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Formation of two microtubule-nucleating sites which perform differently during centrosomal reorganization in a mouse cochlear epithelial cell

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SUMMARY

This report provides evidence for the formation of a cell surface-associated centrosome with two spatially discrete microtubule-nucleating sites that perform differently; the minus ends of microtubules remain anchored to one site but escape from the other. Centrosomal reorganization in the cells in question, outer pillar cells of the organ of Corti, indicates that its pericentriolar material becomes intimately associated with the plasma membrane at the two nucleating sites.

Two large microtubule bundles assemble in each cell. A beam which includes about 1,300 microtubules spans most of the cell apex. It is positioned at right angles to a pillar with about 4,500 microtubules which is oriented parallel to the cell's longitudinal axis.

The beam's microtubules elongate from, and remain attached to, a centrosomal region with two centrioles which acts as a microtubule-nucleating site. However, the elongating microtubules do not radiate from the immediate vicinity of the centrioles. During beam assembly, the minus ends of the microtubules are concentrated together close to the plasma membrane (less than 0.2 μm away in many cases) at a site which is located to one side of the cell apex.

High concentrations of the pillar's microtubules elongating from one particular site have not been detected. Analyses of pillar assembly indicate that the following sequence of events occurs. Pillar microtubules elongate from an apical cell surface-associated nucleating site, which becomes more distantly separated from the centriolar locality as cell morphogenesis progresses. Microtubules do not accumulate at this apical nucleating site because they escape from it. They migrate down to lower levels in the cell where the mature bundle is finally situated and their plus ends are captured at the cell base.

Key words: centrosome, microtubule, cochlea, mouse

INTRODUCTION

The pericentriolar material of the centrosomal microtubule-organizing centre acts as the main microtubule-nucleating site in most animal tissue cells (see Gould and Borisy, 1977; Bornens and Karsenti, 1984; Brinkley, 1985; Vorobjev and Nadezhdina, 1987; Kalnins, 1992; Kimble and Kuriyama, 1992; Kalt and Schliwa, 1993). However, it has become apparent that centrosomal organization is substantially modified as far as several types of epithelial cells are concerned (Achler et al., 1989; Bacallao et al., 1989; Mogensen et al., 1989, 1993; Bré et al., 1990; Tucker et al., 1992; Henderson et al., 1994). There are substantial indications that much of the pericentriolar material is more intimately associated with the apical cell surface and its plasma membrane than it is with the centrioles. Such centrosomal reorganizations facilitate construction of the microtubule arrays which are an important functional feature of polarised epithelial cells (see Fath et al., 1993; Mays et al., 1994). Microtubules project from the apical surfaces of cells and run parallel to the cells' longitudinal axes (rather than radiating from a typical and centrally positioned centrosome in which pericentriolar material occupies a more or less spherical region around the centrioles). Relatively little is known about the important and versatile modes of centrosomal form and action that are expressed to assist assembly of the wide range of microtubule configurations that are functionally crucial for various types of terminally differentiated cells. This report is concerned with a mammalian epithelial cell which offers valuable opportunities for the investigation of such centrosomal versatility.

Certain epithelial cells in the organ of Corti of a mammalian cochlea possess microtubule bundles which are larger than any others described so far for mammalian cells. Some of these bundles are more than 40 μm long and include several thousand microtubules. The cells in question are called supporting cells because they evidently provide mechanical support for the neighbouring sensory hair cells, which are connected to the microtubule bundles via cell junctions (see Gulley and Reese, 1976; Henderson et al., 1995). The three-dimensional layout of microtubules in supporting cells is elaborate and highly asymmetrical (Iurato, 1967; Engström and Ades, 1973; Kimura, 1975; Gulley and Reese, 1976; Slepecky and Chamberlain, 1983; Henderson et al., 1994, 1995). This account deals with centrosomal involvement during control of microtubule assembly and positioning in the supporting cells, which are known as outer pillar cells.

The investigation has revealed that each mature outer pillar

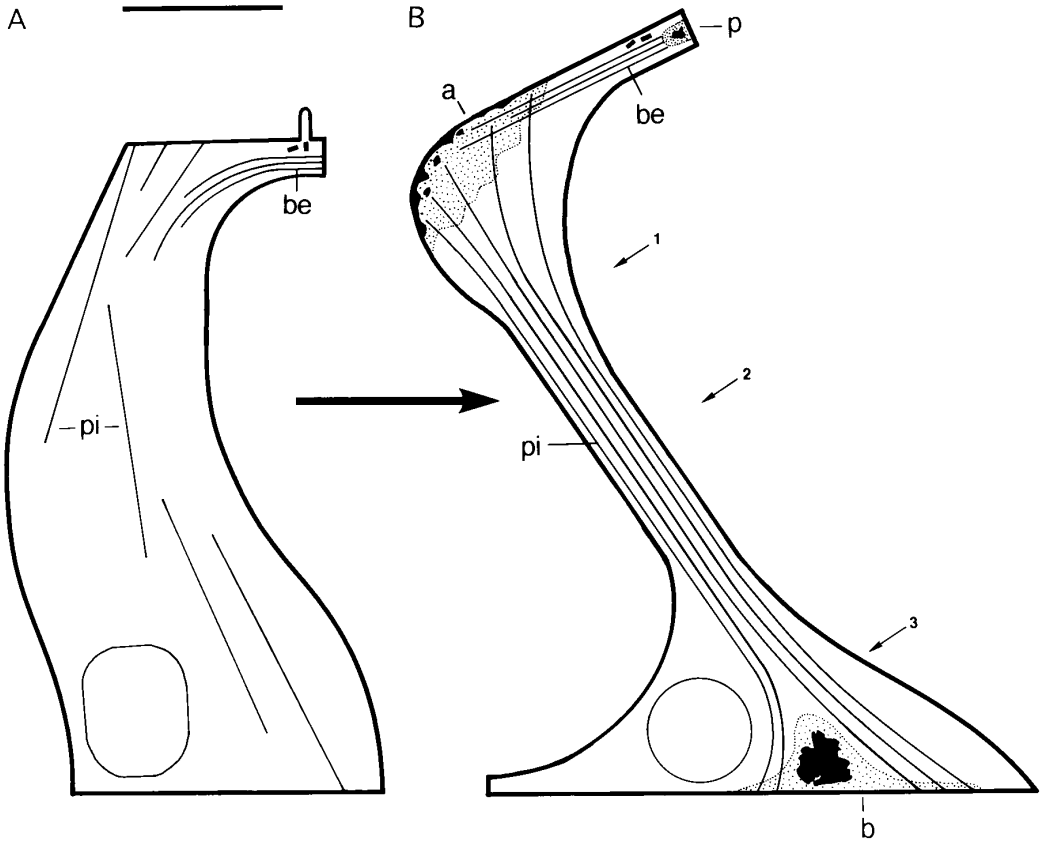

Fig. 1. Schematic diagrams showing the shapes of outer pillar cells and microtubule arrangement. The apical surfaces of the cells are oriented towards the top of the diagram; the outer sides of the cells face towards the right. The flat basal surfaces of cells (towards the bottom of the diagram) indicate the plane of the upper surface of the basilar membrane (not shown) on which they are situated. Black lines inside cells show the orientation of microtubules (which are present in much larger numbers than depicted by the lines) in regions where the beam (be) and pillar (pi) are situated. Black rectangles show the positions of the centrosomal centrioles near the tip of the cell's phalangeal process. Bar, 10 μm. (A) Day 6. The phalangeal process has started to extend from the outer side of the cell apex and one of the centrioles is the basal body of a primary cilium. (B) Day 21. Stippled regions show the shapes and positions of the phalangeal (p), apical (a) and basal (b) surfoskelosomes which anchor microtubule ends to the cell surface; regions where surfoskelosomal material is especially dense and compact have been blocked in black. The number of microtubules/pillar cross-section has been assessed at levels 1-3 when the pillar is assembling on day 8 (see Fig. 12).

cell possesses two microtubule bundles, which are orthogonally positioned and interdigitate where they meet at right angles to each other. A centriole-containing centrosomal microtubule-organizing centre is located at one end of one of the bundles. The other bundle is remotely located with respect to the centrosomal centrioles (8 μm away at its closest point). Furthermore, both ends of both bundles are precisely positioned with respect to certain cell surface regions where they are connected to the plasma membrane and its cell junctions.

To what extent is the centrosome specialised to deal with the challenge of organizing two large orthogonally positioned microtubule bundles? Are there other microtubule-organizing centres? Does the centrosome nucleate microtubules for the largest bundle (of about 4,500 microtubules), which apparently is not located close to it? What mechanisms operate to effect the attachment of both ends of both bundles to the cell surface? These issues have been investigated in a study of the spatiotemporal sequence of events during assembly of the two bundles.

MATERIALS AND METHODS

Outer pillar cells have been examined at 0, 3, 6, 8, 9, 21 and 60 days after the birth of Swiss CD1 mice (the period 0-24 hours after birth

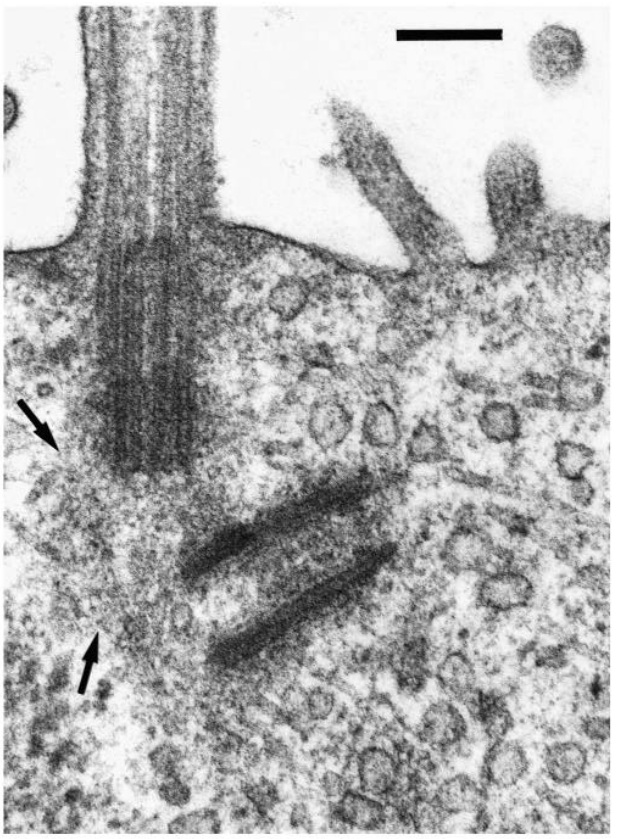

Fig. 2. Pericentriolar material (arrows) concentrated around the centrosomal centriole and basal body on day 0. Bar, 0.2 μm.

= day 0, etc.). Each organ of Corti was prepared for electron microscopy using previously described procedures (Tucker et al., 1992; Henderson et al., 1994). The portions of the organ that have been examined were all selected from the basal regions of cochleas because a graded baso-apical decrease in the number of microtubules/mature pillar cell has been reported for rats and guinea pigs (Iurato, 1967; Kikuchi et al., 1991) and in addition, there is a graded baso-apical advance in the progress of morphogenesis during mammalian cochlear development (Lim and Rueda, 1992; Walsh and Romand, 1992).

RESULTS

Cell morphogenesis and microtubule deployment

No marked differences in cell organization were found when the cells of 21 and 60 day old mice were compared. Hence, the cells of mice which are more than 20 days old will be referred to as being mature.

Each outer pillar cell is more or less columnar in shape in the new born mouse. Subsequently, a cell process extends out laterally from the cell apex (Fig. 1). This phalangeal process projects from the outer side of the cell in terms of the established convention for describing cell arrangement in the organ of Corti (see Lim, 1986). In a mature cell there are two large microtubule bundles which are oriented at right angles to each other (Fig. 1B). One of the bundles extends along the phalangeal process and is attached to its tip. This bundle will be referred to as the beam. The other bundle assembles parallel to the cell's apicobasal axis (Fig. 1) and will be called the pillar.

Beam assembly and elongation progress as the phalangeal process extends. Until day 6, the longitudinal axis of the beam is slightly curved and projects downwards fairly steeply away from the cell's apical surface (Fig. 1A). By day 8, changes in cell shape and microtubule orientation have occurred. As a result, cell shape and microtubule positioning closely resemble that found in mature cells (Fig. 1B). The beam straightens so that it runs parallel to the cell's apical surface along its entire length. The phalangeal process slopes upwards in an outward direction, and the longitudinal axis of the pillar slopes inwards with respect to the plane of the cell base (Fig. 1B).

The mid-portion of the main cell body narrows markedly (between days 6 and 9) as a cell matures (Fig. 1). The opening up of large intercellular spaces is involved. The cell surface of the narrowed portion of a cell becomes separated from that of the surfaces of neighbouring cells against which it was closely applied at earlier stages.

Dense fibrous meshworks called surfoskelosomes (SSSs) (Henderson et al., 1995), which include a certain amount of cell junctional material, anchor the ends of the microtubule bundles to the cell surface of a mature cell (Fig. 1B). A phalangeal SSS connects one end of the beam to the tip of the phalangeal process. The other beam end and the top of the pillar are both embedded in an apical SSS. A basal SSS is involved in attaching the bottom of the pillar to the cell base, which

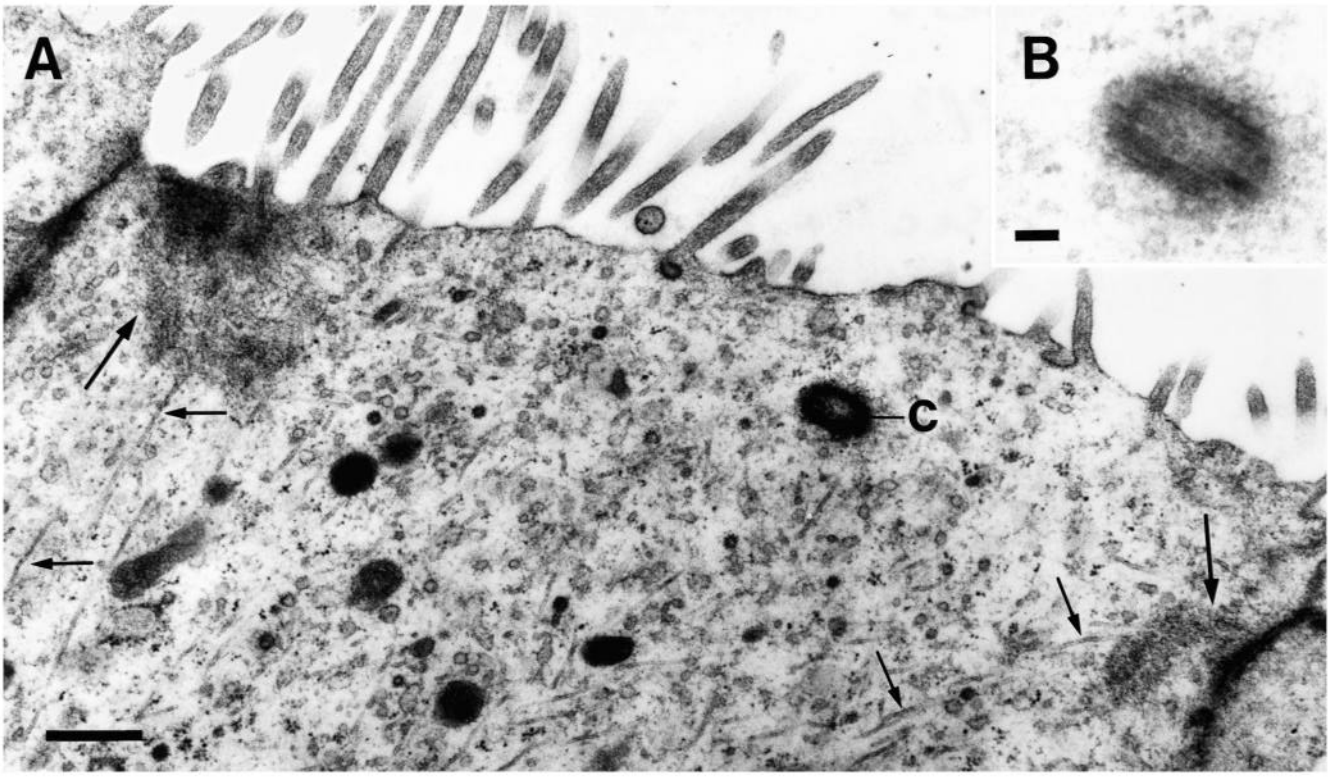

Fig. 3. (A) Longitudinal section through the apical portion of a cell on day 3, which includes one of the centrosomal centrioles (c). Two clumps of dense material (long arrows) are concentrated near the cell's apical surface on opposite sides of the cell. Beam microtubules (short arrows towards the right of the micrograph) and pillar microtubules (short arrows towards the left) project from these clumps. Bar, 0.5 μm. (B) The portion of the micrograph shown in A which includes the centriole has been printed at a higher magnification and at a different level of contrast to show the centriole more clearly. Bar, 0.1 μm.

adheres to a specialised basement membrane called the basilar membrane.

Centrosomal reorganization

Centrosomal organization is similar to that in animal tissue cells generally on day 0 (Fig. 2). Finely divided dense pericentriolar material is concentrated around the two centrioles, which are positioned close to the centre of the cell's apical surface. Only a few microtubules are present in each cell at this stage; most of them are situated in the region around the pericentriolar material. One of the centrioles bears a primary cilium until day 6. The ciliary shaft is resorbed at some point thereafter; it has not been detected after day 6.

A well defined concentration of pericentriolar material in the immediate vicinity of the centrioles is not evident on day 3 or thereafter when assembly of the microtubule bundles is progressing. On day 3, microtubules project from two clumps of dense material with an appearance similar to that of pericentriolar material (Fig. 3). The apical ends of beam microtubules contact the clump which is situated on the outer side of the cell apex in the region from which the phalangeal process will subsequently extend. The apical ends of pillar microtubules are associated with the clump which is located on the opposite (inner) side of the cell's apical surface (Fig. 4). However, the structural organization of both of these microtubule-organizing centres is subsequently modified; two distinct clumps of apically situated microtubule-organizing material are not detectable on day 6 or thereafter (see below).

Centrioles have migrated to the outer side of the cell apex and are positioned close to the tip of the extending phalangeal process by day 6 (Fig. 5). They remain at this location as cell morphogenesis progresses (Fig. 1). There is a substantial concentration of well aligned beam microtubules near the centrioles, which do not radiate from the immediate vicinity of the centrioles, nor do they still project from a pronounced clump of dense material. The outwardly directed ends of most of these microtubules are positioned close to the cell surface (less than 0.2 μm from the plasma membrane in many cases, and less than 50 nm in a few instances) (Fig. 5). Further changes in the organization of this juxta-centriolar centrosomal region occur. Finely fibrous dense material accumulates in the increasingly larger space which separates microtubule ends and the cell surface. In mature cells the separation is about 1.7 μm; the dense fibrous meshwork of the phalangeal surfoskelosome has been intercalated between the ends of the microtubules and cell junctions at the tip of the phalangeal process (Fig. 6).

Much of the cell's apical surface (apart from that associated with the phalangeal process) is situated above the region where the top of the mature pillar is finally situated (Fig. 1B). However, there is only a sparse population of microtubules in this region throughout the period when nucleation of pillar microtubules is occurring (compared with the high concentration of beam microtubules focussed on the surface site near the centrioles) (compare Figs 5, 7). From days 6-9, the apical ends of pillar microtubules no longer project from a clump of dense material; some of them are very closely applied to the apical plasma membrane (Fig. 7). However, most of these contacts are not permanent ones. They are often evident where longitudinal sections cut through the apical surfaces of cells on days 8 and 9 (when pillar assembly is still proceeding) but they have rarely been found in such sections of mature cells (when assembly has been completed).

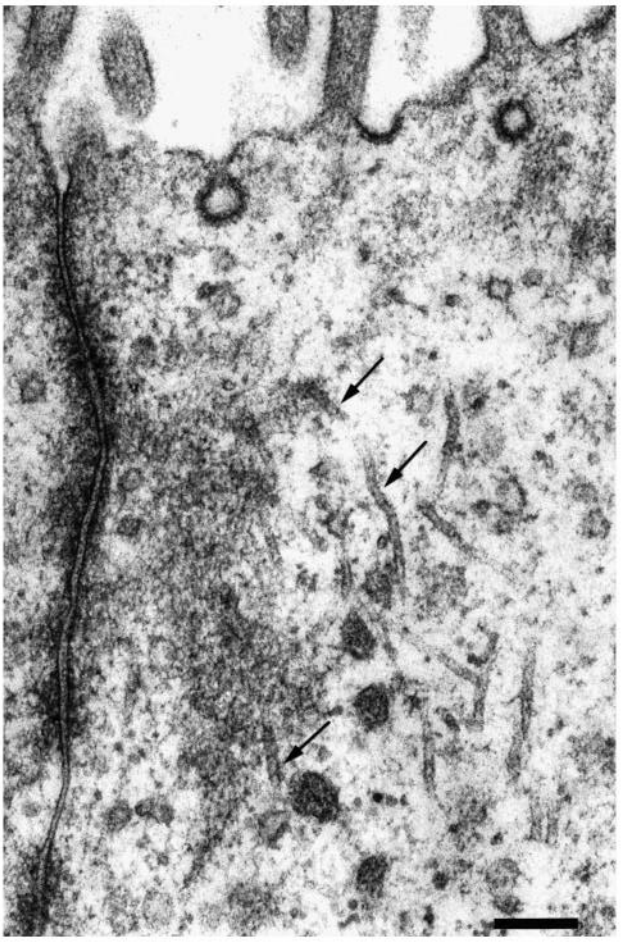

Fig. 4. Longitudinal section through part of the apical portion of a cell on day 3. Pillar microtubules (arrows) project from a clump of dense material which is concentrated against the plasma membrane where the inner side of the cell meets its apical surface. Bar, 0.2 μm.

Beam structure and assembly

There are progressively smaller numbers of microtubules at succeedingly greater distances from the cell surface-associated microtubule-organizing centre at the outer end of the beam (Fig. 5) during beam assembly (Fig. 8). This is also the case after beam assembly is completed. Hence, there is little doubt that microtubules elongate from this microtubule-organizing centre, which presumably acts as a microtubule-nucleating site.

Cross-sections cut 3 μm from the outer cell surface-associated ends of 3 different mature beams included an average of 1332 (1184, 1359, 1452) microtubules. Beam assembly takes a long time; it progresses for at least a week. It has been initiated by day 3 but the beam has not achieved its final length of about 14 μm by day 9.

Very few of the distal (plus) ends of a mature beam's microtubules extend as far as the cell surface on the inner side of the cell; most of them terminate in the apical SSS (Fig. 1B). The mature beam is slightly splayed into several microtubule groupings, which include variable numbers of microtubules where it penetrates the apical SSS. These splayed portions of

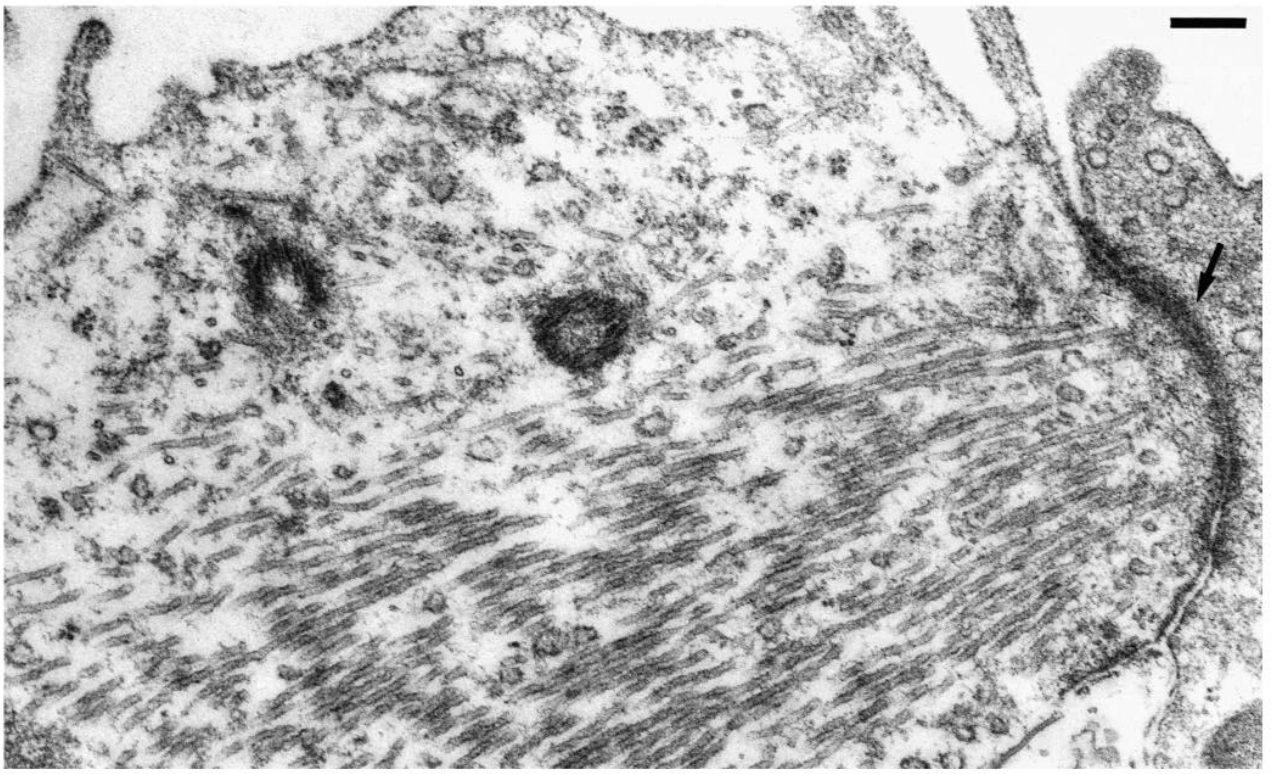

Fig. 5. The outwardly directed ends of most beam microtubules are closely applied to the cell surface at the tip (arrow) of the extending phalangeal process on day 6. Relatively few microtubules radiate from the immediate environs of the two centrosomal centrioles. The apical surface of the cell is towards the top of the micrograph. Bar, 0.2 μm.

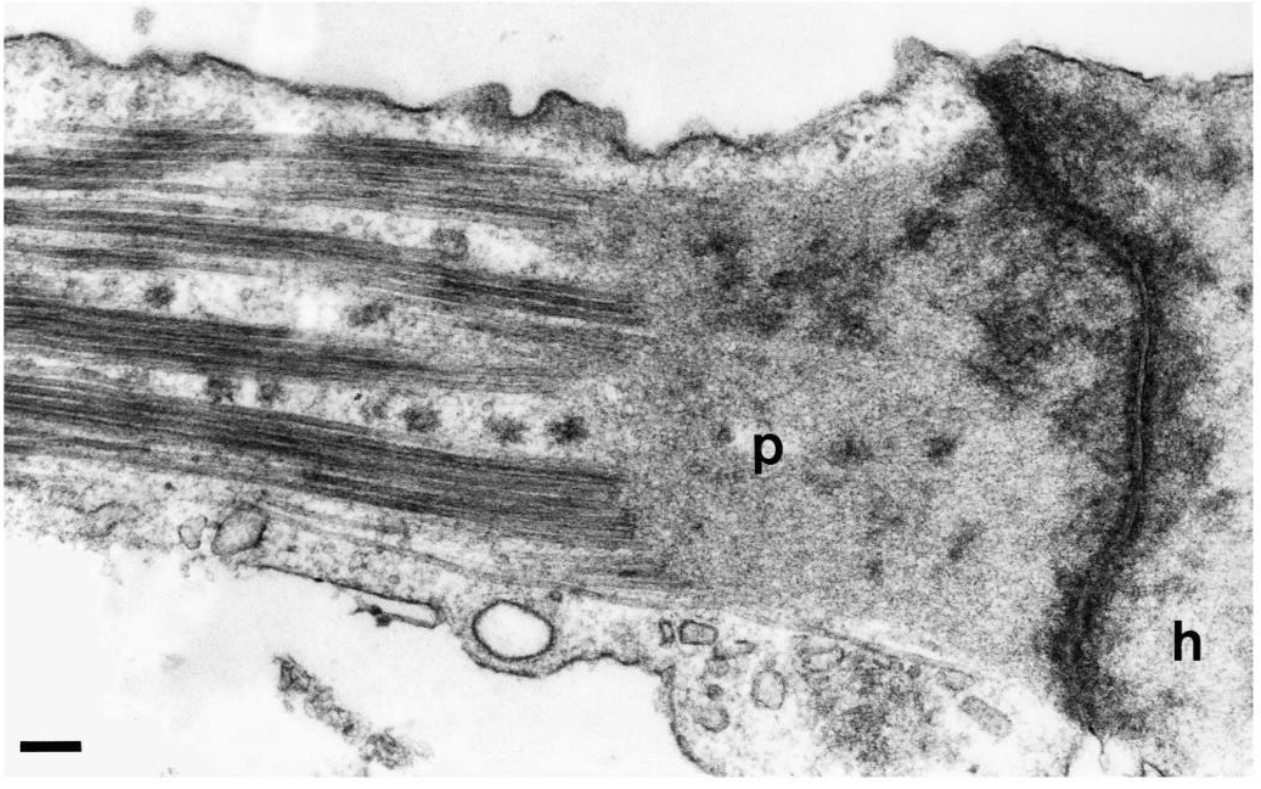

Fig. 6. Longitudinal section through the outer end of a mature phalangeal process on day 21 with its apical surface oriented towards the top of the micrograph. The phalangeal surfoskelosome (p) joins the ends of beam microtubules to the cell surface where cell junctions connect the outer pillar cell to a sensory hair cell (h). Bar, 0.2 μm.

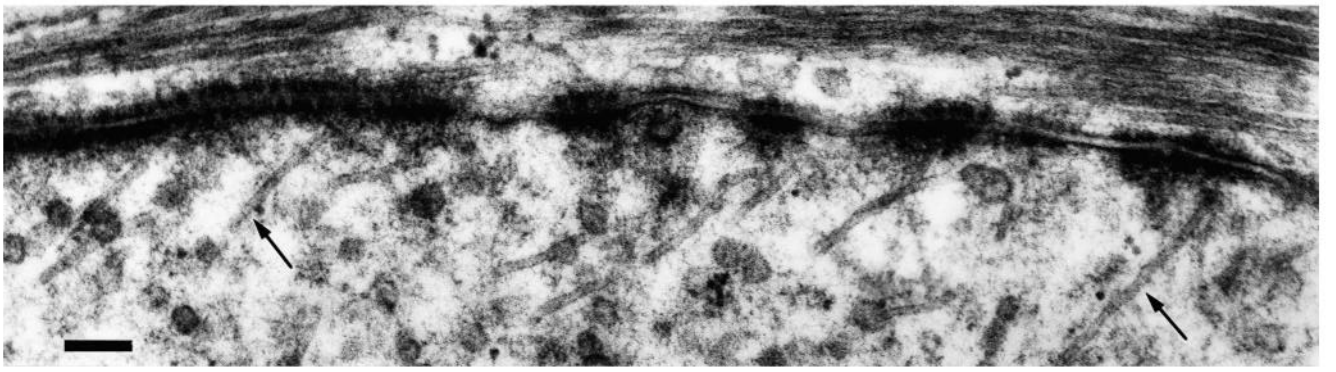

Fig. 7. Longitudinal section through part of the apical surface of a cell above the region where the top of the pillar is assembling on day 8. The apical ends of several microtubules (arrows) are positioned closely against the cell surface. Microtubules in part of the phalangeal process of a neighbouring inner pillar cell (which has extended over the apex of the outer pillar cell at this stage) are included at the top of the micrograph. Bar, 0.2 μm.

the beam interdigitate with similarly splayed groupings of microtubules at the top of the pillar (Fig. 9).

Pillar structure and assembly

By day 6, a relatively sparse array of pillar microtubules extends along the entire length of a cell (Fig. 1A). Cross-sections of 3 different pillars cut at a level 5 μm below the apical surfaces of cells only included 109 microtubules on average (86, 112, 129).

By days 8 and 9 a substantial increase in the number of pillar microtubules has occurred (Fig. 10) and microtubule arrangement is similar to that in mature cells. Microtubules are well aligned along a midportion which extends along much of the pillar's length for a distance of about 26 μm (between levels 1 and 3 in Fig. 1B). Microtubules are less closely packed together where they have a splayed configuration at each end of the pillar which resembles the arrangement in mature cells (Fig. 1B). The basal ends of many microtubules are situated close to the cell base (some within 100 nm of the plasma membrane). In contrast, only a few microtubules are located close to the apical cell surface (Fig. 7).

A mean value of 4485 (5359, 4131, 3965) was obtained for microtubule number/pillar for cross-sections cut through the midportions of 3 different mature pillars (near level 2 in Fig. 1B). There are about 2,600 microtubules at this level on day 8 (see below). Hence, pillar assembly is still progressing on day 8 and, as is the case for beam assembly, its construction probably takes at least one week.

On days 8 and 9 the pillar midportion consists largely of several closely packed groupings of microtubules (Fig. 10).The cross-sectional shaping and arrangement of these microtubule groupings are slightly different in each cell. Such idiosynchrasies permitted individual cells to be distinguished from each other and their cross-sections to be tracked for a short distance (about 2 μm) near the top of the pillar midportion at level 1 in Fig. 1B. Microtubule number increased at successively lower levels from 1934-2247 (Fig. 11), 1987-2334, 2134-2308, and 2377-2763, respectively, along the portions of the 4 pillars that were monitored. The microtubule profiles were not sufficiently well oriented in cross-section to permit accurate counting to be continued at lower levels (due to slight curvature in the pillars' longitudinal axes). Examination of

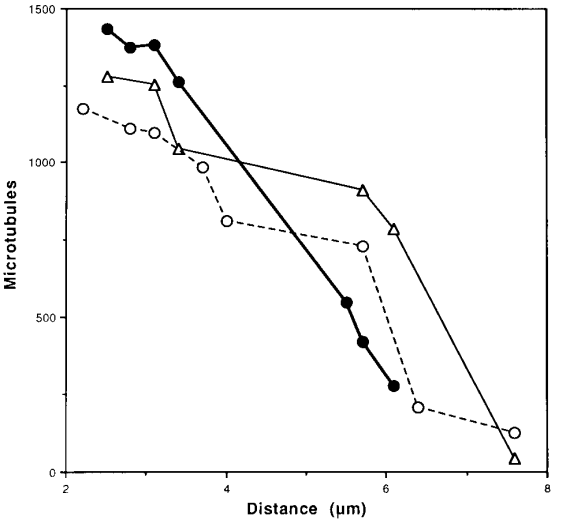

Fig. 8. Microtubule number/cross-section of 3 beams (●,△,○) cut at different distances from their proximal cell surface-associated ends (which are located near the centrosomal centrioles at the tips of phalangeal processes) on day 9.

pillars in another cochlea revealed that the apicobasal increase in microtubule number at the top of an assembling pillars' midportion is not a feature that extends down to the pillar base. There is an increase in the average microtubule number from 2198 to 2617 between levels situated near the top and middle of the pillar midportion and then a decrease to 2295 near the pillar base (levels 1, 2 and 3, respectively, in Fig. 1B) (Fig. 12).

Microtubules are closely packed together along most of the mature pillar midportion's length. However, several longitudinally oriented channels which are devoid of microtubules extend through this otherwise compact grouping of aligned microtubules (Fig. 13). It appears that the spatially discrete microtubule groupings present on days 8 and 9 (Fig. 10) increase in girth as more microtubules assemble and accumulate around them until these groupings make lateral encounters to form a continuum of closely packed microtubules. If this interpretation is correct, the long channels represent regions where such 'lateral fusion' of microtubule groupings has not

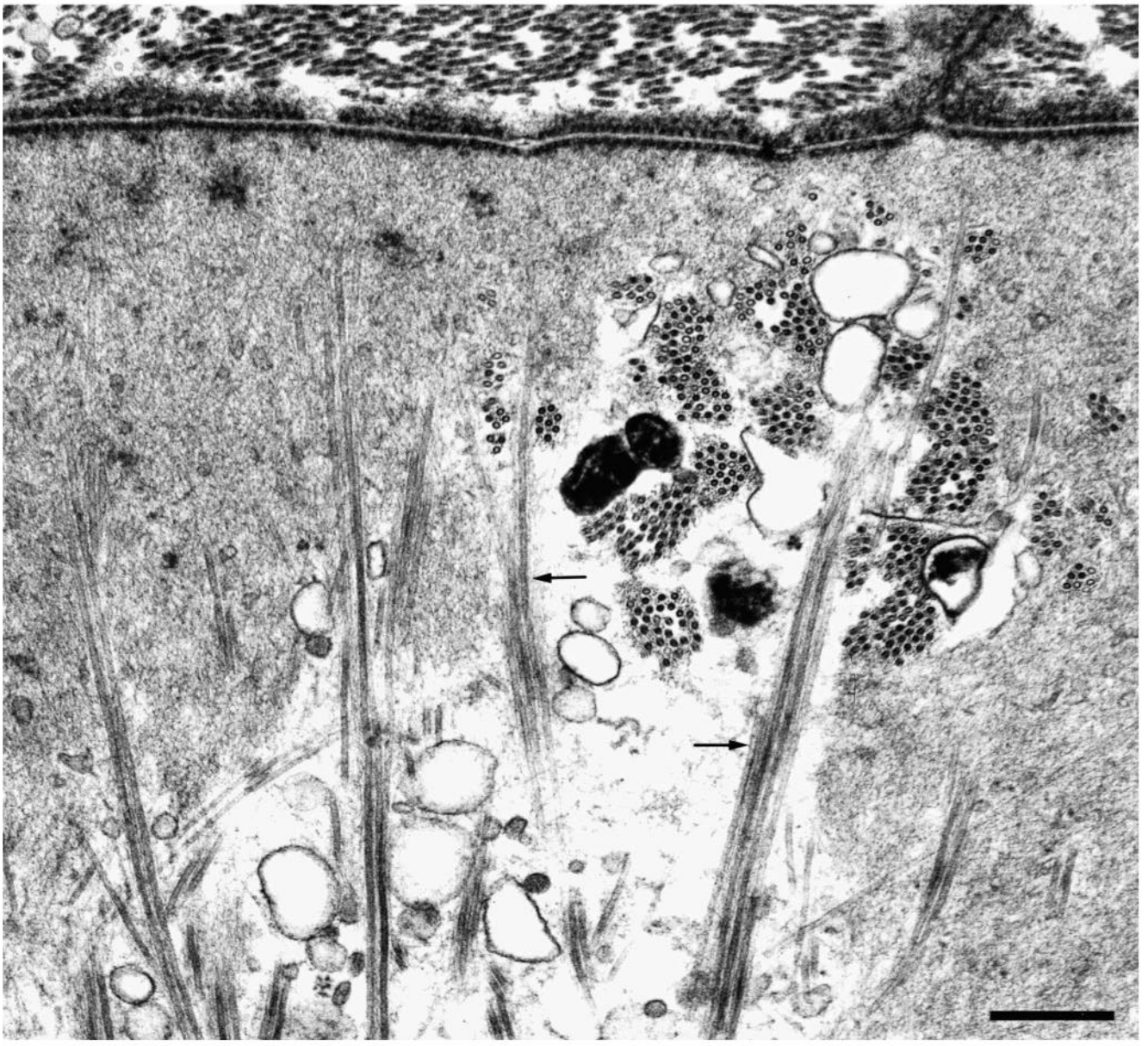

Fig. 9. Longitudinal section through part of the apical surface and apical surfoskelosome of a mature cell on day 21 showing cross-sections of beam microtubules where they orthogonally interdigitate with the longitudinal profiles (arrows) of pillar microtubules. The apical cell surface is oriented towards the top of the micrograph where cell junctions connect it to the undersurface of two inner pillar cell phalangeal processes. Bar, 0.5 μm.

occurred. Presumably, the more widely spaced microtubules, which are not included as members of the groupings during pillar assembly on days 8 and 9 (Fig. 10), represent microtubules that are young relative to those in the groupings.

The apical portions of many of a mature pillar's microtubules penetrate the apical SSS (Figs 1B, 9). Instances in which they extend all the way to the apical cell surface have rarely been detected although such instances are not uncommon during pillar assembly (Fig. 7).

The basal SSS is shaped rather like a witch's pointed hat with a thickened brim (Fig. 14). Most microtubules at the bottom of a pillar do not penetrate the dense central cone-shaped mass of the basal SSS; they are splayed around its sides (Fig. 1B). However, the basal portions of many of the microtubules do pass into the brim, and are embedded in it, where the SSS has a less dense and compact composition than at its centre (Fig. 14). It is only at the edge of the brim that the basal ends of some microtubules are situated close to the plasma membrane (within 100 nm) at the cell base. Such microtubules contact a layer of dense material which coats the cytoplasmic surface of the plasma membrane at the bottom of the SSS (Fig. 14).

Surfoskelosomes

The three SSSs help to anchor microtubule ends by invading regions occupied by the cell surface-associated microtubule-organizing centres where nucleation and capture of microtubules takes place. Assembly of SSSs has started by day 9

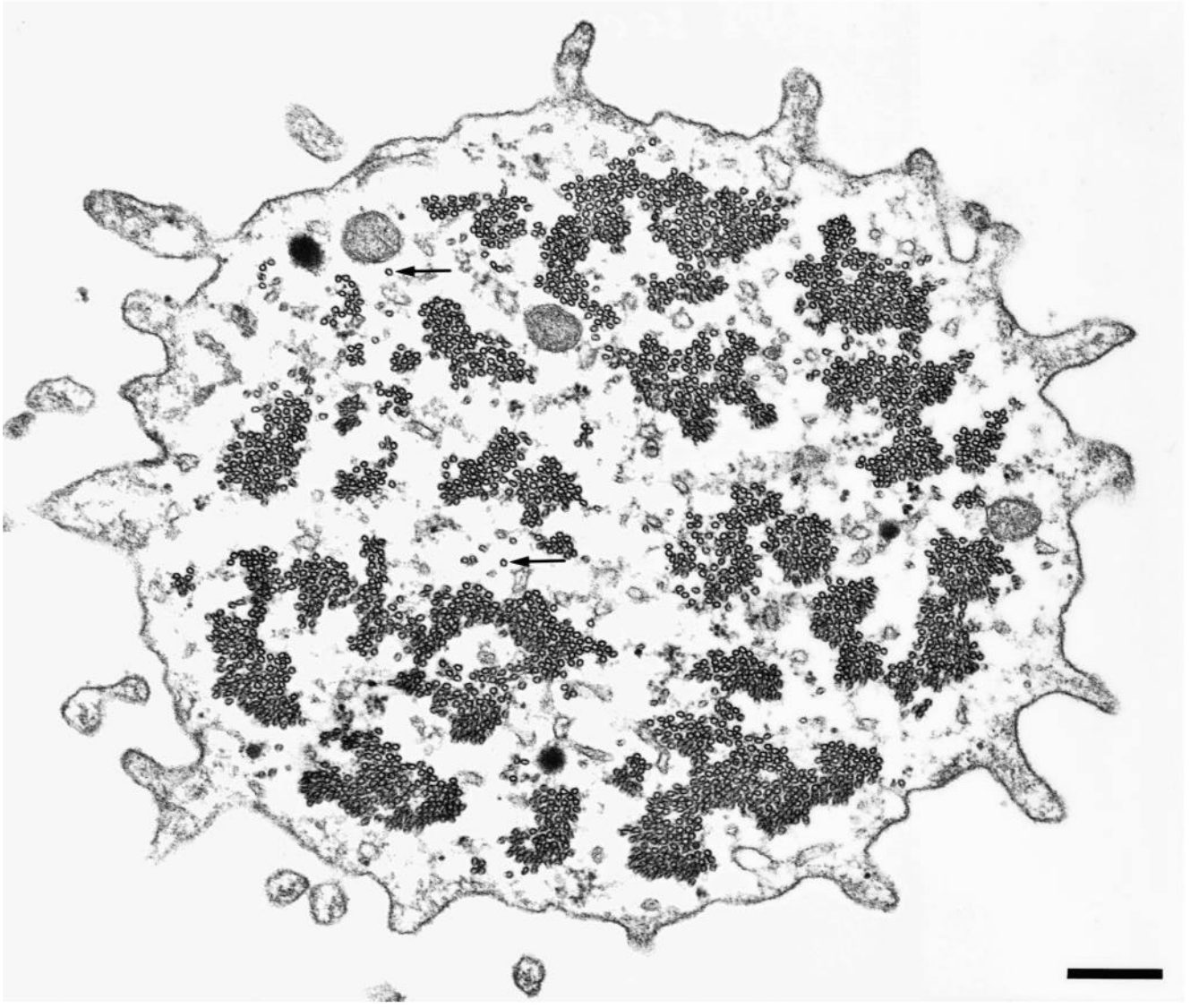

Fig. 10. Cross-section of a cell and the midportion of its pillar cut at level 2 (in Fig. 1B) on day 9. Some microtubules (arrows) are not included in the main closely packed microtubule groupings. The short processes at the cell surface are present for a period following the opening up of a large intercellular space (which surrounds the cell along much of its length) when the surface regions in question separate from those of neighbouring cells against which they were closely juxtaposed on day 8. Bar, 0.2 μm.

(about a week after microtubule assembly has begun but before construction of the two bundles is completed). Numerous small tufts of dense fibrous material project out from the cell surface in regions where the apical and basal surfoskelosomes will finally be located.The procedure during construction of these SSSs is probably similar to that which occurs in inner pillar cells where the tufts apparently increase in size and fuse together as a SSS forms (Henderson et al., 1995).

At the ultrastructural level much of the fabric of the mature SSSs has a compact and apparently amorphous appearance although it is not completely uniform; each SSS includes several irregularly shaped regions of greater density, which have a different composition than elsewhere and/or where its components are more highly concentrated (Figs 1B, 6, 14). However, dense material is relatively loosely packed in the lower portion of the apical SSS and here a fibrous composition is sometimes evident where it is extensively penetrated by the interdigitating microtubules of the pillar and beam (Fig. 9). Mitochondria and certain vesicles are often located in the spaces between groupings of microtubules in the vicinity of the apical and basal SSSs where the pillar and beam have a splayed configuration (Figs 9, 14).

DISCUSSION

Centrosomal reorganization and the differentiation of two nucleating sites

Two large microtubule bundles assemble in each cell and microtubules project from two distinct sites on opposite sides of the cell apex at an early stage in cell morphogenesis. Hence, it is reasonable to suppose that there are two spatially discrete nucleating sites - one for each bundle. During the earliest assembly stages a clump of dense material is concentrated

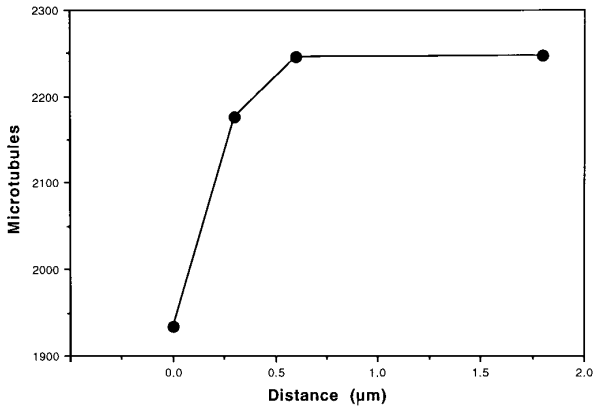

Fig. 11. Microtubule number/cross-section in an assembling pillar on day 9 cut at successively lower points along a short portion of its length (about 2 μm) situated near level 1 (in Fig. 1B).

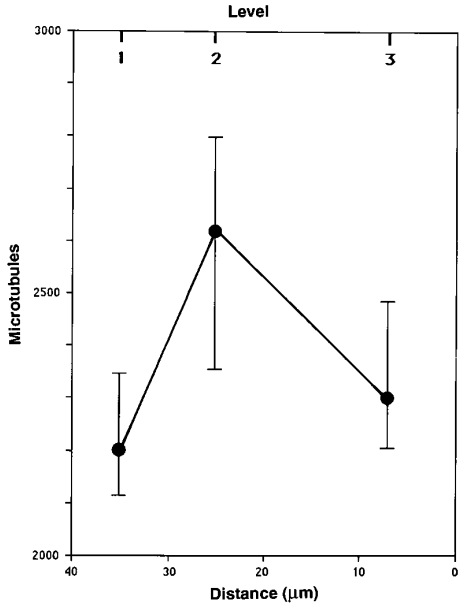

Fig. 12. Microtubule number/pillar cross-section at different levels on day 8. Levels 1-3 correspond to those marked in Fig. 1B. The distances (measured along the longitudinal axis of the microtubule bundle) which separate each level from the bottom of the bundle at the cell base are also shown. Each point shows the mean value for microtubule numbers counted for three different cells at each level. The accompanying vertical line shows the range of each set of counts. Cross-sectional tracking was not sufficiently detailed to determine whether exactly the same cells in the portion of the organ that was used (which included about 25 outer pillar cells) were being assessed at the different levels. Hence, the bundles counted at any one level were often likely to have been different from those counted at another.

against the plasma membrane at each of these sites. The ultrastructural appearance of the dense material in each clump is similar to that of the pericentriolar material (PCM) of centrosomes generally. Most microtubule nucleation by centrosomes is effected by elements within the PCM rather than by any of the centriolar components (Gould and Borisy, 1977). While the nucleation of about 6,000 microtubules is progressing in an

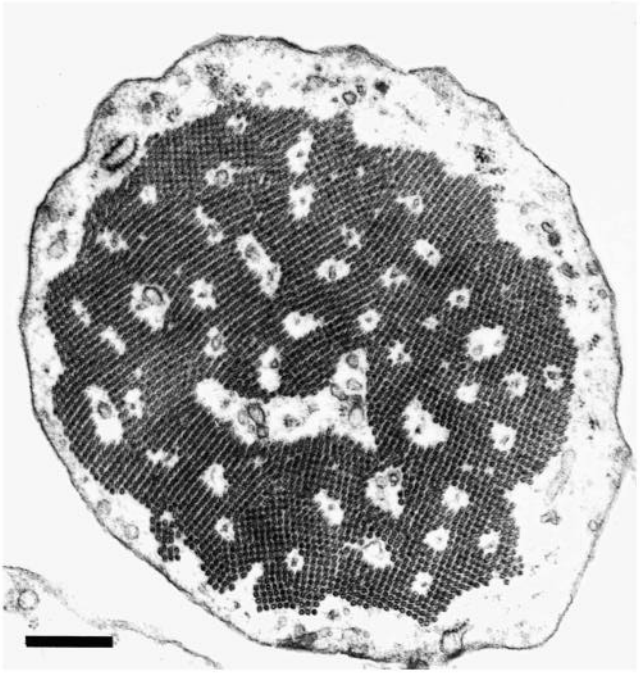

Fig. 13. Cross-section through the midportion of a mature pillar on day 64 cut at level 2 (in Fig. 1B). Bar, 0.5 μm.

outer pillar cell, uncharacteristically small amounts of PCM are associated with the centrioles and few microtubules radiate from the immediate environs of the centrioles. Thus, it seems that a radical reorganization of the centrosome occurs in which PCM is redeployed to two localities where it clumps against the cell surface and then nucleates assembly of the two microtubule bundles (Fig. 15A). This study has provided no information about whether such redeployment involves assembly of new material at the cell surface or the translocation of preexisting material from the pericentriolar region.

Further centrosomal reorganization occurs during the period when microtubule nucleation is occurring. By day 6, the minus ends of many of the microtubules are situated closer to the plasma membrane than was previously the case and well defined clumps of PCM-like material are not clearly evident. Although many details of the organization of PCM remain to be ascertained it is evident that PCM includes a range of molecular components. Microtubule-nucleating elements are probably connected together by several types of structural proteins and functionally modulated by regulatory proteins which are also associated with them (see Tucker, 1977; Kimble and Kuriyama, 1992; Kalt and Schliwa, 1993). It is presumably changes in the organization of the structural proteins which are mainly involved as the clumps of PCM-like material spread out and/or diminish in bulk so that nucleating elements become more closely associated with the plasma membrane (Fig. 15B).

Events during beam assembly provide direct evidence for the sequence of changes summarized in Fig. 15 so far as its nucleating site is concerned. Evidence for the way in which the pillar-nucleating site operates is less clear cut. Large numbers of microtubules do not project from the site while the pillar is assembling. We suggest that the following events are occurring. Microtubules do not accumulate at this site because

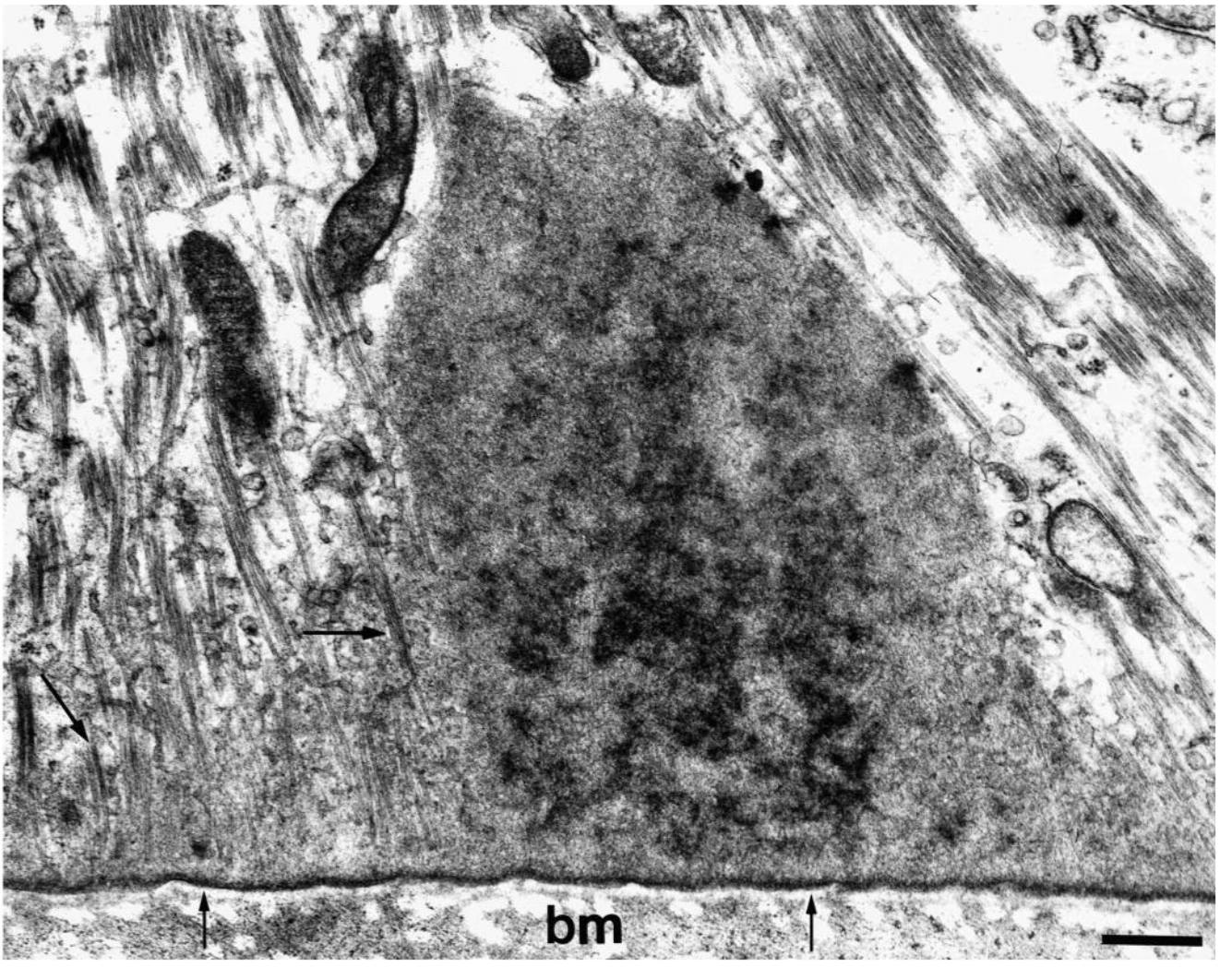

Fig. 14. Longitudinal section through a mature basal surfoskelosome on day 60. Pillar microtubules have avoided the central cone-shaped region of the surfoskelosome but have penetrated its edges (large arrows). The basal plasma membrane is coated with a layer of dense material (small arrows) on its cytoplasmic surface where the surfoskelosome is associated with the basilar membrane (bm). Bar, 0.5 μm.

they are continually being released from it. Only relatively recently nucleated microtubules are attached to their nucleating elements at any one particular point in time (Fig. 15B). After release, microtubules migrate to the cell base where their plus ends are captured. Then they elongate back up towards the apical surface until they encounter the region where the apical SSS is forming.

Variations in the number of microtubules along the lengths of the assembling pillars on days 8 and 9 are compatible with this interpretation. The highest number of microtubules occurs near the pillar mid-level on day 8 where overlap between microtubules that are still migrating downwards and those that are elongating back upwards is to be anticipated. Tracking along individual pillar bundles near the tops of their midportions on day 9 reveals the apicobasal increase in number that is to be expected if some microtubules are elongating up from the cell base while others are descending from the cell apex.

Pillar cell microtubules which have reached the cell base might elongate at either end. Such elongation may be promoted by continued addition of tubulin to the captured plus ends, since such addition can occur after plus end capture by kinetochores (Mitchison, 1989). Alternatively, minus end growth might occur if there is a high tubulin concentration in the cells.

The case for a microtubule-releasing site

The scheme suggested above (Fig. 15) for differentiation of the pillar's nucleating site and its microtubules-for-export-only function is admittedly an elaborate one. However, all the other potential mechanisms which might reasonably be expected to operate are incompatible with the spatiotemporal sequence of events during pillar assembly. For example, the evidence that microtubules elongate upwards raises the question of whether there is a cell surface-associated nucleating site at the cell base. If such a site is responsible for generating all the pillar's 4,500 or so microtubules then high concentrations and numbers of microtubules would be expected at the cell base during the relatively early stages of pillar assembly (as is the case for the beam at the apical surface). Such population of the cell base by microtubules in advance of other cell regions that are finally occupied by the mature pillar has not been detected.

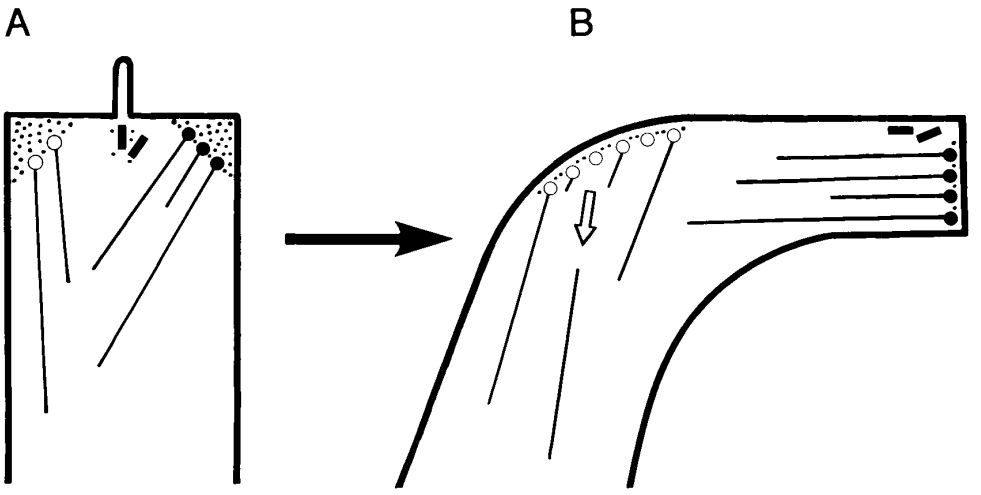

Fig. 15. Schematic diagrams of the apical portions of differentiating cells showing changes during centrosomal reorganization and microtubule assembly, which are indicated by the spatiotemporal sequence of events at the ultrastructural level. Straight lines show the positions and lengths of microtubules. Black rectangles show the positions of centrosomal centrioles. Small dots show the location of pericentriolar material. Circles show the positions of nucleating elements for beam (●) and pillar (○) microtubules. (A) On day 3 microtubules are elongating from two nucleating sites on opposite sides of the cell apex. (B) As assembly of microtubule bundles progresses the two nucleating sites become more widely separated because the phalangeal process has started to extend (towards the right of the diagram). Microtubule-nucleating elements and pericentriolar material are more closely associated with the cell surface at both nucleating sites than is the case on day 3. Centrosomal centrioles have migrated to the tip of the phalangeal process and are located near the beam's nucleating site. Elongating pillar microtubules are released from the pillar nucleating site and its nucleating elements; they migrate downwards (to the base of the cell) as indicated by the arrow.

High concentrations of the pillar's 4,500 or so microtubules do not obviously emanate from one particular cytoplasmic region that can be immediately identified as its nucleating site. Perhaps this is because there is no highly localized site and nucleation progresses throughout a much larger cytoplasmic region, since the pillar occupies much of the cell body. However, the assembly of nearly all large microtubule bundles that have been studied (in a wide range of cell types) usually involves elongation of a high concentration of microtubules from a readily identifiable cytoplasmic site. Such a site often includes a substantial concentration of dense material and/or is associated with some other cell component such as a basal body, centriole, the plasma membrane or the nuclear envelope (see Tucker, 1979, 1984, 1992). It seems unlikely that some initial control of the positioning of pillar microtubules should not also be facilitated by the provision of a well localized site such as the site identified (albeit with difficulty) in this report.

There is no straightforward way of accounting for the temporary presence of microtubule ends at the site identified here as the pillar nucleating site, unless it acts as a microtubule releasing site as outlined in Fig. 15. The suggestion that a microtubule release/translocation/elongation sequence operates for redeployment of certain centrosomally nucleated microtubules is not a new one. There is evidence that such events proceed in certain other cell types (Vorobjev and Chentsov, 1983; McBeath and Fujiwara, 1990; Joshi and Baas, 1993) and in the inner pillar cells which are adjacent to outer pillar cells (Henderson et al., 1994, 1995). Although the final layout of microtubules in outer and inner pillar cells is distinctly different there are some spatial homologies (for example, both types of cells possess a microtubule array that is located near their centrioles and another that is not). It is reasonable to suppose that such resemblances and differences may be established because these two neighbouring cell types effect subtle variations on a common control theme for positioning their microtubules.

Microtubule stability and assembly dynamics

This investigation has monitored events during a very slow microtubule assembly programme, which runs for at least a week. Examination of progress at intervals of a day or more provides no information about the extent to which the lengths of the microtubules may fluctuate as they probe for capturing sites (see Kirschner and Schulze, 1986; Gelfand and Bershadsky, 1991; Holy and Liebler, 1994) at the cell surface where their ends can be capped and stabilized. Presumably, only a relatively small proportion of the microtubules are exhibiting marked changes in length and orientation at any particular stage. If this were not the case, then much greater variations in microtubule number and arrangement than those that have been found should have been detected when the same regions of *different* cells at the same stage were compared. There are indications (Fig. 10) that bundling (and associated cross-connection) may progressively stabilize correctly positioned microtubules while the pillar is being built.

Why does assembly take such an extraordinarily long time? The cells are about 50 μm long. If their microtubules elongate at rates of up to 420 μm/h, as is the case for certain cultured epithelial cells (Cassimeris et al., 1988), then each pillar cell microtubule could probe for its target several hundred times during the course of a week. However, precise positioning of thousands of microtubules is effected in each cell. A very highly ordered array of about 38,000 microtubules elongates for 65 μm at an average rate of 16 μm/h in the ciliate *Nassula* (Tucker, 1970). If the overall rate of microtubule bundle elongation proceeds at a similar rate in outer pillar cells, then construction of the pillar (47 μm) and beam (14 μm) could be completed in about 3 hours. Other factors may be rate limiting. Throughout much of the assembly period, the cells are constructing surfoskelosomes and modifying the organization of cell junctions at the cell surface sites where the ends of microtubules are finally located. They are also changing in shape and altering their contacts with cell neighbours. Supracellular coor-

dination of the deployment and intercellular linkage of thousands of microtubules in thousands of supporting cells is taking place in the organ of Corti (Henderson et al., 1995). This is probably crucial for micromechanical reasons related to the organ's role as a sound receptor. These more global considerations, which transcend parochial aspects of spatial control so far as individual cells are concerned, may involve some especially prolonged fine-tuning of the construction ritual.

Centrosomal versatility and association with the apical surfaces of epithelial cells

Apical positioning of the centrosome is a feature of several types of epithelial cells (see Tucker et al., 1986, 1992; Bacallao et al., 1989; Fath et al., 1993). Microtubules elongate from sites associated with one of the apically situated centrosomal centrioles, which is the basal body of a cilium in each ectodermal cell of certain sea urchin blastulae (Tilney and Goddard, 1970). This results in a configuration which resembles that in several of the relatively simple primitive flagellated unicellular eukaryotes (see Beech et al., 1991). Hence, apically situated centrosomes which nucleate apicobasal microtubule elongation may be a very ancient feature of eukaryotic cell organization. If this is true, then the centrally positioned juxtanuclear centrosome (and the rudimentary primary cilium which sometimes accompanies it) is a more recently evolved feature.

Studies of epithelial cells and their highly polarized organization may be revealing the extent to which an ancient microtubule-organizing mode has evolved by advances in the structural integration of centrosomes and apical cell surfaces. The minus ends of microtubules are distributed across all or most of the cell apex, rather than being focussed on the immediate vicinity of the centriole pair in mouse and rat intestinal epithelial cells (Achler et al., 1989) and polarized Madin-Darby canine kidney cells (Bacallao et al., 1989). This is also the case in certain epidermal cells in *Drosophila* after the centrioles have been disassembled and the nucleating role of the PCM is effected by material which forms numerous small dense plaques attached to the cytoplasmic surface of the apical plasma membrane (Mogensen and Tucker, 1987; Mogensen et al., 1989). A higher level of spatial differentiation of the apical surface/centrosome system is apparently exhibited by outer pillar cells. Nucleating capability is not more or less uniformly distributed across the apical surface but concentrated at two sites which perform differently.

Some juxtanuclear centrosomes may have followed a parallel evolutionary programme of PCM/membrane association. Functional nucleating elements are associated with the nuclear envelope in differentiating muscle cells, which dismantle their centrosomal centrioles (Tassin et al., 1985; Kronebusch and Singer, 1987), and in plant cells, which lack centrioles altogether (see Lambert, 1993).

Pillar cells excepted, ultrastructural details of the ways in which the minus ends of recently nucleated microtubules are associated with the apical cell surfaces of mammalian epithelial cells have not been described. Microtubules at the apices of certain mature mammalian epithelial cells do not have an orderly arrangement and form an apical network (Sandoz and Lainé, 1985; Bacallao et al., 1989; Gilbert et al., 1991; Mays et al., 1994). Such networks are associated with terminal webs (which include actin and intermediate filaments) that are perhaps related to the SSSs of pillar cells in terms of their composition and association with microtubule-organizing regions. SSSs contain β- and γ- non-muscle actin isoforms (Slepecky and Savage, 1994) and α-actinin (Drenkhahn et al., 1985). There are indications that intermediate filaments are also associated with them (Oesterle et al., 1990; Kuijpers et al., 1991).

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