Sorting of actin isoforms in chicken auditory hair cells

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SUMMARY

Most nonmuscle cells of higher vertebrates contain two different actin isoforms, β- and γ-cytoplasmic actin. The β-isoform is with few exceptions the predominant isoform in nonmuscle cells and tissues. Perturbation of the β:γ ratio has been shown to affect the organization of bundled actin filaments indicating that the β- and γ-genes encode functionally distinct cytoarchitectural information. In the present study we localized by immunostaining β- and γ-actin in chicken auditory hair cells. These highly specialized cells serve as model system for studying certain developmental and structural aspects of a complex actin filament system with high architectural precision. We show that γ-actin is the predominant actin isoform in auditory hair cells with an apparent β:γ ratio of ~1:2. γ-Actin is not sorted and occurs in all three actin assemblies of the hair border, i.e. the cores of sensory hairs (stereocilia), the subjacent gel-like actin filament meshwork (cuticular plate) and the zonula adherens ring. In contrast to γ-actin, the β-isoform is specifically sorted to the actin filament core bundle of stereocilia that is extensively crosslinked by fimbrin. In view of recent studies showing that L-plastin, the leukocyte homolog of fimbrin, has a higher binding affinity for β-actin than for γ-actin, a mechanism is proposed for how hair cells might restrict formation of actin filament bundles to a single cellular site (i.e. the stereocilia). The limited level of expression of β-actin in hair cells may help to prevent ectopic bundle formation in other cellular compartments.

Key words: Actin isoform, Hair cell, Stereocilium

INTRODUCTION

Actin is one of the most abundant proteins of nucleated cells where it is centrally involved in diverse cellular functions such as cell motility, contraction, cellular adhesion, and control of cellular shape (Pollard and Cooper, 1986). Actin monomers (G-actin) can polymerize into filaments (F-actin) that form a striking diversity of assemblies reaching from highly ordered contractile fibrils (e.g. myofibrils of muscle) to densely packed noncontractile bundles (e.g. core bundles of microvilli) and gel-like formations (e.g. in the cortex of leucocytes and amoeba). The generation of this structural and functional diversity of the actin filament system can be partly explained by association of actin filaments with different actin-binding proteins, although it is still not clear how different actin-binding proteins are selectively targeted to different actin assemblies within the cell. Also involved in this structural diversity of the actin filament system appear to be the six different isoforms of actin that are expressed in vertebrates (Vandekerckhove and Weber, 1978; Hamada et al., 1982; Gunning et al., 1983; Ng et al., 1985; Erba et al., 1988). According to their isoelectric point (pI) these isoforms are separated into three α-isoforms (pI 5.40), one β-isoform (pI 5.42), and two γ-isoforms (pI 5.44) (Garrels and Gibson, 1976). All α-isoforms and one γ-isoform are restricted to muscle, whereas nonmuscle cells contain the 'cytoplasmic' β- and γ-isoforms (Otey et al., 1986, 1988; Erba et al., 1988; Paterson and Eldrigde, 1984; Rubenstein, 1990; Herman, 1993). Chicken contain a further nonmuscle isoform of actin, type 5

actin (Bergsma et al., 1985). Sorting of actin isoforms has been rather well studied in striated muscle where α-isoforms form the myofibrils, whereas cytoplasmic isoforms are confined to the subplasmalemmal space (Craig and Pardo, 1983; Hall et al., 1981; Otey et al., 1988). In smooth muscle, cytoplasmic actin (β-actin) is also excluded from the contractile actomyosin system (North et al., 1994). Little is known about sorting of the two cytoplasmic actins (β, γ) in nonmuscle cells (Otey et al., 1986). In a variety of cultured cells, β- and γ-actin are associated with all kinds of microfilament structures developed in these cells, however, with a tendency of β-actin to become preferentially concentrated at the plasmalemmal domains involved in cellular migration (lamellae, filopodia) suggesting a role for β-actin in certain aspects of cellular motility (DeNofrio et al., 1989; Hoock et al., 1991). No information is available on isoactin sorting in differentiated nonmuscle cells in situ and it is unclear up to now why most nonmuscle cells express two different actin isoforms at the same time.

Clues to the structural and functional role of the two cytoplasmic actin isoforms (β, γ) may come from studies on the subcellular distribution of these isoforms. To pursue this aim we chose auditory hair cells (Hudspeth, 1989) of the chicken cochlea as a cellular model system. Auditory hair cells have been previously shown to contain in their apical cell pole a highly ordered actin filament cytoskeleton that consists of three different actin filament assemblies in close proximity (Flock et al., 1981, Drenckhahn et al., 1991). These are: (i) bundles of extensively crosslinked actin filaments with uniform polarities that serve as internal supporting scaffold of the stiff rod-shaped

mechanosensory projections named stereocilia (Tilney et al., 1983); (ii) a dense gel-like network of actin filaments denoted as cuticular plate (DeRosier and Tilney, 1989) into which actin filaments of the stereocilium bundle are basally inserted; and (iii) a circumferential array of actin filaments with antiparallel alignment that is laterally attached to the belt-like intercellular adhesion junction (zonula adherens bundle) (Hirokawa and Tilney, 1982). Each of these three different assemblies of actin filaments appears to be associated with only one specific actin crosslinking protein, i.e. the stereocilium bundle with fimbrin, the cuticular plate with spectrin (fodrin) and the zonula adherens ring with α-actinin (Drenckhahn et al., 1991). It is presently not known how fimbrin, fodrin and α-actinin are targeted to only one of these actin filament assemblies. One possibility would be that actin isoforms may participate in this sorting process.

MATERIALS AND METHODS

Immunofluorescence, antibodies

Young chickens (White Leghorn) of 1-14 days of age were decapitated. The cochlea duct was removed as described by Tilney et al. (1989) and immediately quick frozen by plunging it into melting isopentane cooled with liquid nitrogen. The frozen tissue samples were carefully freeze-dried for 12 hours at −40°C and 10^{-5} Torr, and were then immersed for 48 hours at 10^{-3} Torr and 20°C with Epon containing 1.8% (v/v) Epon accelerator. The cochlear duct was transferred to gelatin capsules, oriented and embedded in Epon at 60°C for 24-48 hours. Semithin sections (0.5-1 µm) were mounted on glass coverslips and heated to 80°C for 2 hours. Etching and partial removal of the resin was performed at room temperature (RT) by placing the coverslips for 5 minutes in a 1:1 mixture of methanol containing 10% sodium methoxide, which was prepared from metallic sodium as described by Major et al. (1961). Afterwards, sections were rinsed with a 1:1 mixture of methanol:toluene (1× 5 minutes), acetone (2× 5 minutes), distilled water (1× 5 minutes), and phosphate-buffered saline (PBS: 10 mM sodium phosphate, 140 mM NaCl, pH 7.4) for 1× 5 minutes as described elsewhere (Drenckhahn et al., 1983; Drenckhahn and Franz, 1986). Immunofluorescence labelling was carried out by overlaying each section for 2 hours at RT with 20 µl of the primary antibodies (1-10 µg/ml). Antibodies were rinsed off the sections with PBS (3× 5 minutes) and the sections were then incubated with secondary antibodies for 30 minutes at RT. As secondary antibodies we used goat anti-rabbit IgG, goat anti-mouse IgG and goat anti-mouse IgM labelled with tetramethylrhodamine isothiocyanate (TRITC), fluorescein isothiocyanate (FITC) and Texas Red, respectively (Dianova, Hamburg, Germany). Afterwards the slides were washed again with PBS (3× 5 minutes) and mounted in 60% glycerol in PBS containing 1.5% n-propyl gallate as antifading compound (Serva, Heidelberg, Germany). Negative controls included omission of the primary antibodies and absorption of the primary antibodies with β-actin purified from human erythrocytes (Tilney and Detmers, 1975) and a mixture of β- and γ-actin obtained from the chicken intestinal brush border.

For double immunofluorescence, sections were incubated for 60 minutes at RT with a mixture of the monoclonal β-actin IgG antibody directed against an N-terminal peptide of β-actin (Sigma, Deisenhofen, Germany) and the polyclonal γ-actin antibody. As secondary antibodies a mixture was used containing FITC-labelled goat anti-rabbit and Texas Red-labelled goat anti-mouse IgG (the final dilution of both antibodies was 1:50 in PBS). All sections were incubated for 1 hour at RT with secondary antibodies. The polyclonal γ-actin antibody directed against an N-terminal 15 amino acid sequence of γ-actin was a kind gift from Dr J. C. Bulinski (for specificity of this antibody see Otey et al., 1986). For the visualization of all actin assemblies in hair cells we used a monoclonal pan-actin IgM antibody reacting with all actin isoforms (ProGen, Heidelberg, Germany).

Immunoelectron microscopy

For the ultrastructural localization of actin isoforms cochleae of 14-day-old chicken were fixed at 4°C by immersion with a mixture of 0.1% glutaraldehyde and 2% formaldehyde in PBS overnight. After fixation, tissue sections were treated for 15 minutes with 0.5 mg/ml sodium borohydride in PBS and embedded in the hydrophilic methacrylate resin LR-White (London Resin Co., Woking, UK). Ultrathin tissue sections were collected on gold grids and processed for immunogold labelling with the antibody directed against γ-actin exactly as described in previously published studies (Drenckhahn and Merte, 1987; Drenckhahn and Dermietzel, 1988). Goat anti-rabbit IgG coupled to colloidal gold particles (10 nm in diameter) were purchased from Janssen Pharmaceutica (Beerse, Belgium). For double immunolabelling a mixture of the monoclonal anti-β-actin and the polyclonal anti-γ-actin was used at dilutions of 1:400 and 1:200, respectively. Bound immunoglobulins were then detected by a mixture of goat anti-rabbit IgG coupled to 15 nm colloidal gold particles and goat anti-mouse IgG adsorbed to 6 nm gold particles (Biotrend, Köln, Germany).
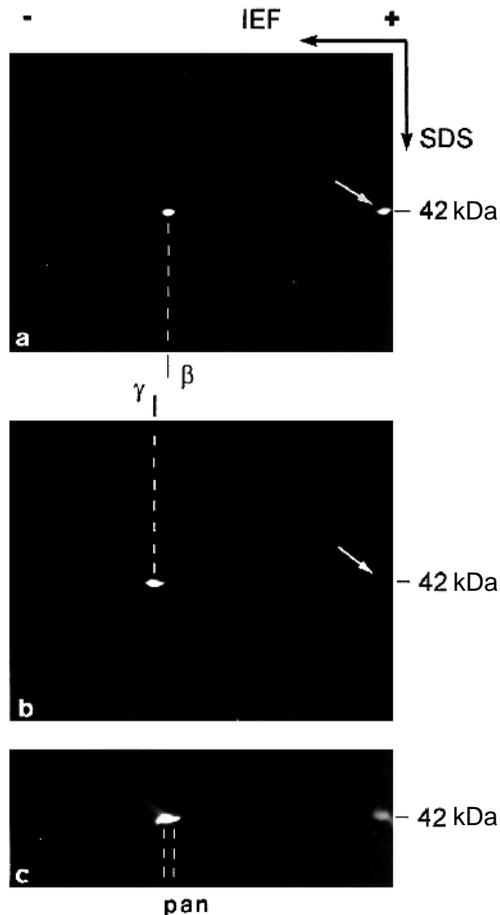
Two-dimensional electrophoresis and immunoblotting

For 2-D gel electrophoresis, the cochlear duct was opened and sensory epithelia (basilar papilla) were isolated as described in detail elsewhere (Tilney et al., 1989). Isolated sensory epithelia from which the tectorial membrane had been removed by 30 minutes incubation at RT with dispase (5 mg/ml) (Boehringer, Mannheim GmbH, Mannheim, Germany) were solubilized in 9.8 M urea, 100 mM DTT, 2% (w/v) NP-40, and 2% ampholytes (pH 4-6: Ampholytes, Serva, Heidelberg, Germany) and then focused to their isoelectric point in urea-acrylamide gels containing 4% ampholyte (75% ampholytes 4-6, 25% ampholytes 3-7). Isoelectric focusing of first-dimension tube gels was performed essentially according to the method of O'Farrell (1975) with the modification of Celis et al. (1992) using the Mini Protean 2-D system of Bio-Rad (Richmond, USA). Tube gels were then mounted on top of 10% SDS-polyacrylamide minislab gels and the second dimension gels were run using the buffer system of Laemmli (1970). Western blotting of polyacrylamide gels onto nitrocellulose sheets (Schleicher & Schüll Inc., Darmstadt, Germany) was carried out according to the method of Towbin et al. (1979). The monoclonal antibody against β-actin was diluted 1:3,000 and incubated overnight at 4°C. Bound immunoglobulins were visualized with peroxidase-labelled goat anti-mouse IgG by exposure to an X-ray film using the enhanced chemiluminescence technique (ECL, Amersham, Braunschweig, Germany). The monoclonal β-actin antibody was then removed by incubation of the nitrocellulose sheets with 100 mM DTT at 56°C for 30 minutes according to the reprobing procedure recommended by Amersham. Afterwards, the nitrocellulose sheets were incubated with the polyclonal γ-actin antibody (diluted 1:5,000) for 12 hours at 4°C. Bound immunoglobulins were detected with peroxidase-labelled goat anti-rabbit IgG (Bio-Rad) and the ECL technique. Western blotting with the monoclonal pan-actin IgM antibody was exactly performed as described for the β-actin antibody.

RESULTS

Biochemistry

Both, β- and γ-actin were identified in the isolated sensory epithelium of the chicken cochlea by 2-D gel electrophoresis and subsequent immunoblotting. The protein spot identified by the monoclonal β-actin antibody was in the expected pI range (~5.42) and showed the same electrophoretic mobility in the

Fig. 1. Identification of β- and γ-actin in the isolated sensory epithelium of the chicken cochlea by 2-D gel electrophoresis and subsequent immunoblotting. The monoclonal β-actin antibody (a) identifies a single protein spot within the expected pI range for β-actin (~5.42) that showed exactly the same electrophoretic mobility as β-actin of human erythrocyte membranes (42 kDa; arrow in a). Reprobing of the same nitrocellulose sheet with the polyclonal antibody directed against γ-actin (b) resulted in a larger, more basic protein spot lying immediately adjacent to the former β-actin signal. The γ-actin antibody did not react with the β-actin band of the erythrocyte membrane (arrow in b). The pan-actin antibody detected β- and γ-actin (c, separate gel), but no further actin spots.

that β- and γ-actin are the only actin isoforms present in the chicken sensory epithelium.

Immunofluorescence

Semithin sections of the cochlea of 1- to 10-day-old chicken incubated with the pan-actin antibody (monoclonal IgM) resulted in a bright staining of all three assemblies of actin filaments in the apical portion of hair cells, i.e. the apical bundle of stereocilia, the cuticular plate and the area of the zonula adherens (Fig. 2d). In contrast, the β-actin antibody (monoclonal IgG) labelled only the stereocilia and small dots in the area of the zonula adherens junctions between hair cells and supporting cells (Fig. 2a). The cuticular plate remained completely unlabelled. This pattern was further confirmed by labelling of serial sections with the pan-actin antibody (IgM) and the β-actin antibody (IgG) again showing that the β-actin antibody does not stain the cuticular plate (Fig. 2c). The staining intensity at the zonula adherens level was more intensively labelled with the pan-actin than with the β-actin antibody. The γ-actin antibody resulted in a similar staining pattern as obtained with the pan-actin antibody indicating that γ-actin is present in all three assemblies of actin filaments (Fig. 2b).

Immunoelectron microscopy

Ultrathin sections of the chicken cochlea labelled with the polyclonal γ-actin antibody and 10 nm immunogold particles resulted in a virtually identical staining pattern as described recently by us for the pan-actin antibody, i.e. strong labelling

second dimension (~42 kDa) as β-actin of human erythrocytes loaded on a separate lane of the same slab gel (Fig. 1a). After removal of the bound immunoglobulins and reprobing of the nitrocellulose sheets with the polyclonal γ-actin antibody a larger, more basic protein spot was identified immediately adjacent to the former β-actin spot (Fig. 1b). The β-actin band of erythrocyte membranes did not react with the γ-actin antibody. The pan-actin antibody identified both the β- and γ-spot (Fig. 1c) but no further protein spots of the gel showing

Fig. 2. Semithin sections of the chicken auditory epithelium immunolabelled for β-actin (A,C), γ-actin (B), and pan-actin (D). (A and B) The same section double-immunolabelled for β- and γ-actin. (C and D) Serial 0.5 μm thick sections. Note restriction of β-actin (A,C) to the stereocilia (S) and the area of the zonula adherens junctions (small arrows), but absence from the cuticular plate (CP). Antibodies to γ-actin (B) and pan-actin (D) label all three assemblies of actin filaments, the zonula adherens (small arrows), the cuticular plate (CP) and the stereocilia (S). The left three hair cells in C and D are cut through planes of the cuticular plates outside of the stereocilium bundle. Bar, 10 μm.
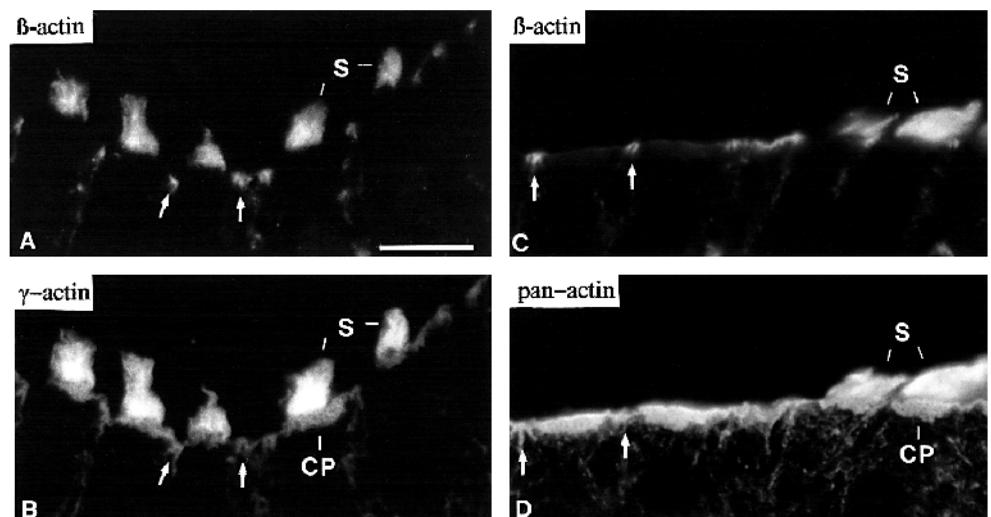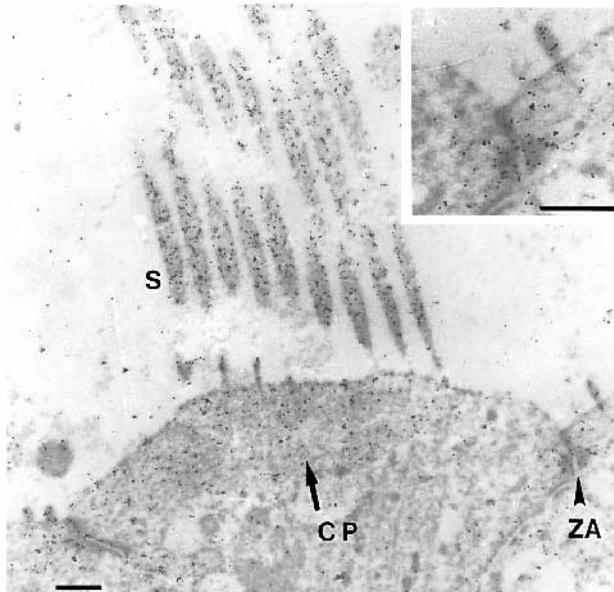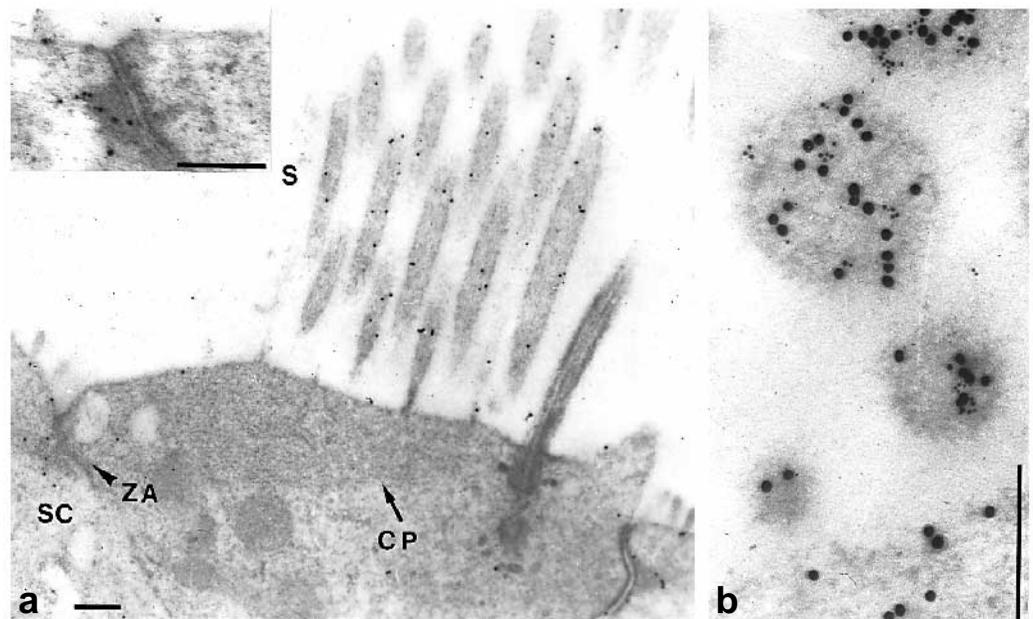
Fig. 3. Electron micrograph of a hair cell apex incubated with the polyclonal γ-actin antibody and 10 nm immunogold particles. Note the dense label overlying the three actin assemblies of hair cells, the stereocilia (S), the cuticular plate (CP) and the zonula adherens (ZA). The inset shows the zonula adherens at higher magnification. The γ-actin label is associated with both the hair cells side and the supporting cell side of the adherens junctions. Bar, 0.2 µm.

of the stereocilia, the cuticular plate and the zonula adherens plaques where the antibody labelled both the hair cell side and the supporting cell side of these junctions (Fig. 3). In contrast, the monoclonal β-actin antibody labelled only the stereocilia but not the cuticular plate and also not the hair cell side of the zonula adherens (Fig. 4a). The dotted stain for β-actin observed in the zonula adherens region at the light microscope level turned out to be caused by labelling of the zonula adherens plaques of only the supporting cells but not the hair cells. A

similar labelling pattern was previously observed by us for myosin II, which we found to be absent from the hair cell side of the adherens junctions but present on the supporting cell side of the junctions (Drenckhahn et al., 1991). Simultaneous labelling of ultrathin sections with the monoclonal β-actin antibody (6 nm gold particles) and the polyclonal γ-antibody (15 nm gold particles) showed coexistence of both actin isoforms in the stereocilia (Fig. 4b). No preferential location of either actin isoform was observed in cross-sectional profiles of the stereocilia.

DISCUSSION

Actin isoforms in hair cells

Recently published data obtained by reverse transcription polymerase chain reaction (RT-PCR) analysis of actin isoforms in the chicken basilar papilla revealed the exclusive expression of β- and γ-cytoplasmic actin mRNA in the sensory epithelium (Pickles, 1993). No PCR products were obtained for type 5-actin, the third cytoplasmic actin isoform expressed in chicken tissues (Bergsma et al., 1985). In the present study we show at the protein level that β- and γ-actin are present in hair cells as well as supporting cells of the chicken cochlea. Within hair cells, γ-actin turned out to be the ubiquitous actin isoform associated with all three major actin assemblies, i.e. the stereocilia, cuticular plate and zonula adherens ring. On the other hand, β-actin displayed a very restricted distribution in being selectively sorted to the stereocilia and absent from the cuticular plate and the adherens ring.

This impression of a predominance of γ-actin in hair cells is in line with the results obtained by 2-D gel electrophoresis of the isolated sensory epithelium in which the γ-actin spot was always significantly larger then the β-actin spot with an apparent β:γ ratio of ~1:2. This ratio is similar to the β:γ ratio determined for the chicken intestinal brush border of 1:1.6 (Vandekerckhove and Weber, 1981), but differs considerably from various other nonmuscle tissues where β-actin is more abundant with a ratio of 1:1 (rat testis) to 3.4:1 rat liver (Otey et al., 1987).

Fig. 4. Electron micrograph of the apex of hair cells (a) showing the restriction of β-actin immunolabel to stereocilia (S). The cuticular plate (CP) is completely unlabelled. Note also the association of β-actin with the zonula adherens plaques (ZA) of only the supporting cells (SC) but not the hair cell side of the junction (also shown at higher magnification in the inset). (b) High magnification showing a cross-sectional profile of stereocilia simultaneously labelled with the monoclonal β-actin antibody (6 nm gold particles) and the polyclonal γ-actin antibody (15 nm gold particles). There is no preferential location of either actin isoform within the stereocilia. Bar, 0.2 µm.

Do actin isoforms encode cytoarchitectural information?

The β:γ ratio appears to be strictly regulated in cells and perturbation of this ratio may have significant effects not only on the organization of the actin filament system but also on cellular shape and size (Schevzov et al., 1992). Increasing β-actin by transfection of cultured myoblasts resulted in well-defined bundles of actin filaments and a larger spread cellular phenotype, whereas cells transfected with γ-actin displayed a more diffuse organization of actin filaments and a smaller, elongated cell morphology. It is difficult to extrapolate from these in vitro studies to cells in situ. However, it appears noteworthy that in chicken hair cells β-actin is also restricted to the only cellular site (i.e. the stereocilia) where actin filaments are assembled into highly organized bundles, whereas γ-actin is the exclusive actin isoform associated with the zonula adherens ring and the gel-like actin filament meshwork of the cuticular plate. Although the observation of a preferential subcellular location does not necessarily prove that both actin isoforms are functionally distinct, for the following reason we favour the idea that the β- and γ-genes might encode functionally distinct cytoarchitectural information.

Sorting of β-actin and fimbrin to stereocilia

In vitro all actin isoforms polymerize into randomly oriented filaments and do not spontaneously associate into bundles or other kinds of highly organized filament assemblies (Pollard, 1990; Fechheimer and Zigmond, 1993). The assembly into either meshworks or bundles is controlled by a variety of actin crosslinking proteins (Pollard and Cooper, 1986; Hartwig and Kwiatkowski, 1991). In stereocilia and the cuticular plate of chicken hair cells, these crosslinking proteins are fimbrin and fodrin, respectively (Drenckhahn et al., 1991). Fimbrin is restricted to the actin filament bundle of stereocilia and fodrin is confined to the actin meshwork forming the cuticular plate. One way to explain isoform-specific control of filament assembly would be to assume different affinities of certain actin-crosslinking proteins for the different actin isoforms present in a given cell type. With respect to the precise colocalization of the actin-bundling protein fimbrin with β-actin in stereocilia, it is tempting to assume that fimbrin has a significant higher binding affinity for β-actin than for γ-actin. Support for this assumption comes from a recently published study in which L-plastin, the leukocyte homolog of fimbrin, was shown to bind in a 1:10 molar ratio to filamentous β-actin but hardly to γ-actin filaments (Namba et al., 1992). Provided that fimbrin binds also preferentially to β-actin then an affinity-sorting process would be one possible way by which the entire β-actin pool of hair cells might be driven into a bundled state. However, such an affinity-sorting mechanism would not explain why the β-actin-fimbrin bundles form selectively in stereocilia of the apical cell surface and do not occur in other locations of the hair cells.

As β- and γ-actin are probably already expressed in hair cells at stages of differentiation before stereocilia grow out from the apical cell surface, one possibility would be that the tips of the nascent stereocilia preferentially bind and nucleate β-actin or β-actin-binding proteins. Selective targeting of β-actin mRNA (but not γ-actin mRNA) to the plasma membrane, as observed in various cultured cell lines, might be important for such a process (Hoock et al., 1991; Hill and Gunning, 1993;

Kislauskis et al., 1993; Hill et al., 1994). The outgrowing filaments would then capture fimbrin molecules which, in turn, would force the filaments into bundles. Preliminary studies of our laboratory indicate that fimbrin is present in hair cells already at day 8 of development. At this stage stereocilia begin to grow out (Tilney et al., 1992). Another factor that could contribute to sorting of fimbrin into stereocilia might be the density of filaments: if filaments are nucleated close together there may be insufficient room for fodrin but not for fimbrin to crosslink.

Functional implications of low βγ-actin ratio in hair cells

The final size and length of stereocilia may be controlled by the number of fimbrin molecules which might be strictly regulated in hair cells. However, the supply of β-actin is obviously also limited in hair cells. This conclusion can be drawn from our observation that stereocilia contain in addition to β-actin a considerable proportion of γ-actin. Whether β- and γ-actin form mixed polymers or whether both isoforms assemble into separate filaments cannot be decided. There is, however, no preferential location of both isoforms throughout the stereocilia suggesting that β- and γ-actin can coassemble during the formation of stereocilia and are not incorporated at different stages of development. That β-actin is kept at lower levels in hair cells than γ-actin may be functionally important. If, for instance, β-actin would be the predominant actin isoform in hair cells (as is the case in most nonmuscle tissues of the body) it would be difficult to explain why fimbrin is preferentially located to the stereocilia and not present in the other actin assemblies of hair cells, given that fimbrin like L-plastin binds preferentially to β-actin. This consideration may also apply for the intestinal brush border where β-actin is also kept at a lower level than γ-actin (Vandekerckhove and Weber, 1981). A limited expression of β-actin in hair cells might thus help to prevent formation of abnormal actin filament bundles in other locations of the cell. That such ectopic bundles can develop under certain circumstances and may be causally related to pathological conditions is implicated by cytoskeletal abnormalities associated with the shaker-2 mouse and the walzing guinea pig. In both genetic disorders abnormal fimbrin-containing actin filament bundles develop throughout and underneath the cuticular plate of vestibular hair cells (Sobin and Flock, 1981, 1983; Sobin et al., 1982).

Obvious experiments to be performed in the future would be to manipulate the expression of β- and γ-actin in hair cells in the embryonic chicken cochlea in culture and to study in detail the sequence of expression of actin isoforms and actin-binding proteins in chicken hair cells during development.

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