pooled into four major fractions, pools 1–4, and these were then analyzed by immunoblotting and tested for their ability to neutralize staining.

**Western Blotting.** No bands could be detected with the monoclonal antibody in any of the four fraction pools from the gradient using Western blots. One of the absorbed polyclonal sera (*IS1*) appears virtually monospecific, staining a major band with a relative molecular mass of 80 kD and a minor component with a relative molecular mass of 55 kD under nonreducing conditions (Fig. 7 b). However, these two polypeptides are only detected at the top of the gradient. The

other absorbed polyclonal serum (*IS2*) stains both these bands and also recognizes a band with a relative molecular mass of 160 kD (Fig. 7 b). None of these three polypeptides are found in the region of the gradient where the peak of the detergent soluble activity detected by the ELISA assay is found.

**Neutralization of Staining with Gradient Fractions.** Only the fraction pool including the 16S region of the sucrose gradient (pool 2; Fig. 7 a) is able to completely neutralize the staining properties of the monoclonal antibody and the two absorbed polyclonal antibodies used in this study (Fig. 8). Pools prepared from higher up the gradient do not inhibit the staining of any of the antibodies.

**Immunoprecipitation.** The results of the immunoprecipitation experiments are shown in Fig. 9. A single major band with a relative molecular mass of 275 kD on 5% gels under reducing conditions is precipitated by the monoclonal antibody from nonionic detergent soluble extracts of the lagena macula. No other bands are detected even on high (12.5%) percentage gels (Fig. 9, lanes 1–4). This 275-kD polypeptide can also be precipitated from the fraction pool including the 16S region of the sucrose gradient using either of the two absorbed polyclonal antibodies or the monoclonal antibody (Fig. 9, lanes 5–9).

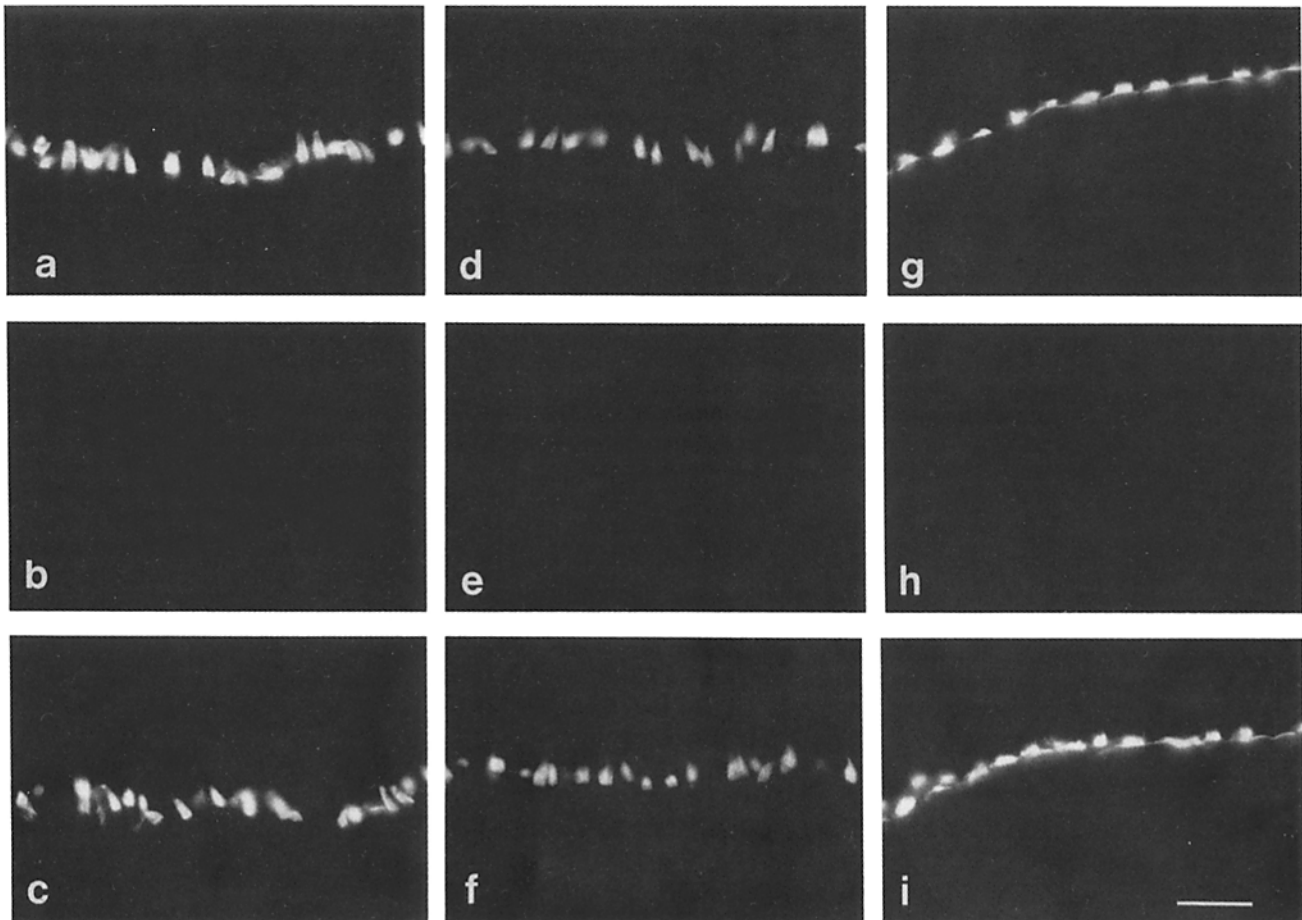
#### **Effects of Antibodies on Stereocilia Bundle Stiffness and Mechanotransduction**

Hair cells in lagena macular cultures are stained by both the monoclonal antibody (Fig. 10 a) and the absorbed polyclonal sera (not shown). The results of five separate experiments in which the effects of the different antibodies on bundle stiffness were tested in vitro are illustrated in Fig. 10 b. The direct addition of unabsorbed polyclonal anti-lagena macula serum causes a 100% increase in the bundle stiffness relative to the preexposure value measured in saline. A nonsignificant decrease of 25% in bundle stiffness is obtained with both absorbed and unabsorbed polyclonal nonimmune sera, and, although the subsequent addition of either absorbed or unabsorbed polyclonal immune sera results in a subsequent 80–90% increase in the stiffness which is significant relative to the value measured in nonimmune serum, the increase is not significant relative to the initial stiffness measured in saline. Both the monoclonal tissue culture supernatant and the purified monoclonal antibodies cause significant (60–75%) increases in stiffness relative to both the initial stiffness measured in saline and the stiffness measured under appropriate control conditions (i.e., in either myeloma-conditioned medium or in saline containing irrelevant mouse IgG). Hair cells in the lagena macular cultures maintain resting membrane potentials of around –45 mV and display a typically rectified, asymmetrical response to sinusoidal stimulation, with deflections of the stereocilia bundle towards the kinocilium producing membrane depolarizations of greater magnitude than the hyperpolarizing responses to deflections in the opposite direction. The unabsorbed polyclonal anti-lagena macula serum does not inhibit mechanotransduction (Fig. 10 c).

#### **Discussion**

The technique of absorbing polyclonal antisera to provide antibodies specific for various structures has been used pre-



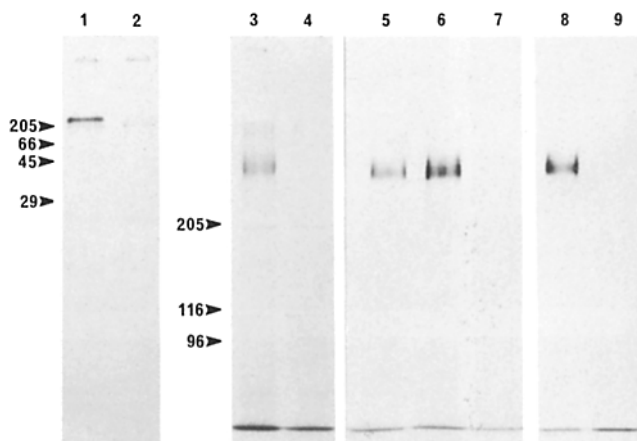


**Figure 8.** Hair cells in the lagena macula (*a-f*) and the basilar papilla (*g-i*) stained with monoclonal anti-hair cell antibodies (*a-c*) and absorbed polyclonal antibodies IS2 (*d-f*) and IS1 (*g-i*). Antibodies were preincubated with either buffer (*a, d, and g*) or detergent soluble lagena macular protein from pools 2 (*b, e, and h*), 3 (*c and f*), or 4 (*i*) from sucrose gradients similar to those shown in Fig. 7 before staining the sections. Note that only pool 2 containing the 16S region of the gradient significantly inhibits the staining. Exposure times for negatives and prints in all panels were the same. Bar, 20  $\mu\text{m}$ .

viously by many investigators: for example, antibodies specific to synaptic basal lamina (Sanes and Hall, 1979) and synaptic vesicles (Carlson and Kelly, 1980; Walker et al., 1982) have been generated by such an approach. Absorbing polyspecific anti-lagena macula sera with tissues that do not contain hair cells provides antibodies that are not just specific for hair cells within the inner ear but are also specific for the apical sensory end of the hair cell. This provides immunological evidence that the apical surface is the most specialized region of the hair cell and also suggests that antigenic components associated with the basolateral membrane are common to other cell types in either the cochlear ganglion, tegmentum vasculosum, or small intestine. A monoclonal antibody has also been obtained that gives a staining pattern at the light microscope level identical to that observed with the absorbed polyclonal sera. The absorbed polyclonal antibodies and the monoclonal antibody all recognize a detergent soluble antigen that behaves as a 16S particle on sucrose gradients and is precipitated by the antibodies from detergent soluble extracts after reductive methylation as a polypeptide with a relative molecular mass of 275 kD on SDS gels under reducing conditions. Although both of the absorbed polyclonals used in this study can be shown to recog-

nize additional proteins using Western blotting, these proteins are not recognized in an ELISA assay and fractions enriched in these proteins do not neutralize the staining capacity of either the absorbed polyclonal sera or the monoclonal antibody. The staining observed with the absorbed polyclonals and the monoclonal presumably reflects the distribution of this 275-kD polypeptide, and, in the following discussion, this will be referred to as the hair cell antigen.

The hair cell antigen can be detected using lightly fixed tissues in the scanning electron microscope, and the antibodies cause increases in stereocilia bundle stiffness when applied to hair cells in culture, indicating that at least one of the epitopes on the antigen is externally orientated on the outer surface of the cell. The antigen is trypsin sensitive, and nonionic detergent is necessary for solubilization, suggesting that it may be an integral membrane protein. The detergent soluble antigen has a sedimentation coefficient of  $\sim 16\text{S}$ . If the particle was roughly spherical, a value of 16S would correspond to a molecular mass of  $\sim 400\text{--}440\text{ kD}$ . The protein precipitated by the antibodies has an apparent relative molecular mass of 275 kD, and therefore the 16S peak observed on the sucrose gradients may result from an interaction of this polypeptide with either itself, another unlabeled polypeptide, or



**Figure 9.** Fluorographs of immunoprecipitates separated on 12.5% (lanes 1 and 2) and 5% (lanes 3–9) polyacrylamide gels under reducing conditions. (Lanes 1–4) Precipitates from a detergent soluble fraction of the lagena macula obtained with monoclonal anti-hair cell antibodies (lanes 1 and 3) and irrelevant mouse IgG (lanes 2 and 4). (Lanes 5–9) Radiolabeled detergent soluble lagena macular proteins were separated on 5–20% sucrose gradients, and the fraction pool containing the 16S region of the sucrose gradient (pool 2 in Fig. 7) was used for immunoprecipitations. The immunoprecipitates obtained with absorbed polyclonal antibodies IS2 (lane 5) and IS1 (lane 6), nonimmune serum (lane 7), monoclonal anti-hair cell antibody (lane 8), and irrelevant mouse IgG (lane 9) are shown. Arrowheads indicate position of standards with molecular masses given in kilodaltons.

the detergent. The 12S shoulder observed in the sucrose gradient profiles would correspond fairly closely to a globular protein in the 260–275-kD molecular mass range. The possibility that the 275-kD polypeptide is a product of limited proteolysis cannot be excluded.

No obvious morphological or histochemical features of the apical ends of hair cells in the avian inner ear have been described that correlate with the observed distribution of the hair cell antigen. Although there are obvious differences between vestibular and auditory organs in the form of the stereocilia bundle, these do not account for the difference in antigen distribution observed between the lagena macula and the basilar papilla.

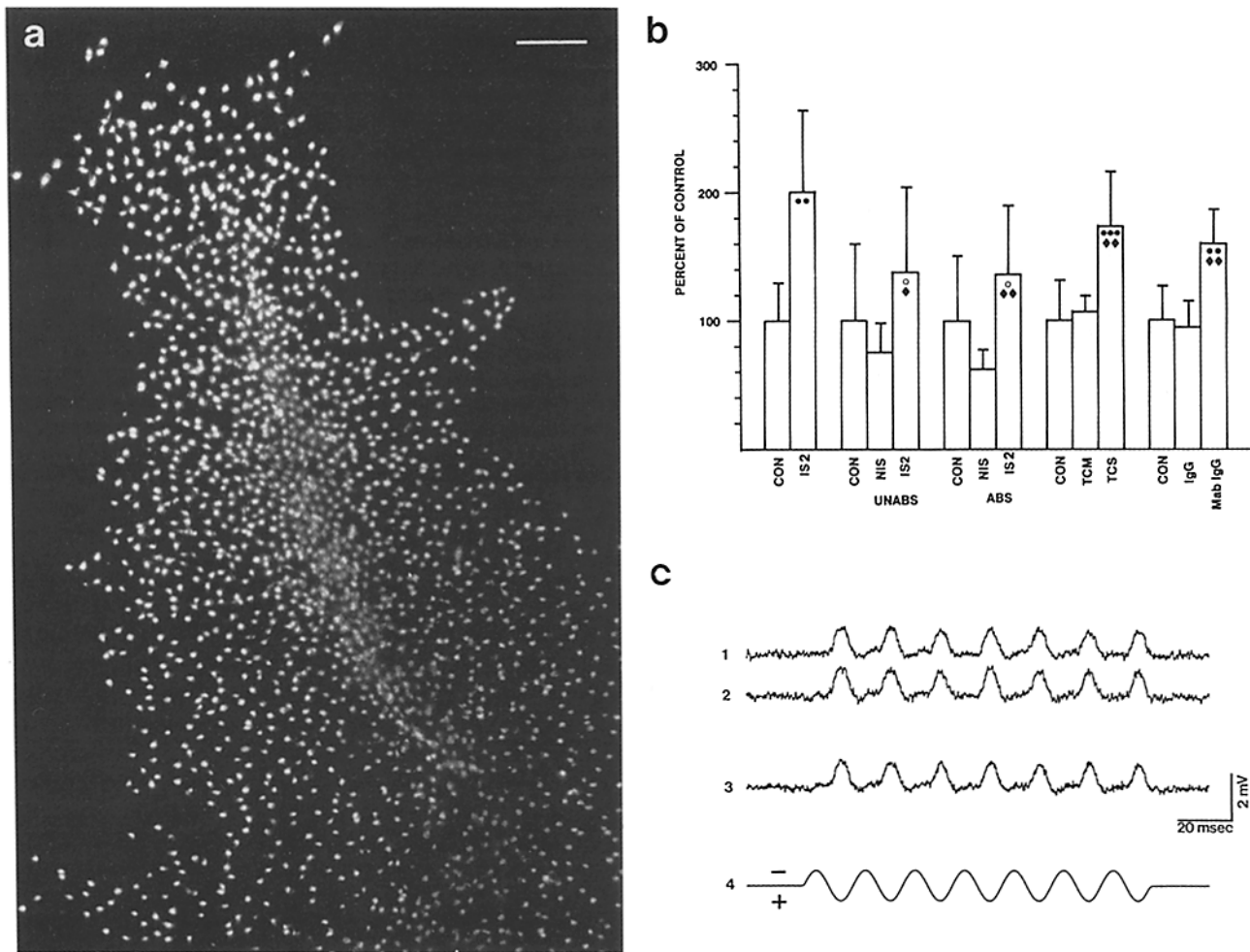
Although specific for the apical ends of hair cells within the inner ear, the absorbed polyclonal sera and the monoclonal antibody also specifically stain the renal corpuscles of *Malpighia* in the kidney. The anti-hair cell antibodies do not stain the central core of the corpuscle where the mesangial cells are located, and the cell type stained in the renal corpuscle is probably the podocyte; however, transmission immunoelectron microscopy is required to verify this suggestion. The apical surfaces of hair cells can be stained by Alcian blue, colloidal iron, and ruthenium red (Santi and Anderson, 1986, 1987; Prieto and Merchan, 1987; Lim, 1986) as can the podocyte (Mohos and Skoza, 1969; Michael et al., 1970; Latta et al., 1975). One of the components responsible for this staining property of the podocyte is likely to be the major sialoprotein of the podocyte membrane, podocalyxin (Kerjaszki et al., 1984). However, it seems unlikely that the anti-hair cell antibodies recognize podocalyxin because podocalyxin antibodies, unlike the anti-hair cell

antibodies, are not specific for renal corpuscles within the kidney and also stain endothelial cells in a number of other different tissues (Kerjaszki et al., 1984). Also, although the avian kidney antigen recognized by the anti-hair cell antibodies has not yet been identified, the molecular mass of mammalian podocalyxin (140 kD) is considerably different from that of the hair cell antigen.

In view of the known, unique susceptibility of sensory hair cells and cells of the kidney nephron to aminoglycoside antibiotics (Hawkins, 1976), it is interesting to demonstrate an immunological identity between the kidney and the inner ear. However, the cells involved in aminoglycoside-induced nephrotoxicity are almost certainly the proximal tubule cells (Kaloyanides and Pastoria-Munoz, 1980) and not those of the renal corpuscle. Unless the effects of aminoglycosides on proximal tubule cells result from a primary lesion at the level of the podocyte, it seems unlikely that the epitope recognized by the anti-hair cell antibody is involved in aminoglycoside toxicity. However, podocyte morphology can be significantly altered by aminonucleoside antibiotics (Ryan and Karnovsky, 1975; Caulfield et al., 1976), as can hair cell morphology by aminoglycoside antibiotics, and it would be interesting to see if aminonucleosides have any effects on hair cells.

The mechanotransducer channels are components of the hair cell that are probably exclusively located on the apical surface, but experimental evidence is contradictory, suggesting that they are either located at the tips of the stereocilia (Hudspeth, 1982) or around their bases (Ohmori, 1988). The unabsorbed polyclonal anti-lagena macula sera do not appear to block transduction, but it is not yet known if any of the antibodies affect either the kinetics or the dynamic range of the transducer channel. Voltage- and patch-clamp techniques are required to investigate in more detail whether the antibodies have any effect on the mechanotransducer channel.

Both poly- and monoclonal antibodies increase the steady-state stiffness of the lagena macular hair cell bundle. The increases caused by the unabsorbed polyclonals, the absorbed polyclonals, and the monoclonal are all of the same order of magnitude, raising the possibility that there is only one antigen involved in this response. The stiffness increase may result from a direct effect of the antibody on the mechanical properties of the bundle or from an indirect effect via a second messenger system. Increases in the steady-state stiffness of mammalian cochlear hair cell bundles can be elicited by the aminoglycoside antibiotic neomycin sulphate, the ionophore A23187, and various lectins (Richardson et al., 1989; Kössl et al., 1990), and it has been suggested that the lectin Con A increases bundle stiffness by reducing the ability of the individual stereocilia within a bundle to slide freely relative to one another. Antibodies against the stereocilia membrane may increase stiffness via a similar mechanism: i.e., by increasing interstereocilia cross-linking and decreasing free shear. The effects of monovalent Fab fragments would provide some evidence for this possibility, but other mechanisms, such as antibody binding causing an increase in intracellular free calcium, may also be operating, and the effects that antibodies may have on stiffness in either calcium-free medium or in the presence of inhibitors of intracellular calcium release also require investigation.



**Figure 10.** (a) 7-d-old lagena macular culture stained with monoclonal anti-hair cell antibodies. (b) Effects of various anti-hair cell antibody preparations and appropriate controls on the steady-state stiffness of stereocilia bundles in 1-d-old cultures of the lagena macula. Values are expressed as a percentage of the mean value obtained in saline (CON) before the addition of the sample to be tested. IS2, polyclonal anti-lagena macula immune serum (1:100 dilution); NIS, mouse nonimmune serum (1:100 dilution); UNABS, unabsorbed; ABS, absorbed; TCM, myeloma-conditioned tissue culture medium; TCS, tissue culture supernatant from hybridoma clone secreting anti-hair cell antibodies; IgG, irrelevant mouse IgG (100  $\mu\text{g}/\text{ml}$ ), Mab IgG, IgG purified from monoclonal tissue culture supernatant (50  $\mu\text{g}/\text{ml}$ ). The standard deviation of the mean ( $n = 10$  for each value) is indicated. Levels of significance in an unpaired  $t$  test are indicated by the symbols in the bars. Circles indicate significance levels for the mean value tested against the preexposure saline control (○ not significant; ●●  $p < 0.01$ ; ●●●  $p < 0.0001$ ) and diamonds indicate significance levels for the mean value tested against the appropriate control (i.e., mouse nonimmune serum, myeloma-conditioned tissue culture medium, irrelevant mouse IgG) (◆  $p = 0.01$ ; ◆◆  $p < 0.01$ ). (c) Effects of unabsorbed polyclonal anti-lagena macula serum on mechanotransduction in 1-d-old cultures of the lagena macula. (1) Response of a cell to 144-nm peak-to-peak amplitude sinusoidal displacement of the stereocilia bundle. (2) Response of the same cell to the same stimulus 3 min after the addition of serum to a final concentration of 1:100. (3) Response of another cell to the same stimulus 30 min after the addition of serum. (4) The stimulus waveform. Downward deflection (+) represents displacement directed towards the kinocilium; upward deflection (−) represents displacement directed away from the kinocilium. The cell in traces 1 and 2 had a resting membrane potential of  $-45$  mV, and the cell in trace 3 had a resting membrane potential of  $-47$  mV.

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