CEP110 and ninein are located in a specific domain of the centrosome associated with centrosome maturation

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Summary

The mammalian centrosome consists of a pair of centrioles surrounded by pericentriolar material (PCM). The architecture and composition of the centrosome, especially the PCM, changes during the cell cycle. Recently, a subset of PCM proteins have been shown to be arranged in a tubular conformation with an open and a closed end within the centrosome. The presence of such a specific configuration can be used as a landmark for mapping proteins in both a spatial and a temporal fashion. Such mapping studies can provide information about centrosome organization, protein dynamics, protein-protein interactions as well as protein function. In this study, the centrosomal proteins CEP110 and ninein were mapped in relationship to the tubular configuration. Both proteins were found to exhibit a similar distribution pattern. In the mother centrosome, they were found at both ends of the centrosome tube, including the site of centrosome duplication. However, in the daughter centrosome they were present only at the closed end. At the closed end of the mother and daughter centrosome tube, both CEP110 and ninein co-localized with the centriolar protein CEP250/c-Nap1, which confirms ninein's centriole association and places CEP110 in association with this structure. Importantly, the appearance of CEP110 and ninein at the open end of the daughter centrosome occurred during the telophase-G1 transition of the next cell cycle, concomitant with the maturation of the daughter centrosome into a mother centrosome. Microinjection of antibodies against either CEP110 or ninein into metaphase HeLa cells disrupted the reformation of the tubular conformation of proteins within the centrosome following cell division and consequently led to dispersal of centrosomal material throughout the cytosol. Further, microinjection of antibodies to either CEP110 or ninein into metaphase PtK2 cells not only disrupted the tubular configuration within the centrosome but also affected the centrosome's ability to function as a microtubule organizing center (MTOC). This MTOC function was also disrupted when the antibodies were injected into post-mitotic cells. Taken together, our results indicate that: (1) a population of CEP110 and ninein is located in a specific domain within the centrosome, which corresponds to the open end of the centrosome tube and is the site of protein addition associated with maturation of a daughter centrosome into a mother centrosome; and (2) the addition of CEP110 and ninein are essential for the reformation of specific aspects of the interphase centrosome architecture following mitosis as well as being required for the centrosome to function as a MTOC.

Key words: CEP110, Ninein, CEP250, c-Nap1, Centrosome

Introduction

The centrosome has been implicated in events as diverse as the organization of the interphase cytoskeleton and mitotic spindle, cytokinesis, intracellular placement of proteins and organelles, and the formation of cilia and flagella (for reviews, see Brinkley, 1985; Mazia, 1987; Tassin and Bornens, 1999; Mack et al., 2000; Compton, 2000). In addition, the centrosome must duplicate itself once each cell cycle. To accommodate these diverse functions, there must be a precise organization of proteins with functional correlates within the centrosome. However, at present, information concerning protein arrangement in the centrosome is limited.

The centrosome in mammalian cells has been thought to consist of an amorphous spherical mass of centrosomal proteins known as the pericentriolar material or PCM organized around a pair of centrioles or centriole duplex (reviewed by Mack et al., 2000). Recently, we have shown that a specific subset of PCM proteins are not arranged in this manner but are rather organized in a highly specific and reproducible configuration within the centrosome. In this report, we will refer to this configuration as the 'centrosome tube'. This configuration appears as either an 'O' or a 'U' profile when the centrosome is stained with specific antibodies and viewed by conventional indirect immunofluorescence microscopy (IIF) (Ou and Rattner, 2000). The 'O' and 'U' profiles can actually be seen in images present in many studies in the literature using a variety of antibodies to stain the centrosomes [(e.g. Young et al., 2000) Fig. 1A; (Mogensen et al., 2000) Figs 1, 2, ninein], although the significance of the images was not apparent. Using digital confocal microscopy, we found that these profiles reflect an underlying tubular protein configuration that is closed at one end and open at the

other (Ou and Rattner, 2000). The centriole proteins CEP250/c-Nap1 and Nek2, which have been shown to associate specifically with the proximal ends of both mother and daughter centrioles (Fry et al., 1998), have been mapped to the closed end of the tube, suggesting that the centriole is preferentially placed towards this end of the tube. Recently, using immuno-electron microscopy, we have found that the parental centriole actually occupies most of the tube lumen. Thus, the tube represents an arrangement of a subset of PCM proteins surrounding the parental centriole (Y.Y.O. and J.B.R., unpublished).

Centrosome duplication usually occurs at the open end of the centrosome tube although occasionally it can also be seen at other sites along the side of the mother centrosome tube. Since centrosome duplication occurs at the site of the daughter centriole duplex, this variation in the duplication sites may reflect the movement seen between parent and daughter centrioles that has been detected in living cells (Piel et al., 2000). Importantly, the tubular configuration of this subset of proteins is duplicated in concert with centrosome duplication. Thus, the ability to visualize the tubular configuration can be used as a light microscope landmark from which individual centrosomal proteins can be mapped both spatially and temporally during centrosome duplication and the cell cycle. Such data may provide insights into the protein dynamics, protein-protein interactions, and protein functions within the centrosome. Furthermore, it may also allow the determination of the precise effects on centrosome structure and duplication of experimental alterations in the abundance of specific proteins. In this study, we carried out fine-mapping and functional studies of two centrosomal proteins CEP110 and ninein.

CEP110 is a novel centrosomal protein that was identified in our laboratory by screening a human cDNA expression library using a centrosome-reactive human autoimmune serum (Guasch et al., 2000). A portion of the gene encoding CEP110 was found to be fused in-frame with a portion of the gene that encodes the protein kinase domain of a receptor tyrosine kinase for the fibroblast growth factor (FGFR1) in a myeloproliferative disorder associated with the chromosomal translocation t(8;9)(p12:q33) (Guasch et al., 2000). The finding that the chimeric CEP110-FGFR1 protein has a constitutive kinase activity led to the proposal that the malignancy that arises from the CEP110-FGFR1 translocation may involve a combination of constitutive activity of the FGFR1 kinase domain and the disruption of normal centrosomal function due to the alteration of the CEP110 protein.

Conventional IIF using antibodies raised against CEP110 suggests that CEP110 has a unique cell-cycle-dependent distribution when compared with most other centrosomal proteins (Guasch et al., 2000). CEP110 is detected in the G1 centrosome. However, following centrosome duplication and separation, CEP110 is observed in association with only one of the two centrosomes, presumably the mature one. This pattern persists until the onset of prophase, at which time CEP110 is also detected at the second centrosome. Whereas many centrosomal proteins such as pericentrin and γ-tubulin rapidly accumulate at the centrosome as the cell approaches mitosis (Dictenberg et al., 1998; Khodjakov and Rieder, 1999), CEP110 declines at this time and reappears during the transition from telophase to G1. The distinctive distribution pattern of CEP110 led us to speculate that this protein may have a function in early centrosome organization and in centrosome maturation.

To obtain more information about the role of CEP110 within the centrosome, we fine-mapped its location within the centrosome during the cell cycle. We show that CEP110 was associated with both the open and the closed ends of the tubular conformation of the mother centrosome at G1. Subsequently, CEP110 was also present at the region where the onset of centrosomal duplication occurs. In daughter centrosomes with a complete tubular configuration of proteins, CEP110 was present only at the closed end. We compared the distribution of CEP110 to that of other centrosomal proteins and found that ninein mapped to regions similar to those of CEP110. Ninein is a coiled-coil protein of 220-240 kDa (Bouckson-Castaing et al., 1996; Hong et al., 2000) that previously has been localized to the centrioles and shown to function as a microtubule minus-end capping and anchoring protein (Piel et al., 2000; Mogensen et al., 2000). The cDNA encoding the human homologue of ninein was initially identified in our laboratory while screening a cDNA expression library with the autoimmune serum M4491 (Mack et al., 1998). The present study indicates that both CEP110 and ninein appeared at the open end of the daughter centrosome tube only after cell division, when the daughter centrosome started to function as a mother centrosome. The change in distribution of these two proteins following cell division suggests that protein addition at the open end of the centrosome tube is a hallmark of centrosome maturation. In addition, both proteins co-localized with the centriolar protein CEP250/c-Nap1 (Mack et al., 1998; Fry et al., 1998; Ou and Rattner, 2000) at the closed end of the centrosomal tube, which suggests that both CEP110 and ninein are associated with the centrioles in both mother and daughter centrosomes. Finally, microinjection of antibodies against CEP110 into HeLa cells at metaphase disrupted the reassembly of the tubular configuration of proteins within the centrosome seen throughout interphase, prevented the centrosomal localization of many centrosome proteins, and interfered with the centrosome's ability to function as a MTOC. Centrosomal architecture was not disrupted if antibodies were microinjected into cells with fully formed centrosomes but the MTOC function of the centrosomes was disrupted. Taken together, our findings suggest that protein addition at the open end of the tubular configuration of the daughter centrosome is required for the completion of centrosome maturation. Furthermore, both CEP110 and ninein are required for the reformation of the tubular configuration of proteins within the centrosome following cell division as well as being required for the centrosome to function as a MTOC.

Materials and Methods

Cell culture

HeLa cells were cultured in J-MEM medium (Gibco/BRL) supplemented with 10% fetal calf serum (Gibco/BRL) and 1× antibiotics at 37°C in 5% CO_2. PtK2 cells were grown in D-MEM (Gibco/BRL) supplemented with 10% fetal calf serum and 0.1 mM non-essential amino acids. Cells were either grown on coverslips or harvested by mechanical release from the tissue culture substrate and re-grown on coverslips in fresh medium for 4 hours prior to use. For microinjection experiments, cells were collected by trypsin/EDTA (Gibco/BRL) treatment and re-grown overnight on coverslips in fresh medium.

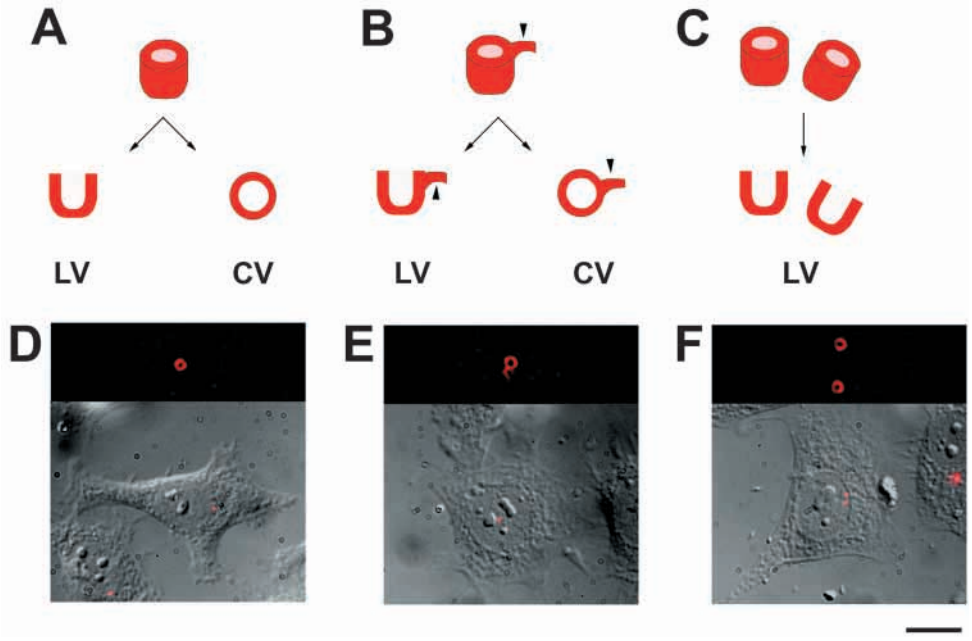

Fig. 1. Typical views of the centrosomes as seen at different time points during the cell cycle when stained by an autoimmune serum (for details, see Ou and Rattner, 2000). (A-C) Diagrammatic representation of the centrosome before duplication (A), the centrosome in the process of duplication (B), and the centrosomes after duplication and separation (C). Arrowheads denote the site from which a new tubular centrosome (daughter centrosome) is formed. The upper panel shows the 3D view of the centrosome. The lower panel illustrates both longitudinal and cross-sectional views of the centrosome tube shown at the upper panel. CV, cross-sectional view; LV, longitudinal view. Note that the longitudinal view represents the middle sections whereas the cross-sectional view represents the top end of the centrosome tube. (D-F) Overlay of whole cell DIC images and the fluorescent centrosomes (red spots) in HeLa cells at the cell cycle stages G1 (D), S (E) and G2 (F). The inserts show high magnification views of centrosomes from the corresponding cells. Note the centrosomes seen at D-F provide the basis for the diagrammatic views of the centrosomes shown in A-C. Bar, 10 μM (for DIC images only).

Antibodies

The centrosome-reactive autoimmune serum M4491 was obtained from the serum bank of the Advanced Diagnostic Laboratory at the University of Calgary. Characterization of this serum has been reported previously (Mack et al., 1998; Ou and Rattner, 2000). Anti-CEP110 antibodies were generated by immunizing rabbits with the recombinant protein as previously described (Guasch et al., 2000). Anti-ninein and anti-CEP250/c-Nap1 antibodies have been described previously (Ou and Rattner, 2000). All the antibodies were affinity-purified as described previously (Guasch et al., 2000) and their specificities were confirmed by western blot analysis using whole cell extracts of HeLa cells (Mack et al., 1998; Guasch et al., 2000; Ou and Rattner, 2000). Anti-pericentrin antibodies were purchased from Babco (Richmond, CA). Anti-γ-tubulin antibodies were purchased from Accurate Chemical & Scientific Corp. (Westbury, NY). Cy^3-, Cy^5- and $Alex^{488}$-labelled secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA), Sigma (St Louis, MO) and Molecular Probes (Eugene, OR), respectively.

Microinjection protocol

Microinjections were performed under phase contrast using a Leitz microinjector. All antibodies and normal control sera were diluted in sterile Dulbecco's phosphate buffered saline (D-PBS). For control experiments, nonspecific rabbit or mouse IgG (protein A sepharose-purified) were diluted to 1.5 μg/μl and the normal pre-immune rabbit or mouse sera were diluted at 1:5 in D-PBS. Antibodies specific to both CEP110 and ninein were diluted with D-PBS and used at a final concentration of 1.5 μg/μl. In some experiments, the injected cells were incubated with 0.1 μg/ml of Colcemid (GibcoBRL) and then reversed from the Colcemid block by washing three times and incubating with Colcemid-free medium until fixation.

Digital confocal microscopy and deconvolution

Digital confocal microscopy (immunofluorescence microscopy in conjunction with digital optical sectioning) was performed as described previously (Ou and Rattner, 2000). Briefly, images were obtained by using the Leica PL APO 100×/1.40-0.7 oil objective lens and a 1.995× magnification tube attached to a CCD camera. Deconvolved images were obtained using Vaytek (Fairfield, IA) Digital Confocal Microscope 4.0 for DOS software. The deconvolved images were rescaled to cover the entire 255-value gray range (Hendzel et al., 1998). The images were further processed and aligned using Adobe PhotoShop 5.0. In some cases, whole cell images were taken using DIC microscopy to illustrate the position of the centrosome(s) within the cells.

Results

CEP110 maps to two regions of the centrosome

Staining the centrosomes with a human autoimmune serum that is reactive to a family of centrosomal proteins (M4491) or monospecific antibodies against particular centrosomal components allows the visualization of the tubular configuration of proteins within the centrosome (Ou and Rattner, 2000). Fig. 1A-C summarizes the types of images typically seen for unduplicated centrosomes (Fig. 1A), centrosomes in the process of duplication (Fig. 1B) and centrosomes that have duplicated and separated (Fig. 1C) (Ou and Rattner, 2000). These representations are based on the findings that centrosomes display either an 'O' or a 'U' profile, which represents the cross-sectional and longitudinal views of a closed tube, respectively (Ou and Rattner, 2000). To provide a context for these images, Fig. 1D-F shows low magnification overlays of whole cell DIC images and the fluorescent images (red) of the centrosomes in HeLa cells at the cell cycle stages G1 (Fig. 1D), S (Fig. 1E) and G2 (Fig. 1F). Inserts show high magnification views of the centrosomes from these cells. Such images provide the basis for the three types of centrosome profiles illustrated in Fig. 1A-C.

To fine-map the distribution of CEP110 prior to the onset of

centrosome duplication, mitotic cells were collected by selective detachment, re-plated onto coverslips and incubated for 4 hours prior to use. The G1 status of the cells was confirmed by co-staining the cells with an antibody against CENP-F as described previously (Ou and Rattner, 2000). In unduplicated mother centrosomes, CEP110 was observed at two regions with respect to the centrosome tube, one at the closed end and the other at the open end (Fig. 2a). Staining at the open end of the tube appeared as two foci in optical sections passing longitudinally through the centrosome tube (Fig. 2a) and as an 'O' profile in cross-sectional views of this region. These images indicate that, in addition to the closed end, CEP110 is distributed within a narrow ring at the open end of the tube (Fig. 2b).

In random cultures, centrosomes initiating duplication can be identified by the appearance of a structure at the margin of the centrosome (Ou and Rattner, 2000). When cells displaying this stage of duplication were stained for CEP110, this protein was found at the site of duplication (Fig. 2c,d) as well as at the open and closed ends of the mother centrosome tube as described above (Fig. 2c,d). CEP110 was localized to a single site within the daughter centrosome throughout the centrosome duplication process. Thus, in late S phase cells that had two separated centrosomes, the mother centrosome had two regions of CEP110 staining, whereas the daughter centrosome had only one site (Fig. 2e). These observations indicate that CEP110 is a component of the closed end of the centrosome tube in both the mother and daughter centrosomes but it populates only the open end of the mother centrosome tube.

A population of CEP110 co-localizes with the centriole component within the centrosome

In our previous study, we demonstrated that the centriole components CEP250/c-Nap1 and Nek2 are usually located at the closed end of the mother centrosome tube and also at the closed end of the daughter centrosome tube with a complete tubular configuration (Ou and Rattner, 2000). Further, we showed that, following centriole separation at G1-S, the daughter centriole moves along the mother centrosome and comes to reside at the site of centrosome duplication (Ou and Rattner, 2000). Since a subset of CEP110 reactivity was also found at these locations, we next determined whether CEP110 co-localizes with the centrioles by triple-staining cells with the autoimmune serum M4491, the antibody to CEP110 and an antibody to CEP250/c-Nap1 [a protein confined to the centrioles (Mack et al., 1998; Fry et al., 1998; Ou and Rattner, 2000)]. Fig. 3 illustrates that CEP110 co-localized with CEP250/c-Nap1 at the site of the centriole component in the mother centrosome (Fig. 3a,b, arrowhead). This relationship is also seen at the site of the centrioles in the forming daughter centrosome (Fig. 3a, arrow) and daughter centrosomes with a fully-formed daughter centrosome tube (Fig. 3b, arrow). These observations indicate that CEP110 is a centriole-associated protein.

Ninein and CEP110 co-localize to similar centrosomal domains

The distribution of the centrosomal protein ninein during the cell cycle (Piel et al., 2000; Mogensen et al., 2000), as well as our preliminary mapping data using antibodies to centrosomal proteins (Ou and Rattner, 2000), suggested that ninein might have a localization within the centrosome similar to that of CEP110. Recently, ninein has been found, by immunofluorescence, to associate primarily with the mother centriole and has been localized, by immunoelectron microscopy, to both the appendages associated with the mother centriole and to the minus ends of microtubules (Piel at al., 2000; Mogensen at al., 2000).

We examined the distribution of ninein within the tubular configuration of the centrosome during the cell cycle, and found that ninein and CEP110 localized to similar sites (Figs

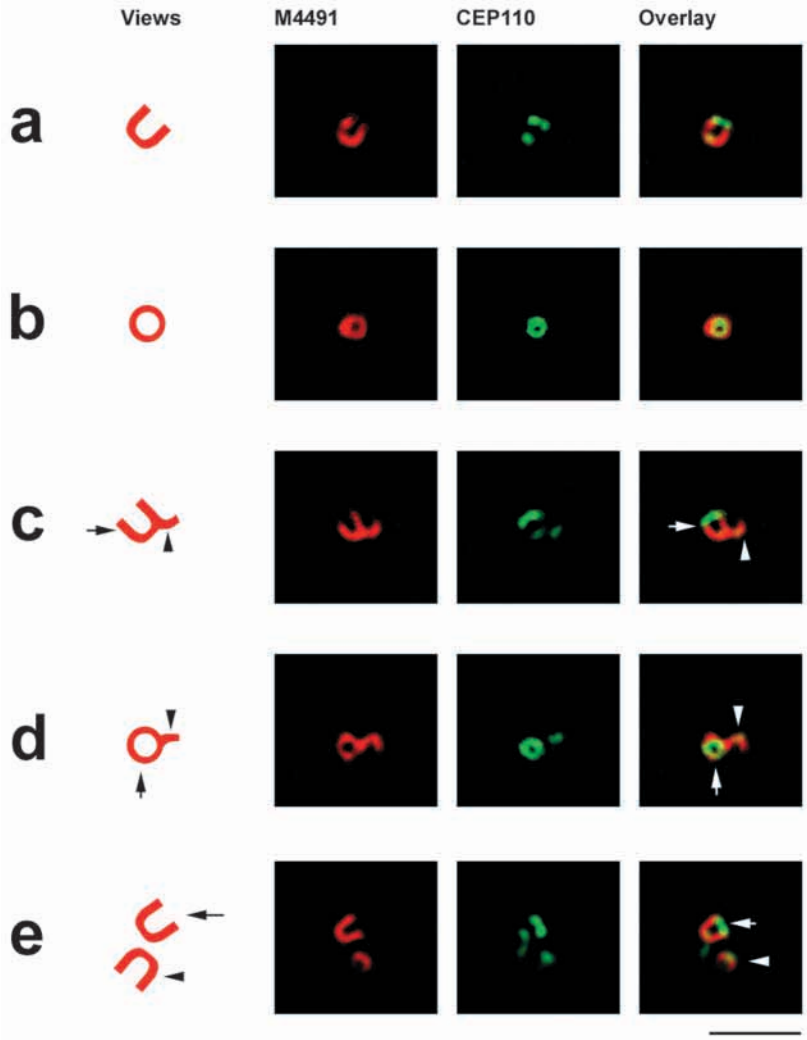

Fig. 2. Localization of CEP110 within the centrosome tube. HeLa centrosomes were stained with the autoimmune serum M4491 (red) and an anti-CEP110 antibody (green). The diagram on the left shows the views of the centrosomes seen in the images on the right. (a,b) The centrosomes before duplication. (c,d) The centrosomes in the process of duplication. (e) The centrosomes after duplication and separation. The arrow denotes the mother centrosomes when the centrosome is still in the process of duplication (c,d) or when duplication and separation are completed (e). The arrowhead denotes the daughter centrosomal bud (c,d) or the fully-formed daughter centrosome (e). Bar, 5 μM.

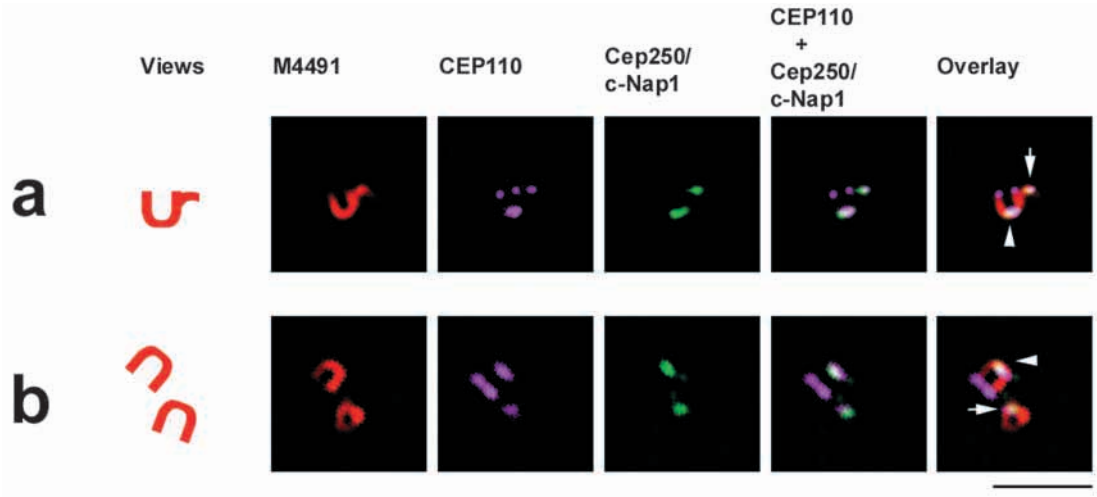

Fig. 3. The relationship between CEP110 and Cep250/c-Nap1. HeLa centrosomes were triple-stained with the autoimmune serum M4491 (red), the anti-CEP110 antibody (purple) and an antibody against the centriolar protein Cep250/c-Nap1 (green). (a) Centrosome in the process of duplication. (b) Centrosomes after duplication and separation. The diagrams on the left illustrate the views of the centrosomes seen in the images. The arrow denotes the co-localization of CEP110 and Cep250/c-Nap1 at the daughter centrosomal bud (a) or the well-formed daughter centrosome tube (b). The arrowhead points to the co-localization of the two proteins at the mother centrosomes when centrosome duplication is still going on (a) or after duplication and separation (b). Bar, 5 μM.

4, 5). Thus, both CEP110 and ninein mapped to the site of CEP250/c-Nap1 in the centriole of mother, daughter and duplicating centrosomes, as well as being located within a ring at the open end of the tube in mother centrosomes. Interestingly, when the open end of the tube was visualized in cross-section, CEP110 was positioned closer to the central lumen of the centrosome, while ninein was more abundant at the periphery of the tube (Fig. 5). These observations indicate that, although CEP110 and ninein display similar staining patterns at the open end of the centrosome, these two proteins localize to distinct subdomains.

Microinjection of anti-CEP110 or anti-ninein antibodies into metaphase HeLa cells disrupts the reassembly of the centrosome

The tubular configuration of a subset of proteins within the centrosome is reassembled at the end of mitosis, at which time CEP110 appears at the open end of the daughter centrosome. If CEP110 is required during the post-mitotic reassembly of the centrosome, we would expect to see abnormal organization of proteins within the centrosome in cells where the CEP110 distribution is perturbed. To test this hypothesis, we microinjected anti-CEP110 antibodies into metaphase HeLa cells and examined the centrosome morphology in cells that had been fixed at 6, 12 and 24 hours following microinjection, and stained with the autoimmune serum M4491 as well as with Alexa^{488}-labelled anti-rabbit secondary antibody to visualize

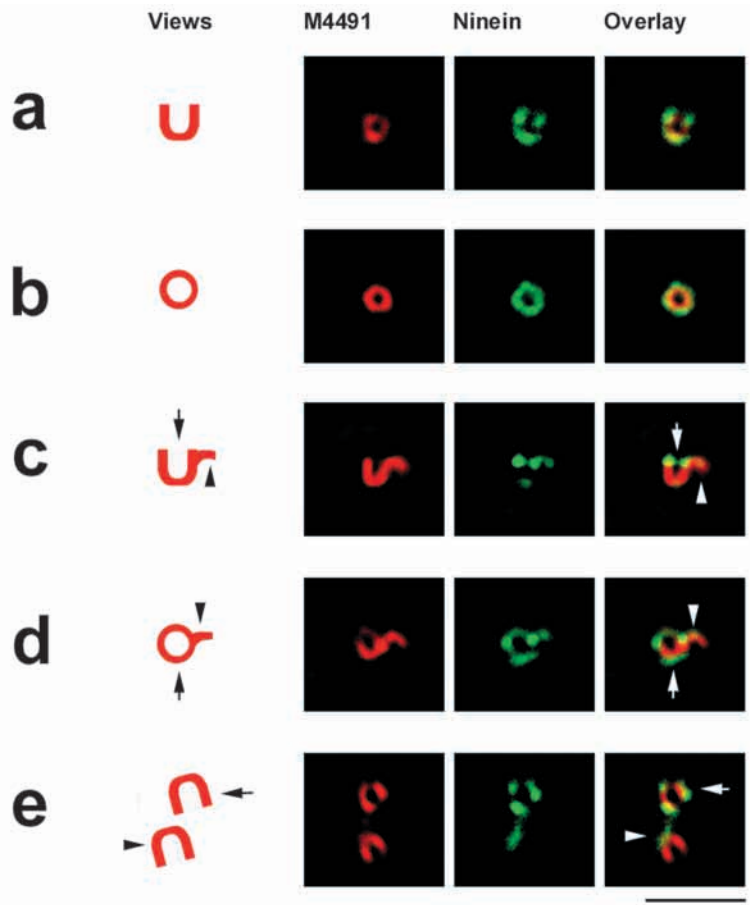

Fig. 4. The distribution of ninein within the centrosome tube. The centrosomes were stained with the autoimmune serum M4491 (red) and the anti-ninein antibody (green). The diagrams on the left illustrate the views of the centrosomes seen in the images. (a,b) Centrosomes before duplication. (c,d) The centrosomes in the process of duplication. (e) The centrosomes after duplication and separation. The arrow points to the mother centrosome when the centrosome is in the process of duplication (c,d) or duplication is completed (e). The arrowhead denotes the daughter centrosomal bud (c,d) or the well-formed daughter centrosome (e). Bar, 5 μM.

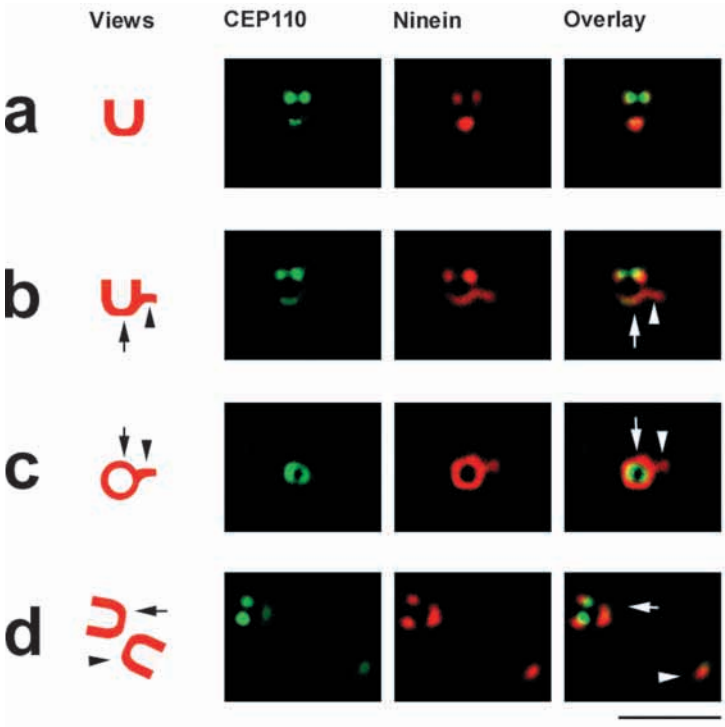

Fig. 5. Co-localization of CEP110 and ninein. The centrosomes were co-stained with anti-CEP110 (green) and anti-ninein (red) antibodies. The diagrams on the left illustrate the views of the centrosomes seen in the images. (a) The centrosome before duplication. (b,c) The centrosomes in the process of duplication. (d) The centrosomes after duplication and separation. The arrow points to the mother centrosome when the centrosome is in the process of duplication (b,c) or duplication is completed (d). The arrowhead denotes the daughter centrosomal bud (b,c) or the well-formed daughter centrosome (d). Bar, 5 μM.

We also injected anti-CEP110 antibodies into early interphase cells that were connected by an intercellular bridge but had a reformed nucleus. In this case, more than 95% (n=40) of the injected cells had centrosomes with a normal tubular morphology when examined at 6 and 12 hours after injection (e.g. Fig. 9e,f). These results were comparable with those obtained from sham-injected cells (data not shown). We conclude that both CEP110 and ninein are required for the reassembly of the centrosome following mitosis. Further, although antibody injection can disrupt centrosome tube reassembly following mitosis, it is ineffective in disrupting mature centrosomes or inhibiting centrosome duplication.

cells that were microinjected. Control cells were injected with pre-immune serum or protein A-affinity purified rabbit immunoglobins. At each time point, aberrant centrosome tube morphology could be observed in cells that had been injected with the anti-CEP110 antibodies (e.g. Fig. 6a-c). At 24 hours after microinjection, 17% (n=42) of cells had multiple foci of centrosomal proteins scattered in the cytoplasm (Fig. 6a), 26% had incompletely formed or irregularly shaped centrosome tubes (Fig. 6b) and the remainder, 57%, had a normal or nearly normal tubular conformation (Fig. 6c). Interestingly, in a majority of the injected cells, the staining intensity with M4491 appeared weaker than that in sham-injected control cells (compare Fig. 6c and d). In cells microinjected with control antibodies (n=60), more than 95% of cells had centrosomes with a normal tubular morphology (e.g. Fig. 6d). Microinjection of metaphase HeLa cells (n=28) with anti-ninein antibodies resulted in defects in centrosome tube assembly that were comparable with those obtained in the anti-CEP110 injected cells (data not shown).

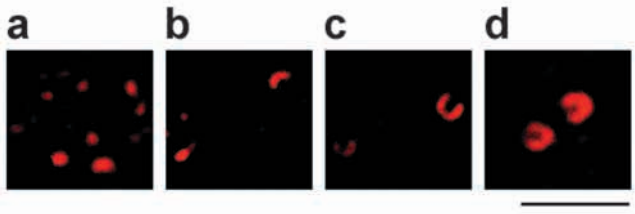

Fig. 6. Microinjection of anti-CEP110 antibodies into mitotic HeLa cells disrupts the centrosome reassembly following mitosis. The cells were fixed at 24 hours after the injection and stained with the autoimmune serum M4491. (a-c) Centrosome morphology in cells injected with the anti-CEP110 antibody. (d) Centrosome morphology in cells injected with the nonspecific IgG. Bar, 5 μM.

Disruption of centrosome organization following the injection of anti-CEP110 antibodies affects the localization of a number of centrosomal proteins

The finding that the tubular configuration within the centrosome is disrupted following the injection of anti-CEP110 antibodies raises questions as to the fate of specific centrosomal components under these conditions. To address this question, HeLa cells injected with anti-CEP110 antibodies at mitosis were fixed 24 hours post injection and triple-stained with the autoimmune serum M4491, a secondary antibody (against anti-CEP110 antibody to reveal the injected cells) and each of the following antibodies: anti-CEP250/c-Nap1, anti-pericentrin, anti-γ-tubulin and anti-ninein antibodies. The results demonstrated that, in cells in which the assembly of the tubular configuration of a subset of proteins within the centrosome was disturbed, all these centrosomal components (CEP250/c-Nap1, pericentrin, γ-tubulin and ninein) were dispersed in the cytosol (e.g. Fig. 7).

Injection of anti-CEP110 or anti-ninein antibodies into PtK2 cells affects the microtubule organizing ability of the centrosome

Our results indicated that the presence of both CEP110 and ninein are required for the proper reassembly of the tubular configuration of the centrosomes and consequently the localization of specific centrosomal proteins to the G1 centrosome. To determine whether the absence of CEP110 and ninein also affects the ability of the G1 centrosomes to function as a discrete MTOC, we assayed for microtubules in PtK2 cells following antibody injection using two protocols. In the first protocol, mitotic or post-mitotic cells were injected with either anti-CEP110 or anti-ninein antibodies, fixed 6 hours post-injection and stained with an anti-β-tubulin antibody. In the second protocol, the cells were injected in the same manner,

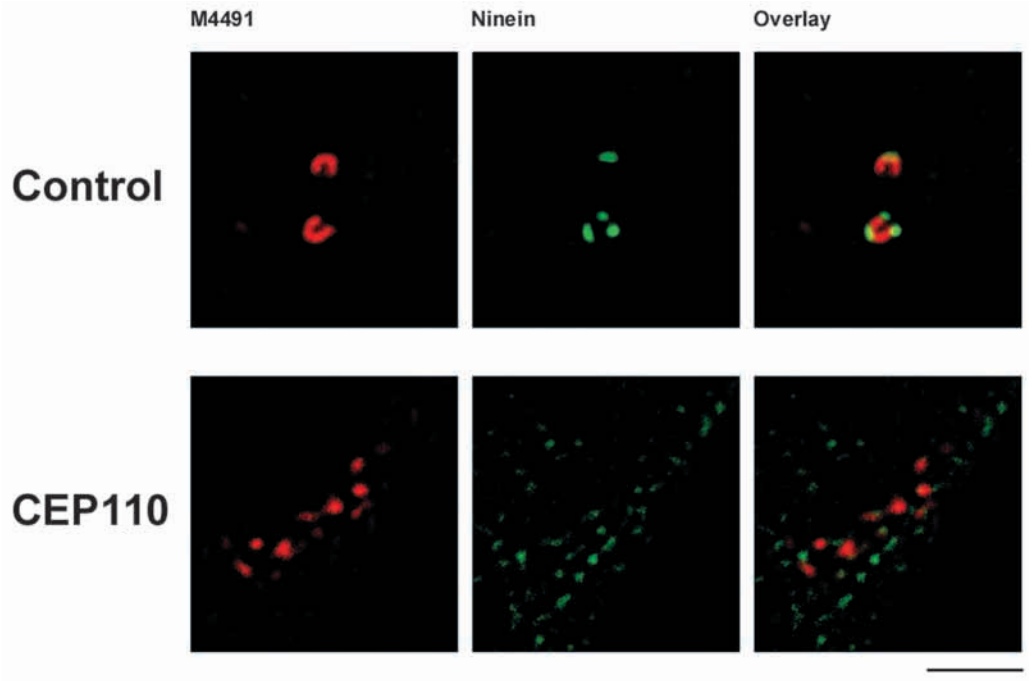

Fig. 7. Comparison of control and CEP110 injected cells stained with M4491 for the centrosome and an antibody to ninein. The CEP110-injected cells shows a disrupted centrosome tube organization as well as a dispersed ninein distribution. Bar, 3 μM.

but 0.1 μg/ml of colcemid was added to the culture 6 hours after the injection. The cells were incubated for 60 minutes at 37°C in the presence of the drug. Subsequently, the drug was removed from the culture by washing with fresh medium and incubating for an additional 20 minutes before fixation. The cells were then stained with the anti-β-tubulin antibody. The drug treatment protocol was used to ensure that any microtubules detected upon fixation were formed subsequent to antibody injection. The results were comparable using both protocols and either anti-CEP110 or anti-ninein antibodies (Figs 8, 9). When mitotic cells were used, microtubule formation at the centrosomes was observed in cells injected with normal serum (Fig. 8a-b). However, no localized microtubule formation was detected in cells injected with anti-CEP110 antibodies (Fig. 8c-d). Similarly, post-mitotic cells injected with anti-ninein antibodies also showed altered MTOC

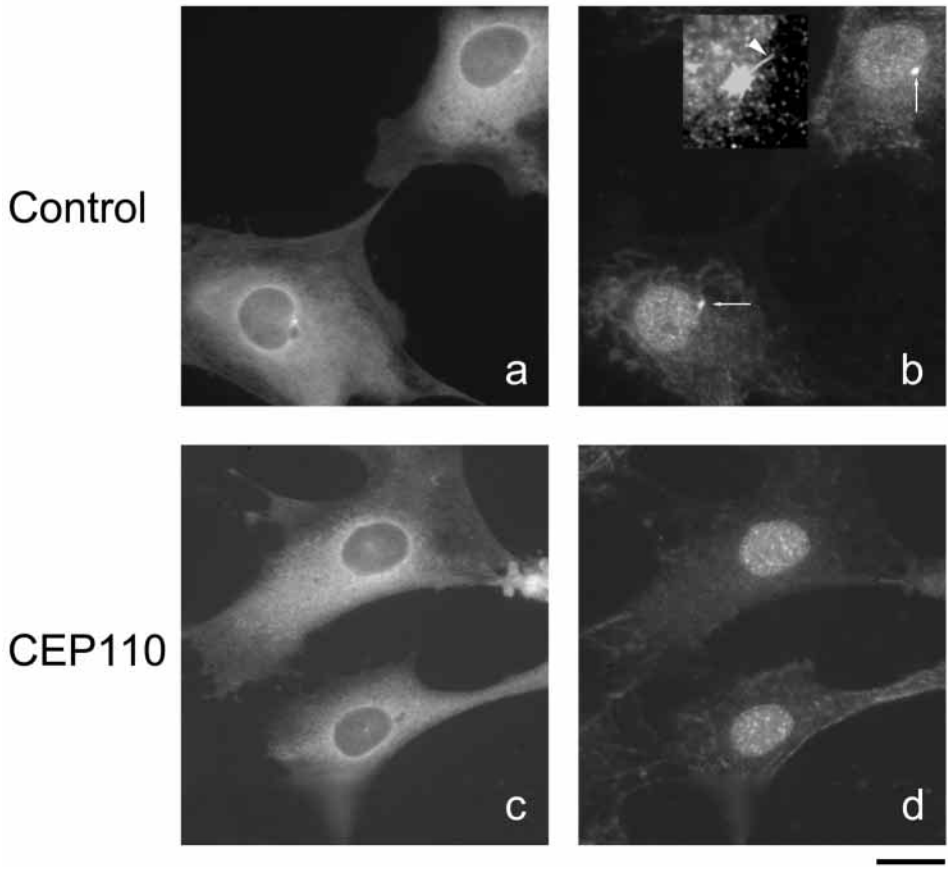

Fig. 8. Loss of centrosome MTOC function in PtK2 cells injected with anti-CEP110 antibodies at mitosis. Control, cells injected with normal serum (a,b); CEP110, cells injected with anti-CEP110 antibodies (c,d). (a,c) The injected cells revealed by staining with secondary antibodies. (b,d) The injected cells were stained with anti-β-tubulin antibodies to illustrate microtubules. Arrows denote microtubule nucleation from the centrosomes in the control cells. (Inset) Higher magnification of the centrosome from the upper cell. The arrowhead points to microtubules. Panel b has been printed with high contrast to better illustrate the microtubules growing from the centrosome. Abundant microtubules are present in the cytoplasm of control cells and increase over time following drug reversal. Bar, 10 μM.

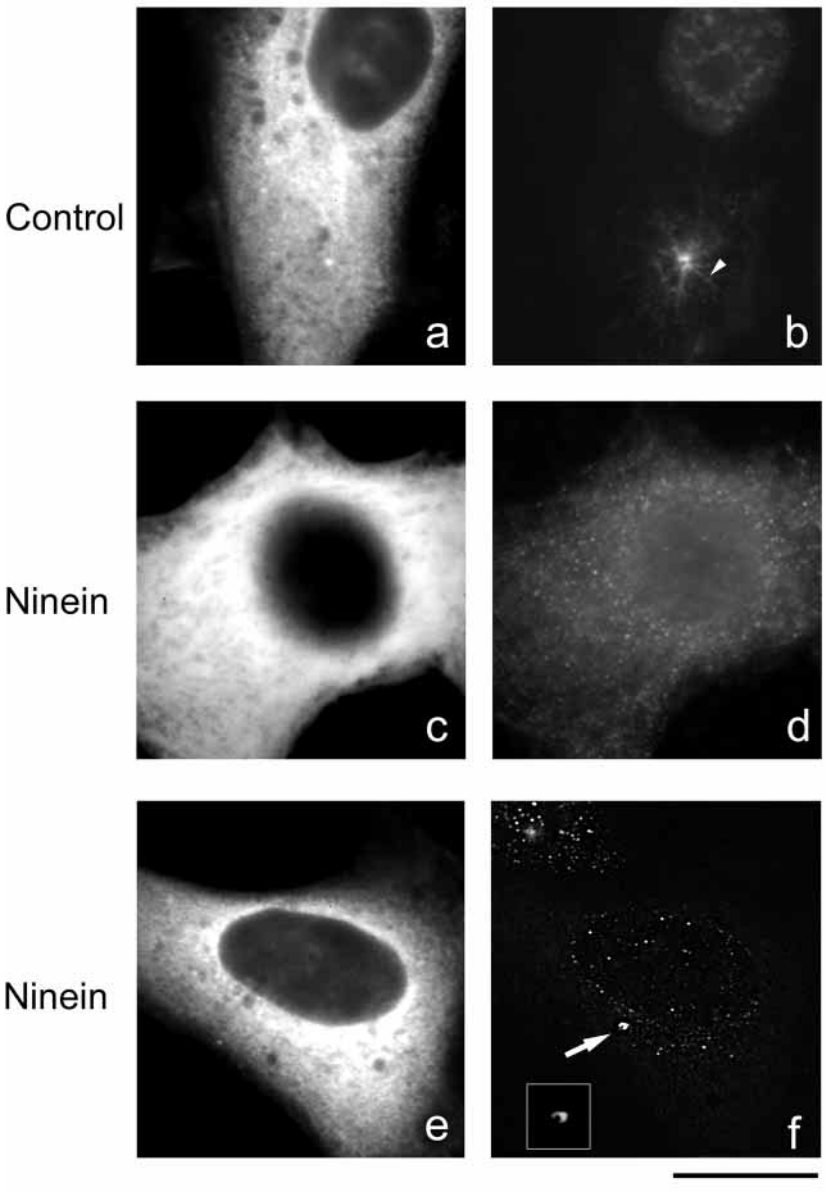

Fig. 9. Loss of centrosome MTOC function in post-mitotic PtK2 cells injected with anti-ninein antibodies. Control, cell injected with normal serum (a,b); ninein, cell injected with anti-ninein antibodies (c-f). (a,c,e) The injected cells revealed by staining with secondary antibodies. (b,d) The injected cells were stained with anti-β-tubulin antibodies to illustrate microtubules. The arrowhead points to microtubules nucleated from the centrosome in the control cell. (f) The injected cell was stained with M4491 for the centrosome. The arrow points to the centrosome. (Inset) Enlarged view of the centrosome. Bar, 10 μM.

function (Fig. 9). We conclude that the presence of CEP110 and ninein are essential not only for the organization of the daughter centrosomes following mitosis but also for the centrosome's ability to function as a MTOC.

Discussion

Understanding the structural-functional correlates that exist within the centrosome requires a detailed knowledge of the placement, interactions and dynamics of centrosomal components. Such information has historically been obtained using conventional immunoelectron microscopy techniques. However, such studies have been limited by the inability to visualize the full extent of the centrosome in conventional thin sections. Our approach has been to exploit our ability to visualize a tubular conformation of a subset of proteins within the centrosome by using this conformation as a reference for the localization of other proteins. This approach allows the identification of protein-protein relationships in a manner that previously has not been possible. A summary of our CEP110 and ninein localization results is presented in Fig. 10.

CEP110 and ninein distribution denotes centriole association and a region associated with centrosome maturation

In this study, we have expanded our previous localization data by mapping CEP110 in relation to the tubular configuration of a subset of proteins within the centrosome. We demonstrated that CEP110 is localized to two regions throughout the life of the mother centrosome, one at the position of the centriole components CEP250/c-Nap1 and Nek2 and another at the open end of the centrosomal tube. Since CEP250/c-Nap1 has been shown to localize to one end of the centriole (Fry et al., 1998), CEP110 must have a similar localization within the centriole. Further, we showed that CEP110 appeared at only one site (the site of CEP250/c-Nap1) within the daughter centrosome from the time of its initial formation until prophase (Fig. 10). Our ability to refine and expand the protein localization data obtained by conventional IIF using the techniques described in this study highlights the usefulness of employing an architectural reference and confocal microscopy to map centrosomal components.

We also investigated the position of ninein with respect to the centrosomal tube. Ninein has previously been shown by immunofluorescence microscopy to be associated primarily with the mother centriole during G1 and both centrioles during S-G2 (Piel et al., 2000). Further, it has been localized to the appendages surrounding the mother centriole and also to the minus-ends of microtubules by immunoelectron microscopy (Mogensen et al., 2000). Ninein, like CEP110, is found at the site of the centriole component CEP250/c-Nap1 in both mother and daughter centrosomes and at the open end of the mother centrosomal tube. Although the detection of ninein in association with centrioles confirms previous findings (Mogensen et al., 2000), this is the first report of ninein at a second site, the open end of the centrosome. It should be noted that the distribution of ninein to two regions of the centrosomes could be seen in previous localization studies as a three-dot pattern (Mogensen et al., 2000), the significance of which was not obvious before our experiments. As shown in our study, one dot maps to the region containing CEP250/c-

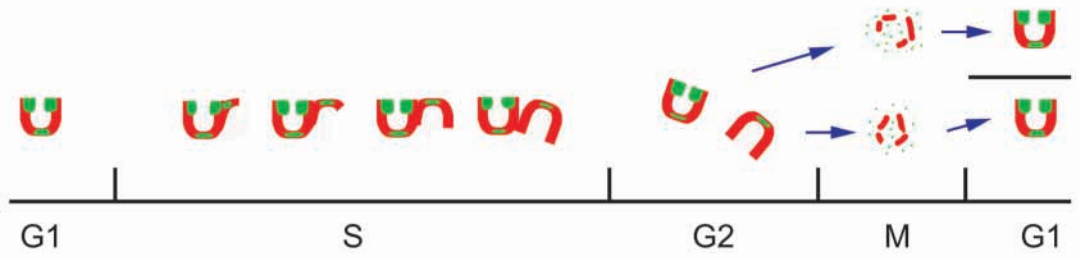

Fig. 10. Summary of the distribution of CEP110 and ninein during the cell cycle and their relationship to centrosome duplication. Red, the centrosome tube; green, CEP110 and ninein.

Nap1, while the other two dots map to the open end of the centrosomal tube.

Since both CEP110 and ninein localize to the site of CEP250/c-Nap1 in mother and daughter centrosomes throughout the cell cycle, these two proteins are likely to be constitutive components of this centriole region. Importantly, the addition of these proteins to the open end of the daughter centrosome tube coincides with the transition of the daughter centrosome to a mature centrosome which is capable of duplication and acts as a microtubule organizing center. It is possible that the tubular structure of the daughter centrosome must be opened during mitosis so that CEP110 and ninein can be added and maturation of the centrosome completed (Fig. 10). Thus, our study, for the first time, identifies a specific region of the centrosome that is modified in its protein composition during the process of centrosome maturation, a process that is completed following cell division.

The identification and localization of two populations of both CEP110 and ninein within the centrosome has some functional implications. First, the function of CEP110 and ninein is, at least in part, centriole-based. Second, previous studies have suggested that ninein plays a role in microtubule anchoring at the centriole and acts as a cap for microtubule ends (Mogensen et al., 2000). If so, the presence of ninein at the open end of the centrosomal tube suggests that microtubule anchoring or capping functions may occur within this region as well, at least at some point during the cell cycle. The absence of ninein from the daughter centrosomes would therefore limit their ability to anchor or cap microtubules at the open end of the tube. Such a relationship may explain in part why previous studies (e.g. Piel et al., 2000) failed to identify microtubules in association with immature centrosomes. The observations described here raise the possibility that ninein plays a larger, and perhaps more diverse, role within the centrosome than previously thought, and that this role, in part, is a hallmark of mature centrosomes.

Centrosome targeting signal and protein association at the centrosome

Protein localization data, such as those presented here, raise important questions about the mechanisms that target proteins to the centrosome and, more specifically, the mechanisms that localize proteins to specific regions within the centrosome. In the search for a centrosomal targeting sequence, Gillingham and Munro identified a 90-residue PACT domain in the centrosomal proteins pericentrin and AKAP450, as well as in two other proteins of unknown function from *Drosophila* and fission yeast (Gillingham and Munro, 2000). This domain was capable of targeting a reporter protein, green fluorescent protein, to the centrosome. Both pericentrin and AKAP450 are very large proteins predicted to form a coiled-coil over most of their length (Doxsey et al., 1994; Dictenberg et al., 1998; Witczak et al., 1999), and the PACT domain is outside of both their coiled-coil regions and their protein kinase A binding site.

Examination of the protein sequences of CEP110 and ninein failed to reveal the PACT domain. In the case of CEP110, we have previously shown that the centrosome targeting signal is located within one of its coiled-coil regions. This region spans 170 amino acids at the C-terminus and contains a leucine zipper motif (Guasch et al., 2000). Leucine zippers, initially identified in transcription factors, have been shown to mediate homo- or heterodimerization of proteins and to play a role in protein-DNA binding (Landschulz et al., 1988). This motif has also been found to mediate protein-protein interactions in a wide array of proteins and play pivotal roles in regulating the activities of these proteins (e.g. Dubay et al., 1992; Vrana et al., 1994; Park and Seo, 1995; Leung and Lassam, 1998). However, our deletion experiments indicate that, in the case of CEP110, the leucine zipper motif is not sufficient on its own to support targeting of green fluorescent protein to the centrosome, which indicates that flanking amino acids are also involved (Y.Y.O. and J.B.R., unpublished). It is likely that different centrosomal proteins contain different centrosomal targeting motifs. This variation could, for example, explain why some proteins are positioned throughout the entire centrosome (e.g. pericentrin) whereas others are more domain-specific (e.g. CEP110).

The mapping data for CEP110 and ninein reported in this study raises the possibility that they interact in vivo. We have been unable to detect such an interaction using an in vitro TNT and immunoprecipitation assay. However, we did find that both CEP110 and ninein bind to kendrin (Y.Y.O. and J.B.R., unpublished). Thus, these proteins can be added to a growing list of pericentrin/kendrin-binding proteins, which include protein kinase A (Diviani et al., 2000), cytoplasmic dynein (Purohit et al., 1999), pcm-1 (Li et al., 2001), calmodulin (Flory et al., 2000) and γ-tubulin (Dictenberg et al., 1998; Young et al., 2000). Interestingly, we found that it is the centrosomal targeting region of CEP110 that interacts with kendrin, and this is the first motif identified to interact directly with kendrin/pericentrin (Y.Y.O. and J.B.R., unpublished).

CEP110 and ninein are essential for centrosome assembly following mitosis

Assembly of the tubular architecture within the centrosome occurs twice during the cell cycle: once during centrosome duplication and once following cell division. Our

microinjection studies clearly indicate that both CEP110 and ninein are required for the reassembly of the tubular architecture of the centrosome following cell division. The findings that CEP110 and ninein are associated with the CEP250/c-Nap1 site within the centrioles when the tubular configuration of the daughter centrosome is established and that neither CEP110 nor ninein is present at the open end of the tubule at this time raise the possibility that this centriole-associated population of CEP110 and ninein may be especially critical for the assembly of the tubular conformation of proteins. The simple interpretation of our microinjection results is that the microinjected antibodies prevented the centriolar CEP110 and ninein from participating in the normal formation of the tubular architecture of the centrosome. This interpretation is consistent with the observations that the centriole duplex functions to organize a centrosome in some cell types (Bobinnec et al., 1998) (for reviews, see Marshall, 1999; Karsenti, 1999) and that centrosome duplication is initiated at the site of the daughter centriole (Ou and Rattner, 2000). Furthermore, by acting as a microtubule anchoring protein, the centriole-bound CEP110 or ninein may act as a focus for the collection of centrosomal proteins transiting along microtubules. This is supported by our results that show that, in cells injected with an anti-CEP110 antibody, centrosomal material is scattered throughout the cytosol and microtubule assembly at the centrosome is disrupted. Thus, it appears that the assembly of the tubular conformation within the centrosome, centrosome protein clustering, and microtubule formation are interdependent.

Our inability to disrupt the centrosome structure by antibody microinjection at the G1 stage may indicate that both CEP110 and ninein are required for the assembly of the tubular conformation of proteins, but not for its stability once it has formed. Further, factors such as epitope accessibility, the abundance of existing protein, the rate of turnover of CEP110 and ninein in the centrosome, and the presence of a mature mother centrosome structure may contribute to our inability to disrupt daughter centrosome formation following antibody microinjection into interphase cells. However, it should be noted that our microinjection experiments indicate that both proteins are required for the G1 centrosome to function as a MTOC.

In summary, our study shows that proteins are arranged in a specific manner within the centrosome and this arrangement can be modified in both a spatial and temporal manner to accommodate centrosome function. Specifically, our data establishes that the open end of the centrosome tube is the site of protein addition associated with the final stages of centrosome maturation and CEP110 and ninein are two of the proteins added to that region.

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