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Microtubule minus-end anchorage at centrosomal and non-centrosomal sites: the role of ninein

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SUMMARY

The novel concept of a centrosomal anchoring complex, which is distinct from the γ-tubulin nucleating complex, has previously been proposed following studies on cochlear epithelial cells. In this investigation we present evidence from two different cell systems which suggests that the centrosomal protein ninein is a strong candidate for the proposed anchoring complex.

Ninein has recently been observed in cultured fibroblast cells to localise primarily to the post-mitotic mother centriole, which is the focus for a classic radial microtubule array. We show here by immunoelectron microscopical analyses of centrosomes from mouse L929 cells that ninein concentrates at the appendages surrounding the mother centriole and at the microtubule minus-ends. We further show that localisation of ninein in the cochlear supporting epithelial cells, where the vast majority of the microtubule minus-ends are associated with apical non-centrosomal sites, suggests that it is not directly involved in microtubule nucleation. Ninein seems to play an important role in the positioning and anchorage of the microtubule minus-ends in these epithelial cells. Evidence is presented which suggests that ninein is released from the centrosome, translocated with the microtubules, and is responsible for the anchorage of microtubule minus-ends to the apical sites. We propose that ninein is a non-nucleating microtubule minus-end associated protein which may have a dual role as a minus-end capping and anchoring protein.

Key words: Microtubule, Centrosome, Cochlea, Epithelial cell

INTRODUCTION

Microtubules are essential for many cellular functions including vesicle transport and cell motility, polarity and division. The precise and intricate microtubule patterns deployed in cells are important for these microtubule-dependent processes, and ultimately for normal cell function. The molecular mechanisms responsible for microtubule distribution and maintenance remain to be fully defined. The temporal and spatial organisation of microtubules is at least in part defined by the microtubule-organising centre. In most animal cells, microtubules radiate from a centrally located centrosomal nucleating centre, which consists of a pair of centrioles and pericentriolar material (Bornens, 1992; Bornens and Karsenti, 1984; Brinkley, 1985; Kellogg et al., 1994; McIntosh and Euteneuer, 1984). Microtubules are nucleated by γ-tubulin complexes within the pericentriolar material and elongation occurs by plus-end addition of heterodimers of α and β tubulin (Berns and Richardson, 1977; Gould and Borisy, 1977; Oakley and Oakley, 1989). The minus-end of a microtubule is usually anchored in the pericentriolar material, but whether it remains associated with the γ-tubulin complex once nucleated remains to be resolved (McIntosh and Euteneuer, 1984; Shu and Joshi, 1995).

Many differentiated cells including polarised epithelial cells such as intestinal, kidney, retinal pigmented, hepatocyte and certain mammalian cochlear cells display non-centrosomal non-radial microtubule arrays (Achler et al., 1989; Bré et al., 1987, 1990; Gilbert et al., 1991; Ihrke et al., 1993; Meads and Schroer, 1995; Rizzolo and Joshi, 1993; Troutt and Burnside, 1988; Tucker et al., 1992, 1995; see also Mogensen, 1999). Here the majority of the microtubules are associated with the apical cell surface while relatively few radiate from the centrosome. Studies until recently have suggested that nucleating material relocates to the apical cell surface of polarised epithelial cells where it provides new sites for microtubule nucleation (Bré et al., 1990; Tucker et al., 1992, 1995). More recently, further evidence from studies on cochlear epithelial cells suggests that centrosomal nucleation is retained in these cells and that a microtubule release and capture mechanism is responsible for the construction of the apical cell surface associated non-radial microtubule arrays (Mogensen et al., 1997; Mogensen, 1999).

Epithelial cells of the mammalian cochlea provide an ideal opportunity to study microtubule nucleation and anchorage during tissue morphogenesis in situ as they retain their centriole-containing centrosome and some cell types (pillar cells) assemble large apico-basal arrays of several thousand

microtubules (Henderson et al., 1994; Mogensen et al., 1997; Tucker et al., 1992, 1995). In these cells, γ-tubulin and pericentrin are present at the centrosome, but the majority of the microtubules elongate from non-centrosomal apical sites (Mogensen et al., 1997). Genetic, biochemical and structural findings provide compelling evidence for γ-tubulin (as part of a complex) being the nucleator of microtubules (Horio et al., 1991; Joshi et al., 1992; Moritz et al., 1995, 1998; Moudjou et al., 1996; Oakley and Oakley, 1989; Shu and Joshi, 1995; Stearns et al., 1991; Stearns and Kirschner, 1994; Sunkel et al., 1995; Tassin and Bornens, 1999; Vogel et al., 1997; Zheng et al., 1995). Pericentrin has been shown also to play an important role in microtubule nucleation, probably indirectly by providing a lattice for the assembly and organisation of γ-tubulin complexes (Dictenberg et al., 1998; Doxsey et al., 1994; Purohit et al., 1999). The cochlear studies suggest that the γ-tubulin complexes do not play a major role in microtubule anchorage as they are absent from the apical sites. The existence of two centrosomal microtubule minus-end associated complexes, a γ-tubulin nucleating complex and an anchoring complex responsible for the anchorage of microtubule minus-ends released from the γ-tubulin complex, was therefore also proposed following analyses of the cochlear epithelial cells (Mogensen et al., 1997; Mogensen, 1999). The novel concept of two functionally distinct centrosomal complexes is further analysed in this investigation with respect to the centrosomal protein ninein.

Ninein is a coiled-coil protein which localises to the centrosome in most cells which display the classic radial microtubule array. It has a putative GTP binding site, four leucine zipper domains and a potential EF-hand-like domain and may oligomerise and exist in macromolecular complexes (Bouckson-Castaing et al., 1996; and unpublished observations). Ninein has been shown recently to accumulate primarily on the mother centriole in several post-mitotic cells (Piel et al., 2000). In mouse fibroblast (L929) cells, both centrioles possess associated γ-tubulin, and nucleate similar numbers of microtubules in microtubule-repolymerisation experiments, but only the mother centriole is located at the focus of the radial microtubule array during G_1. This suggests a role for ninein in stabilising rather than in nucleating microtubules.

In order to clarify this issue, we conducted immunofluorecence and immunoelectron microscopical analyses of centrosomal ninein in L929 cells during the cell cycle progression, using cells stably expressing a GFP-centrin fusion protein. Due to centrin's striking concentration in the distal lumen of each centriole and in the early pro-centriole buds, this protein represents a very precise marker of the centrosome in living cells and of the cell cycle progression (Piel et al., 2000). Furthermore, cochlear supporting cells were used to investigate whether ninein, like γ-tubulin and pericentrin, is restricted to the immediate vicinities of the centrioles or whether it is located at the non-centrosomal apical sites and thus possibly implicated in microtubule anchorage. Localisation of ninein in both L929 and cochlear supporting cells indicates that ninein is a microtubule minus-end associated protein which is not directly involved in microtubule nucleation, but is important for the stability, positioning and anchorage of microtubules.

MATERIALS AND METHODS

Antibodies

Antibodies to ninein polypeptides Pep1 and Pep3 were generated in rabbits, affinity purified as described previously (Bouckson-Castaing et al., 1996) and were used at dilutions of 1:250 and 1:1000, respectively. Monoclonal antibodies to γ-tubulin (Sigma) and α-tubulin (Amersham) and a rabbit polyclonal antibody to γ-tubulin (Sigma) were used at dilutions 1:1000, 1:500 and 1:5000, respectively. YL1/2 (diluted 1:100) (Serotec) was also used to detect α-tubulin.

Cy3, Cy2 and FITC-conjugated goat anti-rabbit IgG (H+L) diluted 1:400 (Jackson ImmunoResearch Laboratories) and Alexia 488 goat anti-rabbit and Alexia 594 goat anti-mouse (Molecular probes) diluted 1:1000 were used as secondary antibodies. Goat anti-rabbit IgG-conjugated 5 nm colloidal gold diluted 1:40 was obtained from British Biocell International.

Control experiments, which consisted of the omission of the primary antisera, produced no detectable labelling in cochlea cryostat sections or whole mounts of the organ of Corti.

Immunolabelling of GFP-centrin expressing L929 cells

L929 cells were grown in DME medium (Gibco) supplemented with 10% fetal calf serum. Stable clones expressing the centrin/GFP fusion protein were isolated as described elsewhere (Piel et al., 2000). In order to facilitate the location of centrosomes for immunoelectron analysis, cytoplasts were prepared by cell enucleation as described previously (Piel et al., 2000). A combination of nocodazole (5 μM) and cold (40 minutes on ice) was used when required to depolymerise the microtubules. This treatment depolymerises even the most stable microtubules in L929 cells which resist 1 μM nocodazole, and these do not reassemble when the cells are subsequently incubated in warm media containing 5 μM nocodazole.

For indirect immunofluorescence studies cells were rapidly extracted with 0.2% NP40 in BRB80 (80 mM KPIPES, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) for 30 seconds, followed by fixation in a mixture of 2% paraformaldehyde and 0.25% glutaraldehyde in PBS for 3 minutes. After reducing free aldehydes with 0.1% $NaBH_4$ in PBS, the coverslips were incubated in primary antibodies followed by the appropriate secondary antibody coupled to either cyanine 3 (red channel, Jackson Immunoresearch) or AMKA (blue channel, Jackson Immunoresearch). The green channel was used to record the GFP signal.

After immunostaining, cells were imaged on a Leica DMRXA microscope. Image stacks (200-nm steps) were recorded using a piezoelectric objective positioning device and a MicroMAx CCD camera (Princeton Instruments). With a ×100 1.4 NA objective the final magnification on the chip was 67 nm/pixel. All centrin and ninein images shown in L929 cells are maximal intensity projections, while microtubules are presented as self-luminous reconstructions.

Immunoelectron microscopy

Coated coverslips were prepared by pretreating them with a solution containing 20 μg/ml collagen type I from rat tail (Sigma) and 5 μg/ml fibronectin from bovine plasma (Sigma) for 1 hour at 37°C. Cells attached to glass coverslips were incubated in 1.5 μg/ml cytochalasin D (Sigma) for 30 minutes, centrifuged at 15000 ***g*** for 40 minutes, rinsed with fresh medium and left to recover for 4 hours, all at 37°C. The resulting cytoplasts were subsequently extracted with 0.2% v/v NP40 in BRB80 for 30 seconds and fixed in 2% w/v paraformaldehyde in PBS with 0.05% or 0.25% glutaraldehyde for 10 minutes. They were then quenched in PBS containing 0.1% $NaBH_4$ for 15 minutes, incubated with the anti-ninein Pep3 antibody diluted 1: 100 for 1 hour, washed in PBS, incubated with 5 nm gold-conjugated secondary antibody for 1 hour and washed in PBS. The cytoplasts were then post fixed in 2.5% glutaraldehyde for 30 minutes followed by 1% osmium tetroxide for 30 minutes, both in 0.1 M sodium cacodylate buffer and processed for electron microscope

analysis as described previously (Henderson et al., 1994).

Immunolabelling of organ of Corti whole mounts and isolated cells

Mouse (Swiss CD1) cochlea day 0 to 5 were dissected as described previouly (Mogensen et al., 1997). Organs were fixed in 90% v/v methanol and 10% v/v MES buffer (100 mM MES, 1 mM EGTA and 1 mM $MgSO_4$ at pH 6.9) (Sigma) at –20°C for 3 minutes, rehydrated in PBS containing 1% v/v goat serum (Sigma) for 2 minutes and processed as described previously (Mogensen et al., 1997). Alternatively, organ of Corti were transferred to slides and left to dry onto slides prior to immunolabelling. All organ explants were mounted in Citifluor (Agar Scientific). Co-labelling with the mouse monoclonal antibody to γ-tubulin and the rat monoclonal YL1/2 was carried out in a stepwise procedure.

Isolated pillar cells were obtained by aspirating organs of Corti suspended in the final wash of distilled water through a 200 μl yellow tip. The cells were left to dry onto slides and mounted in Citifluor.

Fluorescent images were recorded with a Bio-Rad MRC 600 Series laser scanning/confocal imaging system operating in conjunction with a Nikon Microphot-SA microscope. For detailed analysis of the labelling, projections of serial optical sections collected at 0.2 μm intervals were obtained. Digital image files were transferred to Photoshop for image handling and printed on an Epson 750 colour printer.

Nocodazole treatment of organ of Corti explants

Three day mice cochlea were dissected as above and organ of Corti explants were attachted to collagen coated coverslips (Becton Dickinson). The organ of Corti explants were treated with 10 μg/ml nocodazole diluted in culture medium from a stock solution of 10 mg/ml DMSO for 1 hour at room temperature followed by 3 hours on ice. They were subsequently fixed and incubated in the Pep3 anti-ninein and anti α-tubulin antibodies and processed as above.

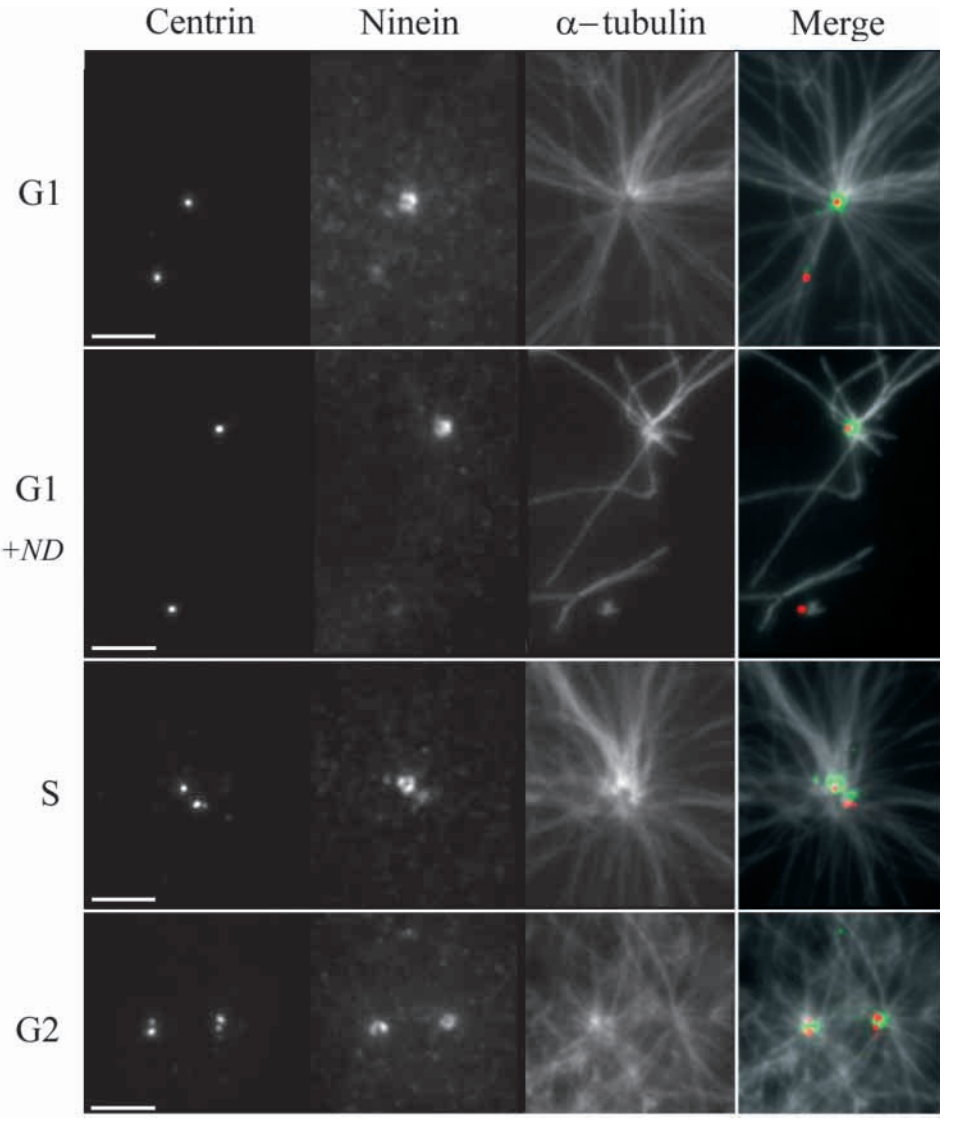

Fig. 1. Ninein associates with the mother centriole in G_1 cultured cells and with both diplosomes in G_2. Synchronised L929 cells expressing GFP-centrin (red in merged image) which concentrates in the lumen of both centrioles, were fixed and labelled with anti-ninein (green in merged image) and anti-α-tubulin antibodies. In G_1 cells, the mother and daughter centrioles can be distinguished by the presence of an aster of microtubules around the mother-centriole only. Ninein staining is conspicuous and organized in several dots, most often three, on the mother centriole whereas it is very weak on the daugther centriole. Note the converging bundles of microtubules abutting in the ninein dots. When G_1 cells are treated for 10 minutes with 1 μM nocodazole (G_1+ND), most of the stable microtubules remain anchored at the mother centriole. During S phase, the centrin-GFP labelling indicates the presence of a bud by a small dot closely associated with each parental centriole (only one is visible in S row, probably due to the orientation of the pro-centriole bud with respect to the parental centriole; but two are evident in G_2 row). An increase in the ninein signal associated with the daughter centriole can be observed. During G_2, the distance between the two GFP-centrin dots in each diplosome has increased and an abundant ninein signal is now seen on both diplosomes. An aster of microtubules can be seen around each diplosome. Bars, 3 μm.

RESULTS

Microtubule organisation and centrosomal ninein distribution in cells which support a radial microtubule array

Ninein localisation was analysed in L929 cells which exhibit a classic radial microtubule array and possess mother and daughter centrioles which are sufficiently separated during G_1 to enable distinction. Furthermore, centrin-GFP expressing L929 cells allowed us to perform double immunofluorescence localisation for ninein (using a Cy3-coupled secondary antibody) and microtubules (using an AMKA-coupled secondary antibody) with respect to the position of the individual centrioles observed in the GFP channel. The

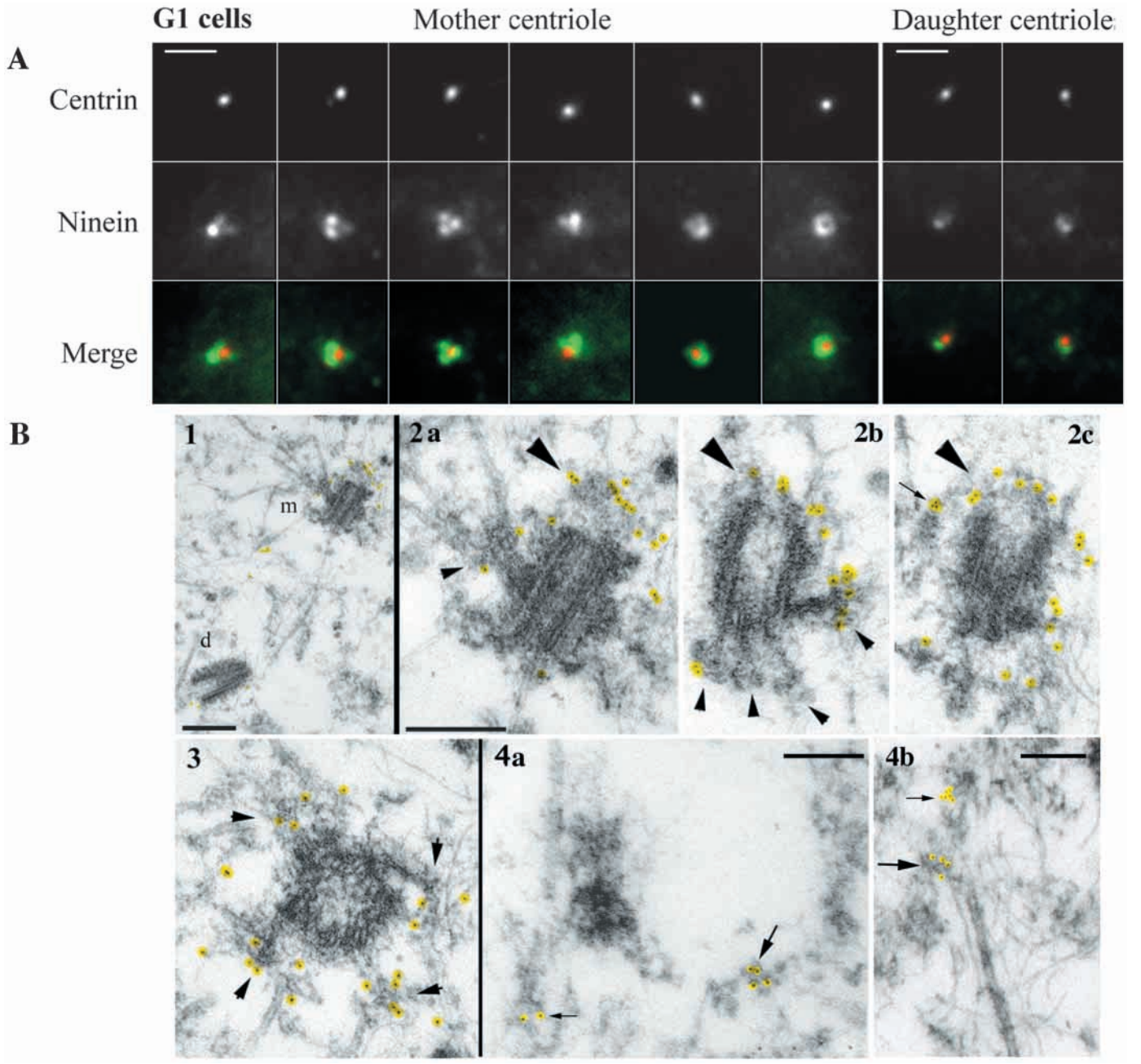

Fig. 2. Ninein distribution within the centrosome of G_1 cells reveals association with the centriolar appendages and microtubule minus-ends. (A) Immunofluorescence staining of ninein on six mother centrioles (left) and two daughter centrioles (right) showing either, from left to right, one strong and two dim dots, two strong and one dim dots, three strong dots, or less defined dots associated with the mother centrioles whereas only a dim dot of ninein is apparent on the daughter centrioles. Bars, 2 μm. (B) Ultrastructural immunogold localisation of ninein (gold particles are indicated by a transparent yellow dot) in 1: low magnification of a centrosome which shows how the mother (m) and the daughter (d) centrioles can be distinguished by the presence of appendages on the former, to which microtubules are associated; 2: sagittal (a), pseudo-sagittal (b) and oblique (c) sections through mother centrioles showing ninein associated with the tip of the centriolar appendages; 3: cross-section of a mother centriole with ninein concentrated on the centriolar appendages and 4: ninein labelling of microtubule minus-ends and small dense aggregates within the pericentriolar material. Bars, 0.3 μm (1), 0.2 μm (2,3) and 0.1 μm (4). The electron microscope analysis in B suggests that the different patterns of immuno-fluorescent dots observed on the mother centrioles in A (left) reflect different organisations of the appendages at the tips of which gold particles accumulate (small arrowheads). A high concentration of ninein is also evident at the proximal-end and thus the minus-end of the mother centrioles (large arrowheads) and the daughter centrioles (not shown). The weakly stained dot of ninein observed in immuno-fluorescence on the daughter centrioles in A (right) probably corresponds to the presence of ninein at the proximal end of the centrioles. The electron microscope analyses also reveal gold particles at the end of microtubules in the vicinity of the centrioles (small arrows) and on small aggregates of dense material (large arrow) within the pericentriolar material.

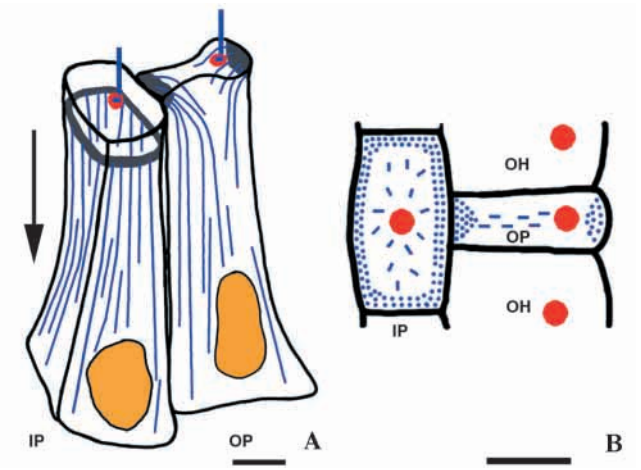

Fig. 3. Schematic diagrams of the microtubule organisation (blue lines) in the inner pillar (IP) and outer pillar (OP) cells when the apico-basal arrays are assembling. Bars, 5 μm.
(A) 3-D representation of the IP and OP cells showing apically located centrosomes (red, with centriole and primary cilium in blue) and the majority of the microtubules associated with an apical peripheral ring of dense material (grey) in the IP and two dense aggregates (grey) at either end of the apex in the OP. The nucleus (yellow) is located near the base of the cells. The arrow indicates the direction of microtubule elongation. (B) Apical view of the pillar cells showing the location of the centrosomes (red), microtubule ends (blue dots) at the apical sites and the transient microtubule population (blue lines). A peripheral ring of about 3000 microtubule ends, located about 1 μm below the centrosome, is evident in the IP. The microtubule ends are concentrated at either end of the apex in the OP. Some 4000 microtubule ends are concentrated at the apical site which is located closest to the IP and up to 7 μm away from the centrosome, while about 1500 are evident at the other site.

centrosomal distribution of ninein during cell cycle progression is shown in Fig. 1. Ninein is associated primarily, if not exclusively, with the mother centriole, which is at the centre of the aster of microtubules during G_1 (Piel et al., 2000). Only the ninein-containing centriole maintains an aster of microtubules after treatment with low doses of Nocodazole, indicating that these are more stable (Fig. 1, second row). Progressive accumulation of ninein is evident on both diplosomes, together with the appearance of two asters of microtubules during S-G_2.

The conspicuous ninein staining on the mother centriole is of particular note, as it is often organised into several dots, most often three, although variations to this pattern could be observed. Several examples are shown in Fig. 2A. We often noted also that the converging bundles of microtubules seemed to abut the ninein-containing dots (see Fig. 1). This suggests that they may correspond to the subdistal appendages which surround the mother centriole, at the tip of which microtubules are often seen to terminate at the EM level (De Brabander, 1982; Gorgidze and Vorobjev, 1995). The specific localisation of ninein within the centrosome of L929 cells was therefore investigated using immunoelectron microscopy of cytoplasts from G_1 cells. We used a pre-embedding immunogold labelling procedure which enables clear identification of microtubules and centriolar sub-structures. Gold labelling is evident within the pericentriolar material as observed previously (Bouckson-Castaing et al., 1996), but distinct associations with microtubule-ends and the subdistal centriolar appendages are also evident. Specific labelling of the tips of the subdistal appendages, which support a fascicle of microtubules, is clearly evident (Fig. 2B). A relatively high concentration of gold particles is apparent within dense material associated with one of the centriolar ends. In sections where the proximal-end of the centriole, and thus the minus-ends of its microtubules, can be defined (due to the position of the subdistal and distal appendages) the gold labelling seems to preferentially associate with the minus-end. Microtubule-ends within the pericentriolar material are difficult to define, but a few microtubules show apparent end-on association with gold labelled dense material (Fig. 2B2c,4a,b). These labelled microtubule-ends are most likely to be the minus-ends as they are situated in relatively close proximity to a centriole.

Microtubule organisation in cochlear supporting cells which display a non-radial array

The construction of large apico-basal microtubule arrays in the supporting inner and outer pillar cells of the organ of Corti involves microtubule nucleation at the centrosome, release,

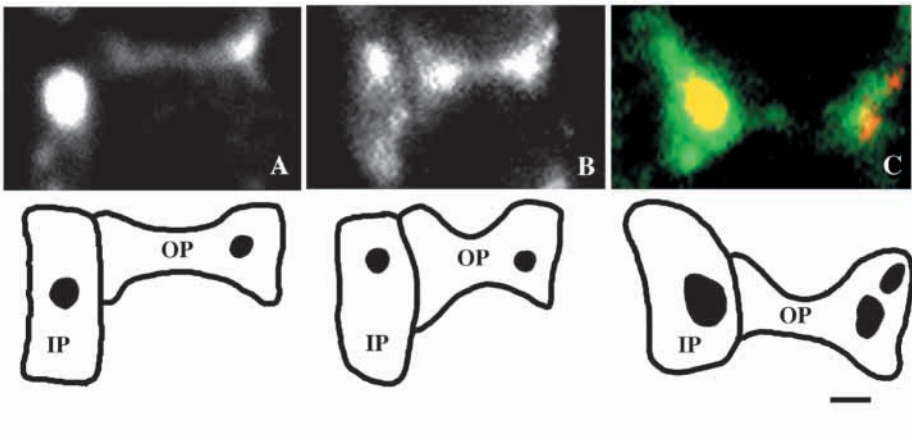

Fig. 4. Projections of optical sections through the apices of inner (IP) and outer (OP) pillar cells labelled for α-tubulin in A and B or double labelled for α and γ-tubulin in C at different stages of assembly of the apico-basal microtubule arrays. Cell outlines together with the centrosome profiles are diagrammatically represented below. Bar, 2 μm. (A) Tubulin labelling is mainly concentrated at the centrosome by early day 1, but there is evidence also of some diffuse labelling in the region between the centrosome and the apical sites in both pillar cells. (B) A concentration of tubulin labelling is apparent at the apical sites and at the centrosome by late day 2. The region between the centrosome and the apical sites, where the transient microtubule population has been reported previously, is now showing distinct staining. (C) Double staining for α-tubulin (green) and γ-tubulin (red) reveals co-localisation (yellow/orange) at the centrosome in the inner and outer pillar cells. There is no evidence of binding of the antibody to γ-tubulin at the apical sites or in the region between the centrosome and the apical sites.

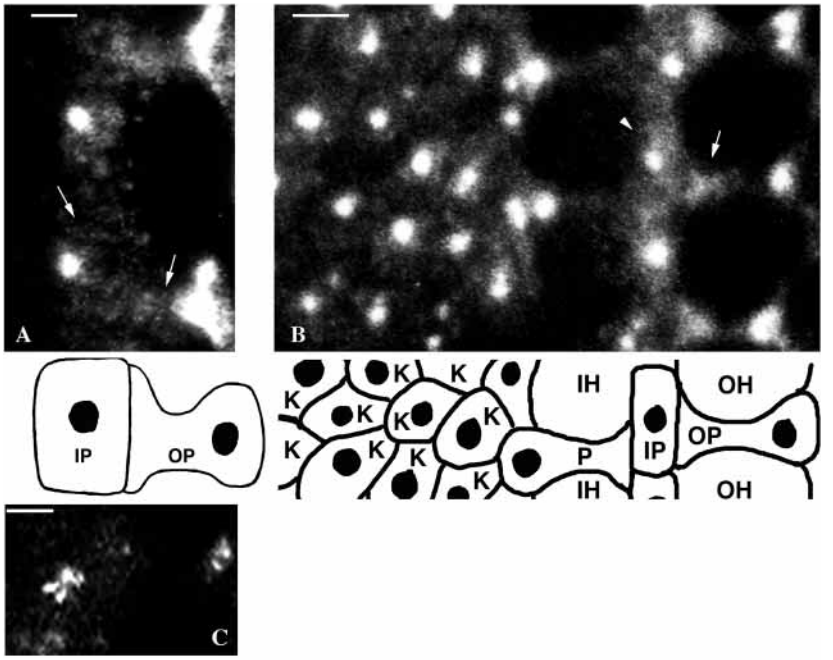

Fig. 5. Projections of optical sections through the apical regions of supporting cells labelled with an antibody to ninein. Cell outlines are diagrammatically represented below. (A) Apical view of ninein localisation at early day 1 in two inner (IP) and two outer (OP) pillar cells. Ninein is mainly concentrated at the centrosome and its immediate environ, but diffuse labelling is apparent also in the region between the centrosome and the apical sites in both pillar cells (arrows). Bar, 2.5 μm. (B) Apical view of ninein distribution at late day 2 in the Kollikers' (K), inner phalangeal (P), inner hair (IH), inner pillar (IP), outer pillar (OP) and outer hair (OH) cells showing distinct presence in the supporting cells (K, P, IP and OP) while little or no ninein in the hair cells (IH and OH). The centrosomes label strongly in all the supporting cells but non-centrosomal labelling is evident also. The apical site where some 4000 microtubule minus-ends are accumulating in the outer pillar cells shows particularly high non-centrosomal concentration of ninein (arrow). The non-centrosomal ninein in the inner pillar cells is evident at the apical sites and in the region occupied by the transient microtubule population (arrowhead). Ninein is distributed diffusely throughout the non-centrosomal apical region in most of the Kollikers' cells. Bar, 5 μm. (C) A single optical section through the apex of an IP and an OP cell at the level of the centrioles showing ninein staining organised into two or more dots at late day 0. Bar, 2 μm.

translocation and subsequent capture at apical non-centrosomal sites (Mogensen et al., 1997; Mogensen, 1999). Developing mouse cochlear epithelia cells provide an excellent system to study this proposal in detail.

Assembly of these apico-basal arrays proceeds over the first 6 days after birth. Only a few microtubules radiate from the apical located centrosome while assembly of the apico-basal arrays is progressing. In the inner pillar cells some 3000 microtubules elongate from a peripheral ring of dense material, which results in the construction of a tube of microtubules (Fig. 3; see also Fig. 7 of Mogensen et al., 1997). In the outer pillar cells up to 4000 microtubules project from dense aggregates located on opposite sides of the apical cell surface and up to 7 μm away from the centrosome (Fig. 3; see also Fig. 3 of Tucker et al., 1995). Evidence suggests that a population of microtubules in transit is present in the inner and the outer pillar cells between the centrosome and the apical sites while microtubule assembly is proceeding (Fig. 3B). Microtubule hook-decoration and immuno-localisation of an antibody to the microtubule plus-end associated protein adenomatous polyposis coli tumour suppressor (APC) (Nathke et al., 1996) reveal uniform polarity of the apico-basal arrays and microtubule minus-ends at the apex (data to be published elsewhere). This means that thousands of microtubule minus-ends are concentrated at the apical sites in the pillar cells. Detection of microtubule minus-end associated proteins would thus be expected.

The microtubule nucleating proteins γ-tubulin and pericentrin have previously been shown to be present at the centrosome while absent from the apical sites where the majority of the microtubules are associated (Mogensen et al., 1997). This suggests that microtubule nucleation is restricted to the centrosome. Here we confirm by double immuno-labelling with antibodies to α-tubulin and γ-tubulin that γ-tubulin is confined to the centrosome. Optical sections through the apex (to a depth of about 3 μm) of inner and outer pillar cells show a distinct concentration of α-tubulin labelling in the centrosomal region at early day 1 (Fig. 4A). Only after day 1 does the α-tubulin labelling become apparent at the apical sites in the inner pillar cell. Likewise, in the outer pillar cell, apical α-tubulin staining is only apparent at about day 2. The intensity of staining at these sites increases with time suggesting that microtubules are gradually concentrating at the apical sites

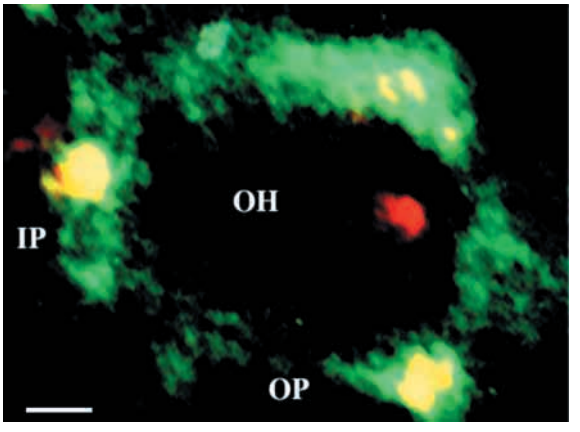

Fig. 6. A projection of optical sections through the apex of an inner pillar (IP), outer hair (OH) and two outer pillar (OP) cells co-labelled with antibodies to γ-tubulin (red) and ninein (green) at day 3. Ninein co-localises with γ-tubulin (yellow) at the centrosome in the pillar cells but is apparently absent from the apical hair cell region. Ninein is also present at the apical sites and in the regions occupied by the transient microtubule population in the pillar cells. Bar, 2 μm.

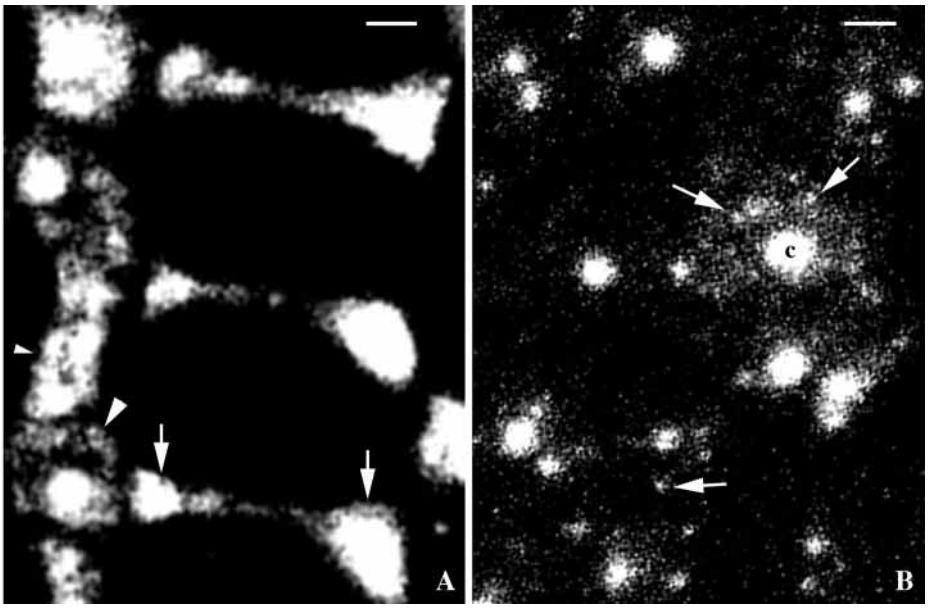

Fig. 7. Projections of optical sections through the apical region of the pillar and Kollikers' cells at day 4 showing ninein localisation at the centrosome and at the apical sites. Bars, 2 μm. (A) At this stage the transient population of microtubules has virtually gone and most of the microtubule minus-ends are concentrated at the apical sites in the pillar cells. A distinct peripheral ring (large arrowhead) of ninein is evident in some of the inner pillar cells while ninein is present throughout the apex in others (small arrowhead). Most of the ninein is concentrated at either side of the apex in the outer pillar cells (arrows), where most of the microtubule minus-ends are concentrated. The centrosome merges with the closest apical site (to the left). (B) In the Kollikers' cells, where microtubules project from multiple small sites scattered over the apical region, distinct small foci of ninein are evident in some (arrows). Ninein is also concentrated at the centrosome (c).

(Fig. 4B). α-tubulin is also evident in the region between the centrosome and the apical sites, where the microtubule population in transit has been reported previously. The intensity of labelling in this region gradually increases until day 3 before decreasing and disappearing by day 4 to 5 (Fig. 4). There is no evidence of γ-tubulin in the region occupied by the microtubule population located between the centrosome and the apical sites or at the apical non-centrosomal sites in the pillar cells (Fig. 4C).

Presence of ninein in centrosomal as well as apical non-centrosomal regions in the supporting cells

Analyses of the apical binding of the anti-ninein antibodies to whole mounts of organ of Corti reveal distinct labelling patterns in the supporting cells which are cell type specific and dependent on the stage of development. The location of the supporting cells can be identified by analysing the apical surface of the organ of Corti (see Mogensen et al., 1997; see also diagram in Fig. 5). Two anti-ninein antibodies raised against Pep1 and Pep3 to non-overlapping regions of ninein were used. They both reveal identical results, labelling the centrosome strongly in the supporting cells while only very weakly or not at all in the sensory hair cells (Fig. 5). Single optical sections through the centrioles of the pillar cells reveal that ninein is organised into two or more dots similar to those observed for the cultured cells (Fig. 5C). However, projections of several optical sections through the apical region reveal that ninein labelling is not confined to the centrosome in the supporting cells, but is

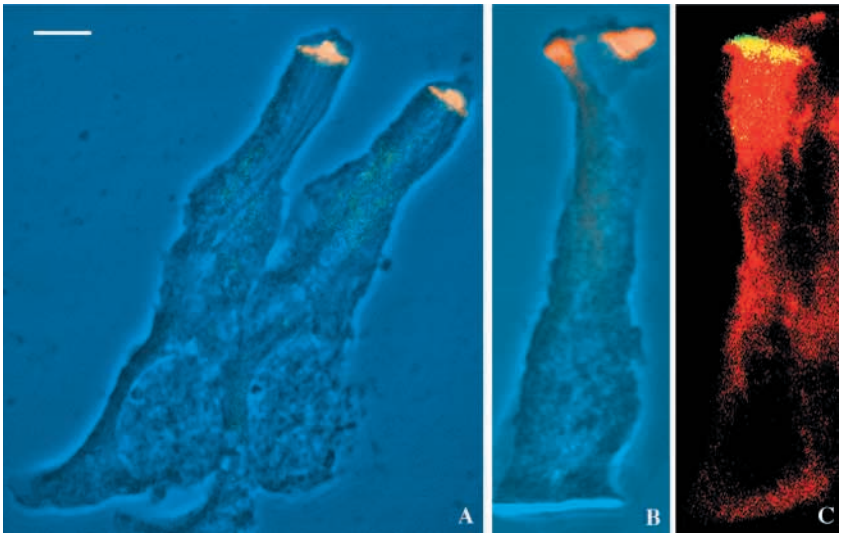

Fig. 8. Isolated inner and outer pillar cells from organ of Corti at day 4, when the transient microtubule population has diminished, showing localisation of ninein in A and B and ninein and tubulin in C. Phase contrast (blue) images have been superimposed on the fluorescent images in A and B. Bar, 5 μm. (A) A projection of optical sections through the lateral length of two inner pillar cells showing ninein (orange) concentrated in an apical band corresponding to the apical sites where up to 3000 microtubule minus-ends are located. A single dot of ninein is evident above the band at the location of the centrosome. Ninein is present at a relatively low level in the cytoplasm in the apical half of the cells. (B) A projection of optical sections through the lateral length of an outer pillar cell showing ninein (orange) accumulation at either end of the apex at the location of the apical sites where up to 4000 microtubule minus-ends are concentrated. Ninein labelling of the centrosome merges with that of the closest site (to the left). Cytoplasmic ninein is apparent in the apical half of the cell. (C) Co-labelling with antibodies to ninein (green) and α-tubulin (red) reveals co-localisation (yellow) at the minus-end of the microtubule array in an inner pillar cell. A primary cilium projects at the top.

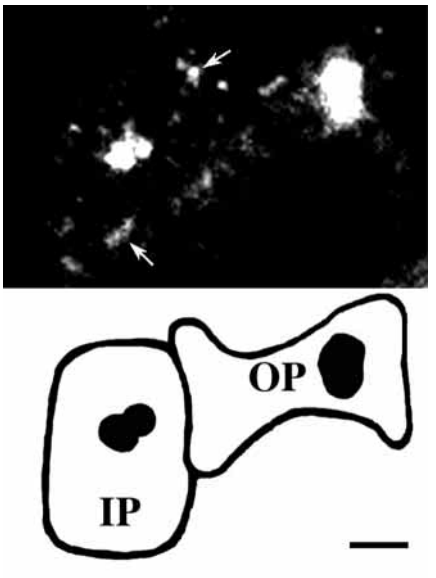

Fig. 9. A projection of optical sections through the apices of an inner and an outer pillar cell at day 3 treated with nocodazole showing a marked reduction in ninein at the apical sites and in the region normally occupied by the transit microtubule population. A high concentration of ninein is evident at the centrosomes while a few aggregates are apparent at the apical sites (arrows). A diagrammatic outline of the inner (IP) and outer (OP) pillar cells is shown below. Bar, 2 μm.

evident also in the apical non-centrosomal region during days 1 to 3 after birth. The non-centrosomal labelling gradually increases in extent and intensity during this period. Ninein is mainly concentrated at the centrosome and its immediate environ in all three supporting cells at early day 1 (Fig. 5A), whereas it is evident throughout the apical region by day 2 (Fig. 5B). The non-centrosomal ninein labelling is particularly prominent at the apical sites where some 4000 microtubule minus-ends are accumulating in the outer pillar cells by day 2, while labelling is evenly distributed throughout the non-centrosomal apical region in the inner pillar cells (Fig. 5B). Both the inner and the outer pillar cells show relatively strong non-centrosomal ninein labelling throughout the apical region by day 3 (Fig. 6). In another cochlear supporting cell type, the Kollikers' cells, diffuse labelling is evident throughout the apical non-centrosomal region during days 2 to 3 (Fig. 5B). These cells resemble typical simple epithelial cells and have microtubules projecting from multiple foci located throughout the apical surface (see Mogensen et al., 1997).

The region between the centrosome and the apical sites, where the transient population of microtubules occurs, labels also with the ninein antibodies. The intensity of the labelling of this region gradually increases and is greatest at about day 3 when the transient population is most prominent (Fig. 6). Analyses of optical sections through the apex of organ of Corti at day 3 double labelled with anti-ninein and anti-γ-tubulin antibodies reveal co-localisation at the centrosome, but the presence of ninein only in the apical non-centrosomal regions (Fig. 6). Double labelling of organ of Corti whole mounts with anti-ninein and anti-α-tubulin antibodies reveals co-localisation at the centrosome, the apical sites and at the location of the transit microtubule population (results not shown). This suggests that ninein is present at the centrosome and at the apical sites where most of the microtubule minus-ends accumulate. It also suggests that ninein is associated with the transient microtubule population during the assembly of the apico-basal arrays in the supporting cells.

Presence of ninein in the apical non-centrosomal sites

The localisation of anti-ninein antibodies was analysed in whole mounts of organ of Corti at days 4 and 5 when the transit microtubule populations have gone and several thousand microtubule minus-ends are concentrated at the apical sites. Optical sections through the apex reveal a distinct peripheral ring in some inner pillar cells while in others the entire apex stains with the antibodies to ninein (Fig. 7A). Not all cells assemble perfect tubular microtubule arrays or develop at the same rate, thus ring-like labelling would not be expected in all cells. Two strongly stained distinct foci are evident at either end of the apex in the outer pillar cells (Fig. 7A). The ninein antibodies also bind to the centrosome with a single dot being evident within the lumen of the peripheral ring in the inner pillar cell in optical projections. The centrosomal labelling in the outer pillar cell merges with that of the closest (by 1-2 μm) apical site, thus only two definable regions of staining can be identified (Fig. 7A). In the Kollikers' cells centrosomal and non-centrosomal ninein labelling is evident as at the earlier stage, but some cell apices reveal distinct small aggregates of staining scattered throughout the apical surface (Fig. 7B).

Ninein distribution was further analysed during this 'post-transit' microtubule stage in isolated inner and outer pillar cells at days 4 and 5. Projections of optical sections through the lateral length of the inner pillar cells reveal labelling of a single apical band 1-2 μm below the apex with a foci above it (Fig. 8A). Two large foci located at either end of the apex label strongly with the ninein antibodies in the outer pillar cells (Fig. 8B). Evidence of weak cytoplasmic labelling in the apical halves of both inner and outer pillar cells is apparent at this stage. The ninein labelling in the pillar cells suggests that it corresponds to the location of the centrosomes and the peripheral ring in the inner pillar cells and the dense aggregates in the outer pillar cells, where several thousand microtubule minus-ends are concentrated. This was confirmed by double labelling with antibodies to ninein and α-tubulin which reveals co-localisation of the ninein and α-tubulin antibodies at the apical minus-end of the apico-basal microtubule arrays (Fig. 8C).

Effect of nocodazole on ninein which co-localises with the microtubule population in transit

The anti-ninein staining patterns vary with the stage of development and closely mimic the distribution of microtubules during this period when assembly is proceeding (see Figs 4, 5, 6, 7).

This suggests that ninein is released from the centrosome and translocates to the apical sites together with the microtubules and that ninein may be associated with the microtubules. If ninein is associated with the microtubules in transit then their depolymerisation should disrupt the distribution of ninein in this region. This was investigated by treatment with the microtubule depolymerising drug nocodazole.

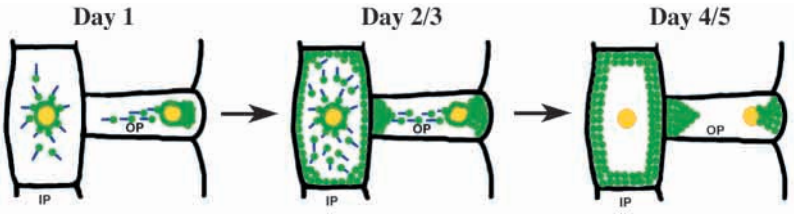

Fig. 10. A diagrammatic representation of the distribution of ninein during assembly of the apico-basal microtubule arrays in the inner (IP) and outer (OP) pillar cells. It is proposed that ninein is released from the centrosome and translocates with the microtubules to the apical sites, where ninein is captured, and anchors the minus-ends of the microtubules. Ninein (green dots) is mainly concentrated at the centrosome at day 1 when microtubule assembly has been initiated and most of the microtubules (blue lines) are concentrated in the centrosomal region. A few microtubules with associated ninein occupy the "transit" region. Ninein gradually increases and is prominent at the apical sites and in the region occupied by the microtubule population in transit at day 2/3. Subsequently, ninein becomes concentrated at the centrosome and at the apical sites as the microtubule population in transit diminishes at day 4/5. In the inner pillar cell a distinct peripheral ring of ninein is evident where some 3000 microtubule minus-ends are concentrated. In the outer pillar cells ninein aggregates at either end of the apex where, respectively, 4000 and 1500 microtubule minus-ends are concentrated.

Exposure to nocodazole at day 3 causes depolymerisation of most of the microtubules in the apical region in most of the pillar cells with a few stable microtubules remaining (result not shown). Nocodazole treatment does not appear to affect ninein's association with the centrosome in the pillar or Kollikers' cells but its localisation in the region of the transit population is lost. Furthermore, a punctate peripheral ring of ninein is evident in the inner pillar cells and a few foci are apparent at the distal apical site in the outer pillar cells (Fig. 9). This suggests that ninein is dependent on the microtubules for its localisation in the "transit" region while it may be stably bound to the centrosome and the apical sites.

DISCUSSION

Microtubule nucleation and anchorage

The centrosome is responsible not only for the nucleation of microtubules, but also for their anchorage. Although substantial evidence points to the γ-tubulin complex as the nucleator of microtubules, it remains to be established whether it also plays a major role in microtubule anchorage. Previous evidence from studies on cochlear supporting cells suggests that other centrosomal complexes may be responsible for microtubule anchorage (Mogensen et al., 1997; Mogensen, 1999). Two functionally distinct microtubule minus-end associated complexes, a nucleating and an anchoring, have been suggested to be fundamental components of the centrosome. This is supported also by recent findings in L929 cells which reveal that the mother and daughter centrioles make distinct contributions to centrosomal activity and behaviour which may be related to their differences in microtubule anchoring and release (Piel et al., 2000). This novel concept of a centrosomal anchoring complex, distinct from the γ-tubulin nucleating complex, has important implications for microtubule organisation in cells generally. Evidence from two different cell systems presented in this investigation suggests that the centrosomal protein ninein is involved in microtubule minus-end anchorage and stability and that it is a candidate for the proposed anchoring complex.

Ninein and microtubule minus-ends

Ninein, which localises to the centrosome in most cells, has been shown recently to accumulate primarily on the mother centriole which is located at the focus of the radial microtubule array during G_1 (Piel et al., 2000). This, together with the higher stability of the mother centriole-associated microtubules, suggests a stabilising mechanism of the aster of microtubules on the mother centriole, distinct from the nucleating activity of the centrosome. Ninein would be an obvious candidate for participating in the stabilisation of the aster.

Immuno-localisation of ninein during the progression of the cell cycle reported here, as well as immunoelectron microscopical analyses of ninein localisation within the centrosome of L929 cells, provide further evidence for microtubule minus-end association and its role in anchorage. Specific localisation of ninein at the tips of the subdistal centriolar appendages provides strong evidence for a role in anchorage (Moudjou et al., 1996; Paintrand et al., 1992) and minus-end association as a fascicle of microtubules projects from these appendages. Microtubule ends within the pericentriolar material also reveal ninein association and those in close proximity of a centriole, which enables prediction of polarity, suggest minus-end affinity. Interestingly, the centriole shows strong ninein association with its minus-end. The proximal end of a centriole which contains the minus-ends of its wall microtubules would be expected to sequester and anchor any minus-end binding proteins (Tassin and Bornens, 1999). Ninein association with the minus-end of the centrioles is therefore significant and indicative of a microtubule minus-end binding protein.

In the cochlear supporting cells ninein is not confined to the centrosome. Ninein is present also at the apical sites where the majority of the microtubule minus-ends are concentrated. The distribution of ninein is dependent on the microtubules and varies with their organisation, producing distinct patterns within the apex in the Kollikers' and pillar cells at different stages of microtubule assembly. Ninein co-localises with γ-tubulin at the centrosome and gradually accumulates at the apical non-centrosomal sites as microtubule assembly progresses. This accumulation coincides with the gradual increase in microtubules at the apical sites. In the inner pillar cells ninein is evident at the end of the tubular array of microtubules localised to the apical peripheral ring where about 3000 microtubule minus-ends are associated. Ninein is present at the end of the two microtubule arrays at either side of the apex in the outer pillar cell where some 4000 and 1500 microtubule minus-ends are concentrated respectively. Multiple focal points of ninein become evident in the

Kollikers' cells once the apico-basal microtubule arrays are established. The distinct localisation of ninein within these three supporting epithelial cells strongly suggests that ninein associates with the minus-end of microtubules.

Ninein and microtubule translocation and anchorage

Evidence from the cochlear supporting cells suggests that γ-tubulin is concentrated at the centrosome. It is apparently not released with the microtubules as it is absent from the population of microtubules in transit. Similarly, γ-tubulin is confined to a single apical focal point in other polarised epithelial cells such as MDCK and those of the retinal pigment epithelium (Meads and Schroer, 1995; Rizzolo and Joshi, 1993). Evidence from neurons also indicates that γ-tubulin may not remain associated with the minus-end of microtubules once released from the centrosome (Baas and Joshi, 1992). In the cochlea ninein, on the other hand, co-localises with the population of microtubules in transit and shows a distinct and dramatic change in its distribution from the centrosome to the apical sites as assembly progresses. This suggests that ninein is released from the centrosome. The progressive shift in ninein localisation mimics that of tubulin, suggesting that ninein is released from the centrosome with the microtubules. Furthermore, it also suggests that ninein translocates to the apical sites with the microtubules (Fig. 10). In agreement with this view, nocodazole-induced microtubule depolymerisation in the pillar cells results in a marked reduction in the non-centrosomal ninein whereas there is no apparent change in the centrosomal localisation. The most marked change in the ninein distribution is in the region normally occupied by the microtubule populations in transit suggesting that ninein is dependent on the microtubules and that it associates with microtubules. The reduced ninein association at the apical sites indicates that not all ninein is firmly attached at this stage and is therefore lost or redistributed when the microtubules depolymerise. Nocodazole treatment did not prove effective beyond day 3 and the predicted increase in anchored ninein at the apical sites at later stages of microtubule assembly could not be verified.

Centrosomal microtubule nucleation and release is not a new concept and has convincingly been shown to be the main mechanism for producing non-centrosomal, free microtubules in the axons and dendrites of neurons and has recently been visualised in epithelial culture cells (Baas, 1998; Keating et al., 1997). However, in polarised epithelial cells, unlike in neurons, microtubules are proposed to be captured at the apical sites following their release. Microtubule minus-end capping, stabilisation and anchorage is evidently an important part of this mechanism. The present findings provide further support for centrosomal microtubule nucleation, release and subsequent translocation and capture at the apical non-centrosomal sites and suggest that ninein provide the essential stabilising and anchoring factor.

Ninein, a non-nucleating microtubule minus-end stabilising and anchoring protein

Ninein is evidently a strong contender for the postulated centrosomal anchoring complex (Mogensen et al., 1997). Ninein's association with the microtubules in transit suggests that it may perform an additional role in capping and stabilising the minus-end of centrosomally released microtubules. This is supported by recent findings from studies on L929 cells which show that ninein preferentially associates with the mother centriole. This is significant as only the mother centriole is able to maintain a radial microtubule array although both centrioles contain γ-tubulin and assemble similar numbers of microtubules in microtubule-repolymerising experiments (Piel et al., 2000). Furthermore, ninein's specific localisation to the tips of centriolar appendages and the minus-ends of microtubules and centrioles within the centrosome, as shown in this investigation, provides further support for its role in microtubule minus-end anchorage.

Whether ninein is directly interacting with microtubules or forms a complex with other anchoring proteins is currently not known. Interestingly, the centrosomal protein R2 (Blomberg-Wirschell et al., 1998) which, like ninein, associates with the tips of centriolar appendages is a potential microtubule anchoring protein. Evidence is clearly emerging which strongly suggests that the centrosome contains separate microtubule nucleating and anchoring complexes.

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