A Human Centrosomal Protein Is Immunologically Related To Basal Body-associated Proteins from Lower Eucaryotes and Is Involved in the Nucleation of Microtubules

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Abstract. Isolation of centrosomes from human cells has revealed a proteic pattern which is both complex and specific. As the most prominent structural element of centrosomes in animal cells, the centriole which is present as two copies, is a highly conserved structure, we have attempted to identify centrosomal proteins on the basis of immunocross-reaction with proteins identified in basal bodies from lower eucaryotes. We report that two antibodies, one raised against the Ca^{+}-binding protein centrin (Salisbury, J. L., A. T. Baron, B. Surek, and M. Melkonian. 1984. *J. Cell Biol.* 99:962–970) and the other directed against a 230-kD protein isolated from the infraciliary cytoskeletal lattice of the protozoan *Polyplastron m.*, decorate the centrosome of human cultured cells, and identify one of the major centrosomal components revealed as a doublet of 62/64 kD. Moreover the nucleation reaction of microtubules, which can be efficiently produced on isolated centrosomes, is blocked by the antibodies, a result which strongly implicates the 62/64-kD protein in this centrosomal activity. We also show that the 62/64-kD protein remains insoluble in conditions (0.5 M KI or 8 M urea) which are capable of extracting most of the centrosomal proteins. Immunocytochemical localization by EM of isolated centrosomes revealed the association of this 62/64-kD doublet with the intercentriolar link and the pericentriolar lattice. Our results suggest that conservation of structure in the centrosome from divergent organisms could be matched by conservation of proteins and activity, evidence for the maintenance of a specific function, which could involve Ca^{2+}, associated with the microtubule organizing centers.

Microtubules are one of the important cytoskeletal components of eucaryotic cells. They constitute the main component of basal body/centriole, ciliary, and flagellar structure, and contribute to cell movement, maintenance of cell shape, and intracellular transport (Dustin, 1978; Schliwa, 1986). Most microtubules show dynamic behavior, capable of rapid assembly and disassembly in both interphase and mitotic cells (Inoué and Sato, 1967; Inoué et al., 1975; Mitchison et al., 1986). The spatial organization of the microtubule network in animal cells depends largely on a specialized structure: the centrosome (for review see Bornens et al., 1990). Centrosome regulation of microtubules includes control of microtubule initiation, number, polarity, and direction (Brinkley et al., 1981*a*,*b*; Kuriyama and Borisy, 1981; Evans et al., 1985).

Even though the centrosome was observed more than a century ago, its chemical composition has not yet been fully determined. Identification of centrosomal proteins has been achieved by immunological or genetical approaches (Kuriyama and Borisy, 1985; Baum et al., 1986; Klotz et al., 1986; Hurt et al., 1988; Steffen and Linck, 1988; Sunkel and Glover, 1988; Whitfield et al., 1988; Kellog et al., 1989; Rout and Kilmartin, 1990; Oakley et al., 1990). The isolation of basal bodies and centrosomes was a crucial step in the study of their structure and functions (Snell et al., 1974; Stearns and Brown, 1979; Mitchison and Kirschner, 1984; Gosti et al., 1986; Bornens et al., 1987; Komesli et al., 1989; Klotz et al., 1990).

As the centriole/basal body is a highly conserved structure, we wondered whether proteins identified in basal bodies from lower eucaryotes would be conserved in human centrosomes. One of them, the basal body–associated centrin, a Ca^{2+}-binding protein (Ca^{2+}-BP)[1] of 20 kD identified in the green algae *Tetraselmis s.* (Salisbury et al., 1984) and in *Chlamydomonas r.* (Huang et al., 1988*b*; there called caltractin), shows important sequence homology with calmodulin and the yeast CDC31 gene product required for spindle pole body duplication (Baum et al., 1986; Huang et al.,

1. *Abbreviations used in this paper*: Ca^{2+}-BP, Ca^{2+}-binding protein; IEF, isoelectrofocusing; PCM, pericentriolar material.

1988*a*). Using the polyclonal anti-centrin antibody, we have identified a 62/64-kD protein which is highly enriched in isolated centrosomes from human lymphoblasts. This novel centrosomal protein has a Ca^{2+}-sensitive behavior in SDS-PAGE and is also recognized by an antibody raised against a 230-kD protein which, together with a 22-kD Ca^{2+}-BP, forms a basal body associated structure in the ciliated protozoan *Polyplastron multivesiculatum* (Vigues and Grolière, 1985). We also report evidence for the involvement of the 62/64-kD protein in microtubule nucleation, and its association with the intercentriolar link and with the pericentriolar material (PCM).

Materials and Methods

Cell Culture

The human lymphoblastic KE 37 cell line (Mayer et al., 1982) was cultured in suspension in RPMI 1640 medium (Eurobio Laboratories, Les Ulis, France) supplemented with 7% FCS at 37°C and 5% CO_2 in air. When KE 37 cells were cultured in unenriched air, the RPMI medium was buffered with 20 mM Hepes, pH 7.3, at 37°C. HeLa cells were cultured in MEM medium containing 10% FCS.

Centrosome Isolation

Centrosomes were isolated from KE 37 cells as previously reported (Bornens et al., 1987) with slight modifications (manuscript in preparation).

Antibodies

Spontaneous rabbit serum 0013 was shown to be directed against the PCM (Gosti et al., 1986); monoclonal antibody CTR453 was raised against human centrosomes (Bailly et al., 1989); anti-α-tubulin is a product of Amersham Corp. (Les Ulis, France), the polyclonal anti-centrin antibody 08/28 raised against *Tetraselmis s.* centrin (Salisbury et al., 1984) and the polyclonal antibody 26/14-1 raised against a TrpE-centrin fusion protein expressed in *Escherichia coli* (Greenwood, T. M., C. Bazinet, A. J. Baron, M. A. Sanders, N. J. Maihle, and J. L. Salisbury. 1990. *J. Cell Biol.* 111:182*a*) were a generous gift from J. L. Salisbury (Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN), the anti-230-kD polyclonal antibody was prepared by one of us (B. Vigues) against electrophoretically purified 230-kD protein from isolated cortical cytoskeleton of *Polyplastron m.*

The IgGs of the anti-230-kD serum were purified by two different approaches. We have used either ammonium sulfate precipitation followed by ion exchange chromatography (DEAE cellulose) or one step protein-A sepharose column. We have checked on SDS-PAGE that the fractions contained only IgG polypeptides.

Immunofluorescence Microscopy

HeLa cells were extracted with PHEM buffer (45 mM Pipes, 45 mM Hepes, 10 mM EGTA, 5 mM $MgCl_2$, 1 mM PMSF, pH 6.9, containing 0.5% Triton X-100 for 1 min, washed in the same buffer without detergent and then fixed with cold methanol at −20°C during 6 min. The first antibody was a mixture of the monoclonal CTR453 and of either the polyclonal anti-centrin or anti-230-kD antibodies. Mixed rhodamine-conjugated goat anti-mouse and fluorescein-labeled goat anti-rabbit (Cappel Laboratories, Cochranville, PA) secondary antibodies were used. The same labeling was done on isolated centrosomes after sedimentation on glass coverslips as described by Evans et al. (1985).

Microtubule Nucleation Test

Microtubule nucleating activity of isolated centrosomes was performed according to Mitchison and Kirschner (1984) using beef brain tubulin purified on phosphocellulose (Weingarten et al., 1975), and monitored by double immunofluorescence with anti-tubulin and anti-centrosome antibodies. To test the effect of the 62/64-kD–reacting antibodies, centrosomes were first incubated during 20 min at room temperature with different dilutions (1/5 to 1/100) of anti-centrin or anti-230-kD antibodies (used as purified IgGs), or with unrelated sera. Tubulin (20 μM final concentration) and 1 mM GTP were added and the temperature was raised to 37°C for 10 min. After glutaraldehyde fixation and sedimentation on glass coverslips, microtubules were visualized with monoclonal anti-α-tubulin and centrosomes with rabbit serum 0013. Second antibodies were mixed rhodamine-conjugated goat anti-mouse and fluorescein-labeled goat anti-rabbit.

The growth of tubulin on fragments of isolated sea urchin axonemes (a generous gift from D. Pantaloni, laboratoire d'Enzymologie, CNRS, Gif-sur-Yvette, France) was performed as with centrosomes. In other experiments, isolated axonemes were first incubated with the anti-230-kD antibody for 20 min at room temperature before addition of tubulin and GTP. After glutaraldehyde fixation, the samples were sedimented on glass coverslips as described for the centrosomes. Only tubulin staining was used in axoneme experiments.

Cell and Centrosome Fractionation

Preparation of KE 37 Triton X-100 Soluble and Insoluble Proteins. Cultured KE 37 cells were recovered by low centrifugation. After one wash step in PBS buffer, cells were extracted for 2 min at 4°C with 1% Triton X-100 in TNM buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$) containing a mixture of protease inhibitors (1 mM PMSF, 10 $\mu g/ml^{-1}$ aprotinin, 1 $\mu g/ml^{-1}$ each of leupeptin, pepstatin, and chymostatin). The Triton X-100 soluble and insoluble fractions were separated by centrifugation at 300 *g* for 8 min (Centrifuge GT4.11; JOUAN SA. St Nazaire, France). The supernatant was precipitated with 10 vol cold acetone and solubilized in boiling Laemmli SDS-PAGE buffer. The Triton insoluble fraction was washed once with TNM buffer and then solubilized in the SDS-PAGE buffer. Protein content of each fraction was quantified according to Lowry method (Lowry et al., 1951) using BSA as standard.

Chemical Extractions of Centrosomes. Centrosome preparations were diluted in the KPipes buffer (10 mM KPipes, pH 7.2) and sedimented at 20,000 *g* (Sigma Chemical Co., St. Louis, MO; 2 MK centrifuge) for 15 min. The pelleted centrosomes were then resuspended in one of the following extraction buffers during 1 h at 4°C: 3D buffer (100 mM Tris-HCl, pH 8.3, 2 mM EDTA, 0.5% DOC, 0.5% NP40, 0.1% SDS); KI buffer (0.5 M KI in KPipes buffer); urea buffer (8 M urea, 2% NP40, 2% ampholines (LKB Instruments, Inc., Bromma, Sweden), 5% β mercaptoethanol: the lysis buffer for isoelectrofocusing (IEF); O'Farrell, 1975). All buffers contained the protease inhibitor mixture. After each treatment, centrifugation at 20,000 *g* for 10 min at 4°C separated the extracted from the pelleted centrosomal proteins in each case. Supernatants and pellets were solubilized in boiling SDS-PAGE buffer for 5 min. The KI supernatant was first dialysed against H_2O at 4°C before addition of the SDS-PAGE buffer.

Trypsin Digestion of the Centrosomal Proteins. 5.10^7 centrosomes were incubated in 50 μl of K.Pipes buffer and different amounts of TPCK-trypsin (Sigma Chemical Co.) were then added to different samples. After 5 min of incubation at room temperature, PMSF was added to give 1 mM final concentration. Each sample was then denaturated by addition of equal volume of double concentrated Laemmli boiling sample buffer and analyzed by electrophoresis and immunoblotting using both anti-centrin and anti-230-kD antibodies.

EM Microscopy

Centrosomes were sedimented on glass coverslips as mentioned above and processed for immunogold staining according to method of Langanger et al. (1984) with some slight modifications. Centrosomes were fixed or not with 0.5% glutaraldehyde in the KPipes buffer during 10 min (equivalent results were obtained), washed 3 times with KPipes buffer, and then treated with 1 mg/ml of $NaBH_4$ for 20 min. After three wash steps with TBS–BSA buffer (20 mM Tris-HCl, pH 8.2, 0.15 M NaCl containing 0.1% BSA), centrosomes were incubated with either anti-centrin or anti-230-kD antibodies diluted in TBS–BSA buffer during 1 h at room temperature. Centrosomes were washed three times with TBS–BSA and goat anti-rabbit antibody coupled to colloidal gold (GAR G5, Janssen Biotech. NV) was added as second antibody diluted 1/5 in TBS–BSA buffer during 1 h at room temperature. The control sample was incubated with the second antibody alone. In other experiments, centrosomes were first extracted with 0.5 M KI in 10 mM KPipes, pH 7.2, for 30 min at 4°C, and then sedimented on glass coverslips for immunogold staining with anti-centrin antibody. After second antibody incubation, samples were washed three times with TBS–BSA buffer, postfixed with 2.5% glutaraldehyde in 0.1 M Na-Cacodylate buffer during 1 h, washed in cacodylate buffer, and followed by incubation of 1 h with 1% os-

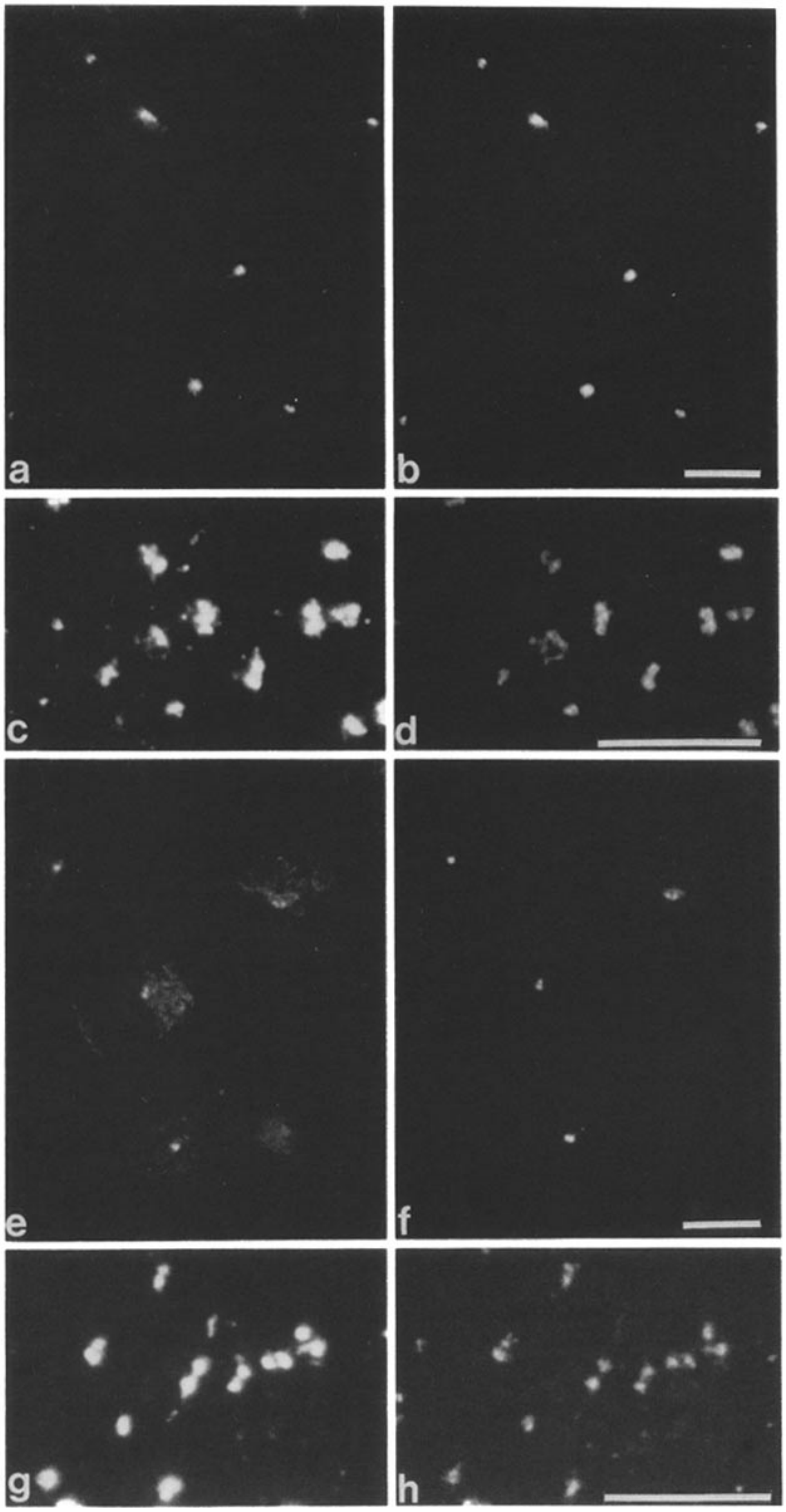


Figure 1. Double immunofluorescence labeling of HeLa cells (*a*, *b*, *e*, and *f*) and of centrosomes isolated from KE 37 lymphoid cells (*c*, *d*, *g*, and *h*) using either polyclonal anti–centrin 08/28 (*a* and *c*) or anti-230-kD protein antibodies (*e* and *g*) and the monoclonal CTR453 antibody (*b*, *d*, *f*, and *h*). Bars, 10 μm.

mium tetroxide and 0.5% tannic acid. Each sample was subsequently progressively dehydrated with ethanol and embedded in Epon. Uranyl acetate was added in the 70% step of dehydration at 0.5%. Sections parallel to the coverslips were observed with an electron microscope (EM 201; Philips Electronic Instruments, Inc., Mahway, NJ).

Electrophoresis and Immunoblotting

The proteins were analyzed by electrophoresis as described by Laemmli (1970) on 6–15% polyacrylamide gel gradients or 8% homogeneous gels, and stained with silver nitrate (Switzer et al., 1979). The EGTA- and Ca^{2+}-containing gels were performed as described by Huang et al. (1988*b*). For

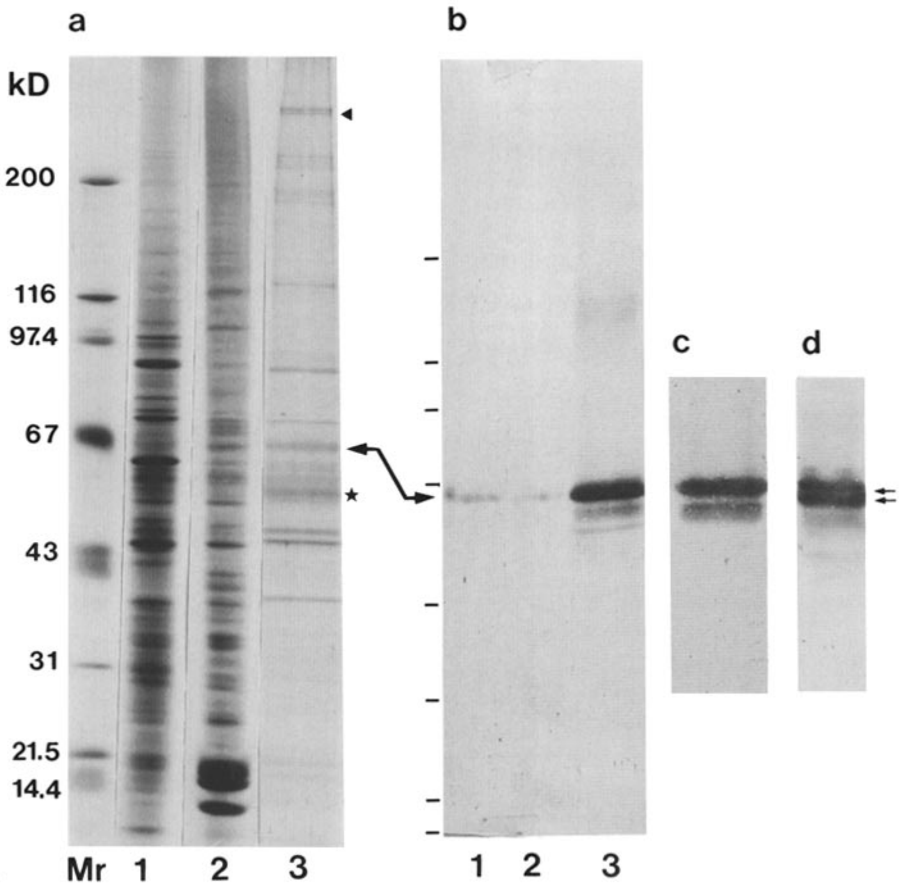


Figure 2. Immunodetection on nitrocellulose filter of a centrosomal 62/64-kD protein by the anti-centrin and the anti-230-kD protein antibodies. (*a*) Silver staining of Triton X-100-soluble (lane *1*, 4 μg), Triton X-100-insoluble proteins (lane *2*, 4 μg) and of centrosomal proteins (lane *3*, 1 × 10^7 centrosomes) from KE 37 after separation on a 6–15% SDS-PAGE gradient. Note the enrichment in the centrosomal antigen p350-kD doublet (*arrowhead*; Gosti et al., 1986). The star indicates tubulins. Molecular mass standards in kilodalton (*kD*): myosin heavy chain 200 kD; β-galactosidase 116 kD; Phosphorylase B 97.4 kD; Bovine serum albumin 67 kD; Ovalbumin 43 kD; Carbonic anhydrase 31 kD; Soybean trypsin inhibitor 21.5 kD; Lysozyme 14.4 kD. (*b*) Immunoblot using anti-centrin antibody 08/28 on the same fractions as in *a* with heavy loading (lanes *1* and *2*, 20 μg; lane *3*, 4.5 × 10^7 centrosomes). Linked arrows between *a* and *b* indicate the 62/64-kD protein in both panels. (*c*) Immunodetection of the 62/64-kD protein in the centrosomal fraction (4.5 × 10^7 centrosomes) by the anti-230-kD protein antibody. No protein was detected with this antibody in fractions 1 and 2 (not shown). (*d*) Detection of the 62/64-kD doublet (*small arrows*) with the anti-centrin antibody 08/28 after separation of centrosomal protein (4.5 × 10^7 centrosomes) on an 8% SDS-PAGE. Several faster bands than the 62/64-kD doublet are detected in an irregular manner from one experiment to the other, suggesting that they are proteolytic products. Both antibodies stain these products in a slightly different manner (compare *b* and *c*).

immunodetection, gel slabs were electrophoretically transferred to nitrocellulose filter according to Towbin et al. (1979) using the semi-dry system (LKB Laboratories). The filters were saturated with TBS buffer (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% Tween 20 containing 5% non-fat dry milk). All washing steps and antibody dilutions were performed in the TBS buffer. The immunoreactive polypeptides were detected with either goat anti-mouse or anti-rabbit antibodies coupled with alkaline phosphatase (Promega Biotec, Madison, WI) using nitroblue tetrazolium and 5-Bromo-4-Chloro-3-Indoylphosphate as substrates in 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$.

Results

Antigens Immunologically Related to Tetraselmis s. centrin and to a Polyplastron m. 230-kD Protein Are Present in Human Centrosomes

Immunofluorescence microscopy with the polyclonal anti-centrin antibody reveal a specific staining of a juxtanuclear region in HeLa cells (Fig. 1 *a*). The use of the anti-centrosome mAb CTR453 in double-immunofluorescence experiments demonstrate the centrosomal localization of the antigen (Fig. 1, *a–d*). Preimmune serum was not available to us but shown to be negative on PtK2 cells in which the immune serum stained the centrosome (Baron and Salisbury, 1988). Our result extends the previous observation by Salisbury et al. (1986) on the presence of a centrin-related protein at the centrosome in mammalian cells. The anti-centrin staining resists nonionic detergent cell extraction and centrosome isolation indicating a tight association of the antigen with the centrosome (Fig. 1, *a* and *c*).

The human centrosomes were also stained, in a manner similar to the anti-centrin antibody staining, by another unrelated antibody directed against a 230-kD protein isolated from the protozoan *Polyplastron m.* (Fig. 1, *e* and *g*). No centrosomal staining was observed using either preimmune serum of the anti-230-kD antibody or secondary antibody alone. The 230-kD protein is not per se a Ca^{2+}-BP but it forms, in conjunction with a 22-kD Ca^{2+}-BP, thin cortical filaments tightly associated with basal bodies in this ciliate (Vigues and Grolière, 1985; Vigues et al., 1984). In double-immunofluorescence experiments on isolated centrosomes with either anti-centrin or anti-230-kD and with CTR453 antibodies, the volumes stained with the anti-centrin and the anti-230-kD antibodies are larger than that stained with CTR453 (Fig. 1, *c*, *d*, *g*, and *h*). Similar results are also obtained on KE 37 lymphoblastic cell line using the anti-centrin and anti-230-kD antibodies (not shown).

Anti-Centrin and Anti-230-kD Antibodies Recognize the Same 62/64-kD Centrosomal Protein

The human antigen recognized by the anti-centrin antibody is identified by western immunoblotting on centrosomal proteins from KE 37 cells. The anti-centrin antibody recognizes

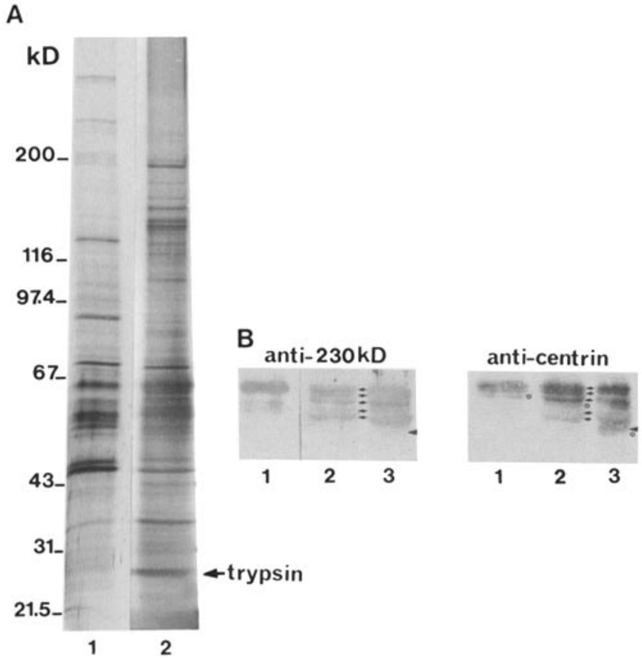


Figure 3. Polypeptides fragments of the 62/64-kD protein after partial trypsin digestion of the centrosomal proteins. (*A*) Silver staining of: lane *1*, control centrosomal proteins; Lane *2*, centrosomal proteins treated with 6 ng of TPCK-trypsin. (*B*) Immunodetection of the 62/64-kD protein with anti-230-kD and anti-centrin (08/28) antibodies. Lane *1*, untreated centrosomal proteins; lane *2*, centrosomes treated with 6 ng of TPCK-trypsin; lane *3*, centrosomes treated with 12 ng of TPCK-trypsin. The overall profile of the polypeptides revealed with both antibodies is comparable (lane *2*, *arrows*). The open circles (○) indicate the polypeptides specifically recognized with anti-centrin antibody. The arrowhead in lane *3* points to a fragment which was detected by anti-centrin rather than anti-230-kD antibodies.

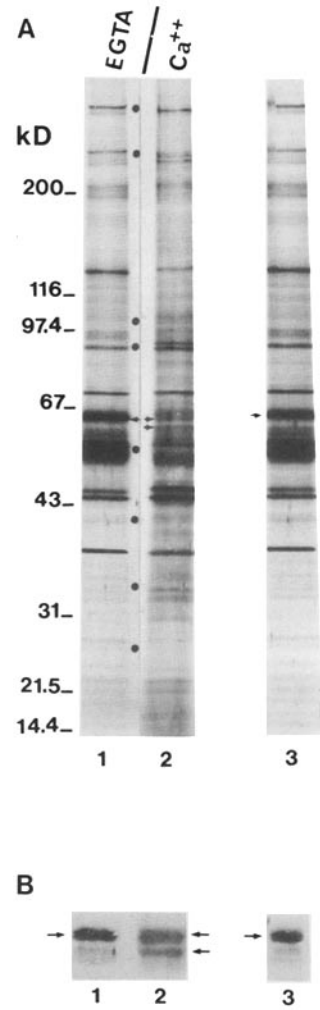


Figure 4. Isolated centrosomes are enriched in proteins which migrate on SDS-PAGE in a Ca^{2+}-dependent manner. (*A*) Silver staining of centrosomal proteins (2×10^7 centrosomes) after separation on a 6–15% gradient of SDS-PAGE in the presence of 2 mM EGTA (lane *1*) or 2 mM Ca^{2+} (lane *2*). 1 mM EGTA or 1 mM Ca^{+} was added to each sample in the presence of protease inhibitors before loading the gels. Several proteins have their mobility changed by Ca^{2+} (*dots*). The 62/64-kD proteins are marked by arrows. Purified calmodulin was used as control for change in migration (not shown). The mol wt standards are as in Fig. 2. Addition of 1 mM EGTA to centrosomes first incubated with 1 mM Ca^{2+} and separated on the EGTA-containing gel reversed the observed effect of Ca^{2+} (lane *3*). (*B*) Immunodetection of the corresponding fractions with the anti-centrin antibody. Note the change in mobility of the reacting bands when centrosomes were incubated with Ca^{2+} (lane *2*), and the reversibility of the Ca^{2+} effect (lane *3*).

a thick band, highly enriched in isolated centrosomes, of 64 kD on a 6–15% SDS-PAGE together with several faint faster bands, suggesting some proteolysis although protease inhibitors were used throughout centrosome preparation (Fig. 2, *a* and *b*). In a more resolutive polyacrylamide gel, this protein is resolved as two polypeptides of 62 and 64 kD, with the same intensities (Fig. 2 *d*). This could also be because of proteolysis, although the 62/64-kD molecules are consistently observed from one preparation to the other. We therefore favor the possibility that they represent at least two isoforms of a single polypeptide. Consequently, we refer to the polypeptide in question as the "62/64-kD" doublet. The copurification of the 62/64-kD antigens with the centrosome fraction is a strong indication that these antigens correspond to the immunofluorescence labeling observed in situ. We further ascertain this conclusion by an independent experiment: preadsorption of the anti-centrin antibody with the 62/64-kD proteins separated on SDS-PAGE and transferred onto nitrocellulose filter abolishes the centrosomal staining (not shown).

The anti-230 kD antibody recognizes a 62/64-kD protein in the human centrosomes in a manner quite similar to what was observed with the anti-centrin antibody (Fig. 2 *c*). For example, both antibodies detect the same 62/64-kD doublet in 8% polyacrylamide gel. Nevertheless, the anti-centrin and the anti-230-kD antibodies exhibit slight differences: they did not recognize the same faint bands migrating ahead of the main 62/64-kD antigens and which could correspond to degradative products (compare Fig. 2, *b* and *c*). This result indicates either that the two antibodies react with two different, although comigrating, antigens or detect different epitopes of the same antigen. To discriminate between these two possibilities, we first attempted to perform 2D gel analysis. This could not be achieved since the antigens recognized by each antibody are insoluble in the IEF buffer. This common solubility behavior of both antigens (see below) is more in favor of the possibility that they are one and the same antigen rather than two comigrating antigens. We however performed a partial digestion of the centrosomes with trypsin in an attempt to compare the proteolytic profile of the antigens as revealed with each antibody. As both antibodies detect differently the spontaneous proteolysis fragments (Fig. 3 *B*, lanes *1*; see also Fig. 2), we are not expecting completely identical profiles after trypsin digestion. However, the major proteolytic fragments of the 62/64-kD protein generated by trypsin are comparable when revealed with each antibody. The only differences concerning the very low relative molecular weight polypeptide which is rather revealed by anti-

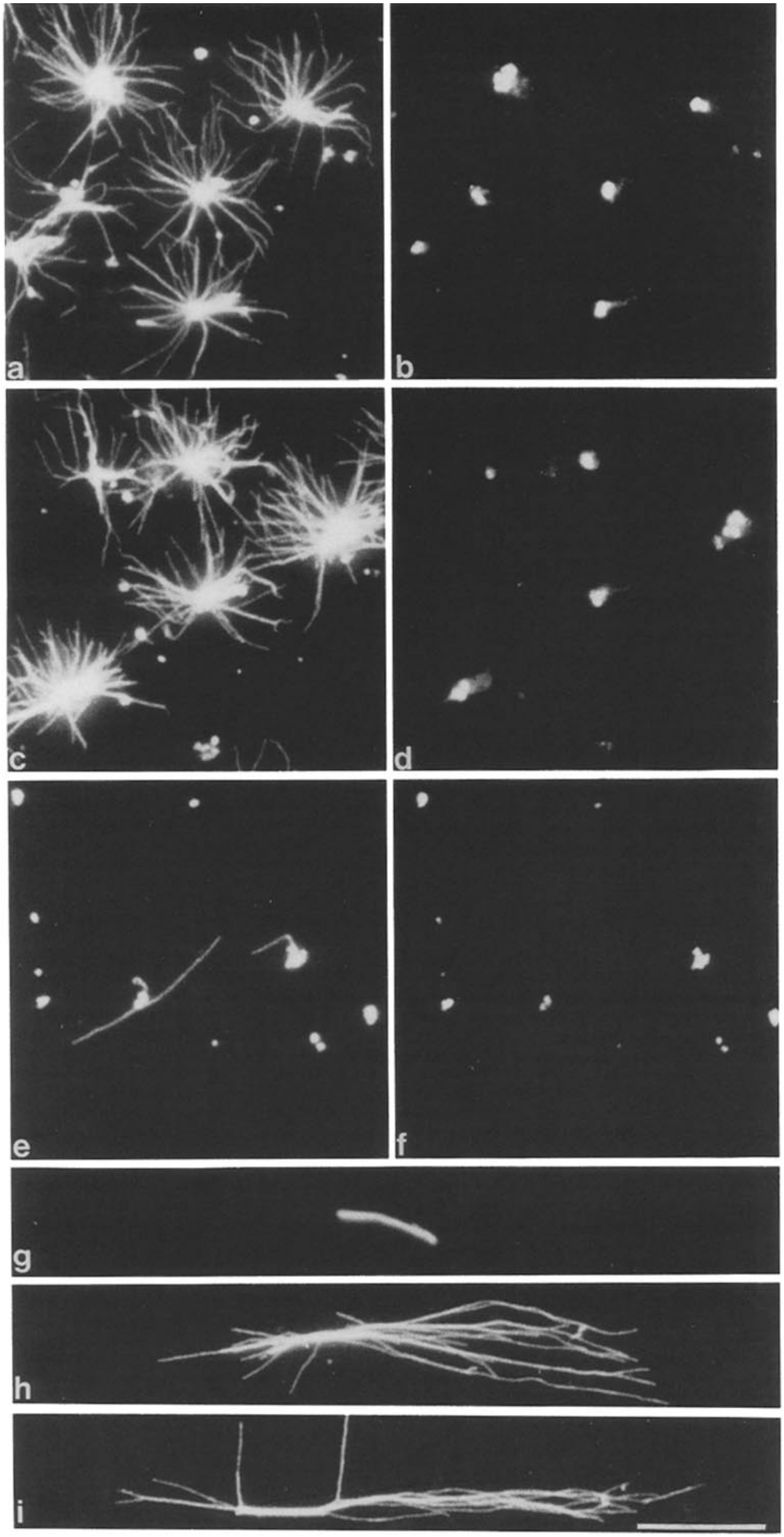


Figure 5. The anti-230-kD antibody prevents in vitro centrosome-dependent microtubule nucleation. Microtubule-nucleating activity of isolated centrosomes was monitored by double immunofluorescence with anti-tubulin and anti-centrosome (*a* and *b*, *control*). To test the effect of the 62/64-kD–reacting antibodies, centrosomes were first incubated with anti-centrin (*c* and *d*) or with anti-230-kD antibodies (*e* and *f*) as described in Materials and Methods. Microtubules were visualized with monoclonal anti-α tubulin (*a*, *c*, and *e*) and centrosomes with rabbit serum 0013 (*b*, *d*, and *f*). Note the specific inhibition of aster formation by the anti-230 kD (*e* and *f*). As this inhibition could bear either on the nucleation or on the elongation step, this antibody was used on fragments of isolated sea urchin axonemes. The preincubation of the axonemal seeds with the anti-230-kD antibody before adding tubulin (*i*) did not modify elongation of microtubules (compare with the control in *h*). In *g*, a seed before addition of tubulin. *g*, *h*, and *i*, anti-tubulin staining. Tested unrelated sera did not affect microtubule assembly on centrosomes (not shown). Bar, 10 μm.

centrin than anti-230 kD when 12 ng trypsin is used (Fig. 3 *B*, lanes *3*). Two other polypeptides are revealed only with anti-centrin (Fig. 3 *B*, ○), a situation which was expected due to the spontaneous degradation of the antigens. Alternatively, the introduction of some variability because of differences in the affinity of anti-230-kD antibody for these cleavage products could explain the differences. These results suggest that both antibodies react with the same antigen (see further arguments below) and that the epitopes specific for each antibody are either different or at least not completely congruent. Interestingly, there is no cross-reaction between the anti-centrin and the 230-kD protein from *Polyplastron m.* and vice versa, although the anti-centrin recognizes the 22-kD Ca^{2+}-BP which interacts with the 230-kD protein in the infraciliary lattice of *Polyplastron m.* On the other hand, neither anti-centrin nor anti-230-kD antibodies cross-react with purified cytoskeletal protein such as desmin, vimentin, cytokeratins, tektins, or lamins (not shown), a control we made since the relative molecular weight of the centrosomal antigen and its insolubility are reminiscent of the intermediate filament proteins (see below). We conclude that epitopes identified in basal body or basal body–associated structures from lower eucaryotes are conserved in human centrosomes, although they are borne by proteins of widely divergent molecular mass.

Analysis of the Electrophoretic Behavior of Centrosomal Proteins in SDS-PAGE in the Presence of Ca^{2+}

Since both centrin and the 230-kD proteins are involved in Ca^{2+}-modulated structures, this prompted us to test for the Ca^{2+}-binding activity of the 62/64-kD proteins. Separation of centrosomal proteins on SDS-PAGE in the presence of EGTA or Ca^{2+}, followed by immunoblotting with anti-centrin antibody, reveal that the mobility of a part of the 62/64-kD protein, is changed in the presence of Ca^{2+} (Fig. 4, lanes 2). Addition of EGTA to the Ca^{2+}-treated sample reversed the Ca^{2+} effect (Fig. 4, lanes *3*), ruling out the possibility that the Ca^{2+}-induced shift corresponds to some irreversible modifications such as proteolysis. This result indicates that at least one of the two polypeptides of the 62/64 doublet has a Ca^{2+}-sensitive electrophoretic behavior. More work will be however necessary to clarify the precise Ca^{2+} effect on each polypeptide.

We have observed that in addition to the 62/64-kD protein, isolated centrosomes are specifically enriched with proteins which change their electrophoretic mobility on SDS-PAGE in the presence of Ca^{2+} (Fig. 4 *A*, *dots*). We have observed that the carbocyanine dye "Stains all" (Campbell et al., 1983) gives a blue coloration of the same centrosomal proteins which change their migration in the Ca^{2+}-containing gels (not shown).

The Centrosomal 62/64-kD Protein Is Involved in Microtubule Nucleation

Looking for a possible function of the 62/64-kD centrosomal protein, we have tested its involvement in the nucleation of microtubules. We have used the in vitro microtubule nucleation assay: the anti-230-kD IgGs blocks the centrosome-dependent tubulin assembly in a dose-dependent manner (Fig. 5, *e* and *f*), whereas the anti-centrin antibody did not (Fig. 5, *c* and *d*). Preincubation of centrosomes with preimmune serum corresponding to the anti-230-kD antibody did not affect the nucleation capacity of human centrosomes. Moreover, other antibodies against centrosomal proteins such as serum 0013 did not inhibit the nucleation of microtubules as already reported by Gosti et al. (1986). We have checked that the inhibition concerns the nucleation step of microtubule assembly: the anti-230-kD antibody did not affect the elongation of microtubules on isolated sea urchin sperm axonemes (Fig. 5 *i*). The discrepancy between the effect of anti-230-kD and anti-centrin antibodies could be related either to their specificity for different epitopes (Fig. 2, *b* and *c* and Fig. 3), to their affinity for the antigen or to their respective concentration of IgG specific to the 62/64-kD protein. However, when tested in more physiological conditions, i.e., in Xenopus egg extracts (Tournier et al., 1989), both antibodies block aster formation (not shown). These results strongly suggest the involvement of the 62/64-kD centrosomal protein in the nucleation step.

The Centrosomal 62/64 kD Is a Highly Insoluble Protein Associated with the PCM

Klotz et al. (1990) have previously shown that centrosomal structure and its parthenogenetic activity are resistant to salt treatment. However, both structure and activity are sensitive to moderate concentrations of chaotropic agents such as urea of KI. A progressive solubilization of several centrosomal proteins is observed as the urea concentration increased (Klotz et al., 1990). We show in Fig. 6 the electrophoretic analysis of optimal extractions of centrosomal proteins by the 3D buffer, the Kl buffer, and 8 M urea (see Materials and Methods). The 3D and Kl buffers treatments give an insoluble fraction of quite comparable pattern (10 major polypeptides) (Fig. 6 *a*, lanes *2* and *4*), and a much simplified pattern with one major protein is obtained after urea treatment as judged by SDS-PAGE (Fig. 6 *A*, lane *6*). The main protein of each insoluble fraction is a protein in the region of 62/64 kD (Fig. 6 *A*, *black arrows*) which correspond to the polypeptides recognized by either anti-centrin or anti-230-kD antibodies (Fig. 6 *B*, *black arrows*). By contrast, the centriolar tubulin is completely extracted in the same conditions (Fig. 6, *A* and *B*, *open arrows*).

Ultrastructural observations of the KI insoluble fraction shows that centriolar structure had completely disappeared, a result expected from the extraction of tubulin (Fig. 6 *B*, lanes *3* and *4*), whereas the PCM and the intercentriolar link remain structured (see Fig. 8 *B*; see also Klotz et al., 1990 for the ultrastructure of the KI and urea insoluble fractions). The correlation between biochemical analysis and EM observations strongly suggest that the identified 62/64-kD centrosomal protein is, to a large extent, associated with the PCM.

To confirm this conclusion, we have immunolocalized the protein at ultrastructural level on isolated centrosomes by the colloidal gold method. Patches of thin filaments within the PCM and along the intercentriolar link are revealed in a comparable manner using either anti-centrin or anti-230-kD antibodies (Fig. 7, *b* and *c*). Gold particles are often observed not directly attached to the centrioles' walls. This corresponds well with observations of immunofluorescence labeling on isolated centrosomes compared to the CTR453 staining (Fig. 1, *c*–*f*). The gold staining is still largely conserved even after

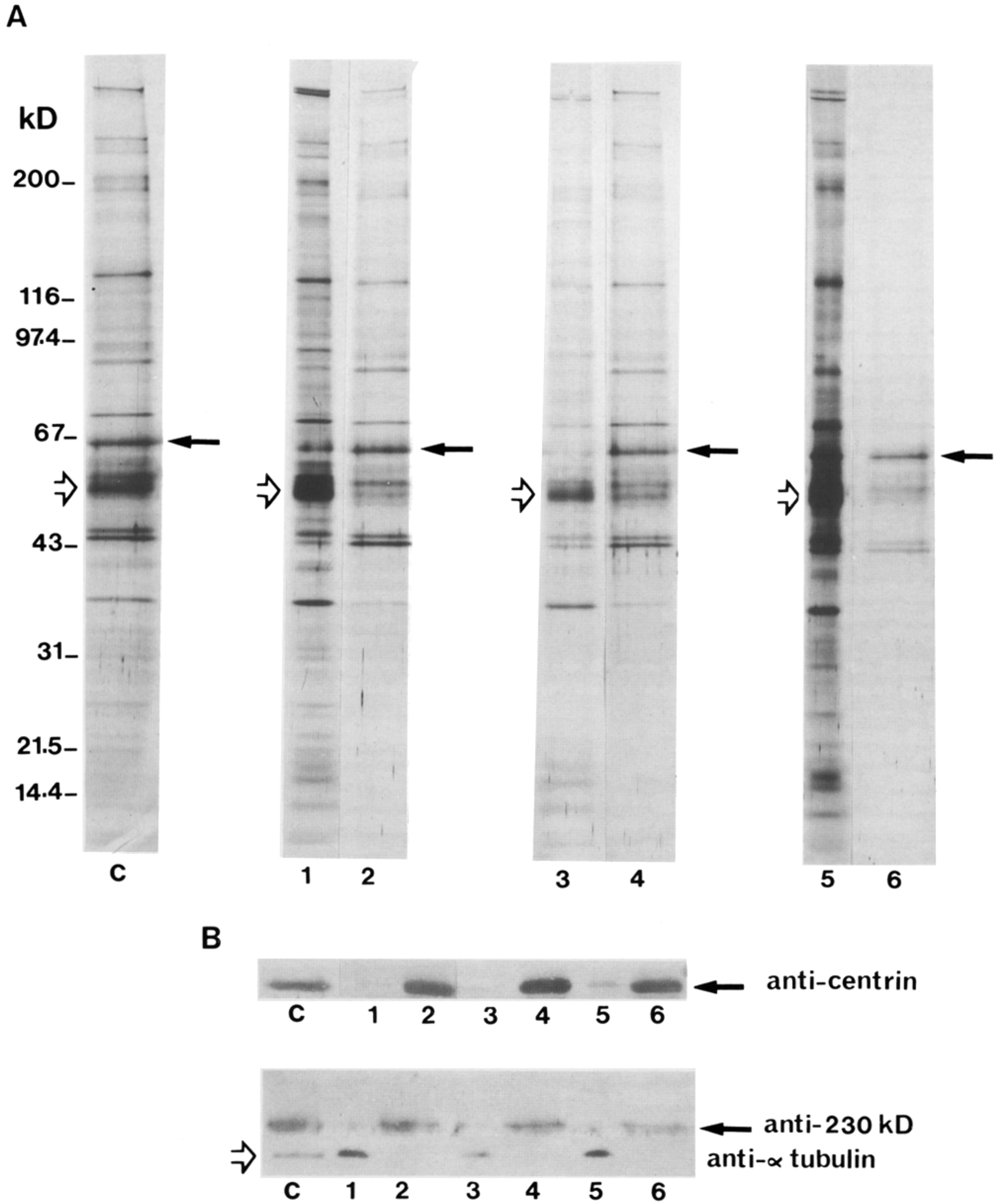


Figure 6. (*A*) Silver staining of 6–15% SDS-PAGE of soluble and insoluble centrosomal proteins after treatment with 3D buffer (lanes *1* and *2*), KI buffer (lanes *3* and *4*), and 8 M urea (lanes *5* and *6*) as described in Materials and Methods. Lane *c* shows centrosomes treated with the KPipes buffer alone (control). 1.6×10^7 centrosomes were used in each case. The 3D and KI insoluble fractions (lanes *2* and *4*, respectively) show a comparable protein composition. The 62/64-kD polypeptide is indicated by the black arrows while the open arrows show the tubulins. Mol wt standards are as in Fig. 2. (*B*) Immunoblotting using anti-centrin antibody 08/28, and anti-230-kD protein antibody (*black arrows*) and anti-α tubulin antibody (*open arrow*) on the same fractions as in *A* except that 3×10^7 centrosomes were used in each treatment. Note the total extraction of the tubulin (lanes *1*, *3*, and *5* corresponding to 3D, KI, and urea soluble fractions, respectively), whereas the 62/64-kD protein revealed by both antibodies was still associated with the insoluble fractions whatever the treatment used (lanes *2*, *4*, and *6*).

KI extraction (Fig. 8), which confirms the biochemical data. Gold particles are insignificant in the subdistal pericentriolar arms (Fig. 7, *d* and *e*, *open arrows*) even though the latter are recognizable within the KI insoluble fraction in which the 62/64-kD doublet is concentrated (Fig. 8 *b*). This could reflect a specific composition of these arms or an inaccessibility of the antigen to the antibodies at this location.

We have tested the nucleation capacity of the insoluble residues after 3D or KI treatment since they mainly contain the 62/64-kD protein. Nucleating activity was never observed in vitro; however these residues induce aster formation when tested in Xenopus egg extract (not shown). We cannot however discriminate between a better renaturation in Xenopus eggs extract of the insoluble residue which could then indicate that the 62/64-kD protein is the major protein involved in the nucleation process, and a complementation of the insoluble residue with Xenopus-nucleating proteins present in the extract.

Discussion

The present study has identified a new centrosomal protein in human cells which is immunologically related to the basal body–associated Ca^{2+}-BP, centrin. Several lines of evidence support the argument that this novel protein of 62/64 kD is a genuine centrosomal component: (*a*) it reacted with two unrelated antibodies both of which specifically decorate centrosomes in situ and after their isolation; (*b*) it is highly enriched in isolated centrosomes; (*c*) it is a part of the most insoluble fraction of the PCM as observed by biochemical and electron microscopic approaches and by immunocytochemical localization; (*d*) notably, the 62/64-kD protein is shown to be involved in the nucleation of microtubules.

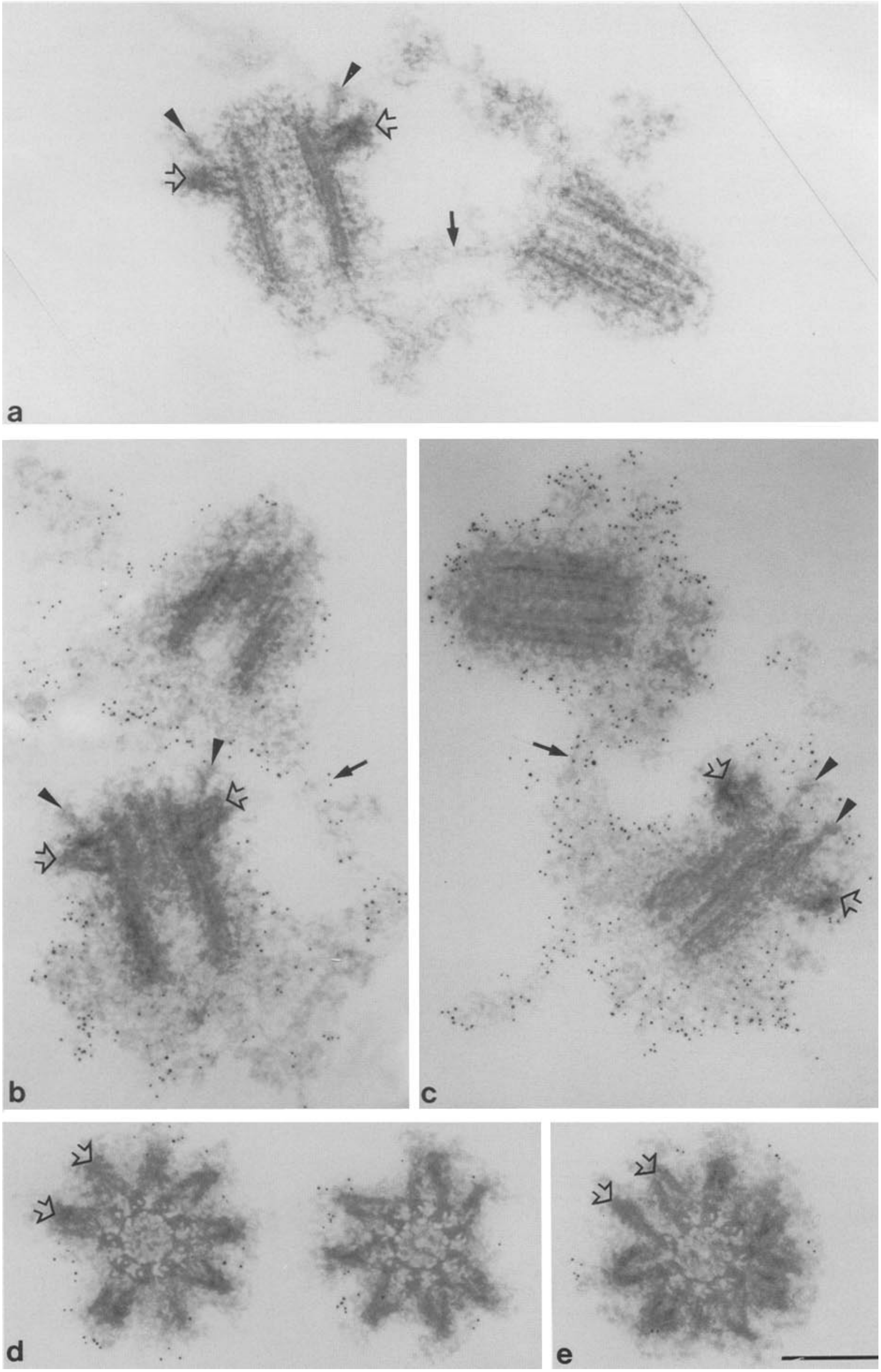
Our results in human cells are at variance with results on PtK2 cells where the anti-centrin antibody specifically decorates the centrosome, and immunoprecipitates a 165-kD protein from cell extracts (Baron and Salisbury, 1988). These results suggest that homologous basal body/centrosome–associated proteins from algal and mammalian cells have different relative molecular weights. However, the centrosomal nature of the 165-kD protein in PtK2 cells must yet be confirmed directly.

The presence of a centrin related Ca^{2+}-BP protein in mammalian centrosomes and the demonstration that it is involved in microtubule nucleation has important implications when considering the roles played by centrin in the basal body apparatus of the green algae (Wright et al., 1985, 1989; Sanders and Salisbury, 1989). The Ca^{2+}-BP centrin is the major component of the contractile nuclear/basal body connector, the distal fiber which links the two basal bodies (Schulze et al., 1987) and constitutes a stelatte structure in the transition zone between basal bodies and flagella in *Chlamydomonas r.* It has been shown that centrin mediates microtubule severing during flagellar excision in this algae (Sanders and Salisbury, 1989). The identification of the 62/64-kD protein which have a Ca^{2+}-sensitive behavior in SDS-PAGE and involved in microtubule nucleation in human centrosomes suggests that Ca^{2+} could also play a role in the control of centrosomal structure or function or both as it is for the basal body apparatus of green algae (Salisbury et al., 1984, 1987). We have observed that Ca^{2+} promotes a structural reorganization of the PCM and a significant decrease of the intercentriolar distance which is controlled by the length of the intercentriolar link which contains the 62/64-kD centrin homologue protein (manuscript in preparation).

The other antibody which reacts with the 62/64-kD centrosomal protein was raised against a 230-kD *Polyplastron m.* protein. Together with a 22-kD Ca^{2+}-BP, this 230-kD protein was identified as a component of the infraciliary lattice, that is a continuous layer of thin filaments that interconnect baren kinetosomes present in the cell cortex of *Polyplastron m.* Individual microtubules are observed that emerge from the proximal part of these kinetosomes towards the cell periphery. Other microtubules originating from the same sites are oriented towards the interior of the cell (Vigues et al., 1984). The role of this structure in microtubule nucleation or stabilization has not yet been directly demonstrated. Garreau et al. (1988) showed that the infraciliary lattice in Paramecium displays Ca^{2+}-dependent contractile properties reminiscent of those of centrin-based rootlets in green algae (Salisbury et al., 1984). Our results on human centrosomes should prompt further investigations of these kind of structures.

To date, only a few basal body or centrosomal proteins have been shown to be implicated in microtubule nucleation or stabilization. A group of high molecular mass proteins (190–210 kD), isolated from the *Polytomella* basal body complex, are shown to participate in a structure from which microtubules are initiated (Stearns and Brown, 1979). Toriyama et al. (1988) have isolated a 51-kD protein from sea urchin eggs which was shown to congregate into granules able to induce aster formation in vitro. This protein was localized in both centrosphere and the mitotic spindle (Ohta et al., 1988), and shared high homology and functional features with the yeast elongation factor EF1α (Ohta et al., 1990). The basic nature of the 51-kD protein (isoelectric point $\sim$9.8) may however in itself explain its in vitro nucleating activity. Dinsmore and Sloboda (1988, 1989) have reported that the Ca^{2+}/calmodulin-dependent phosphorylation of a 62-kD protein, associated with sea urchin mitotic spindle, was correlated with a complete depolymerization of mitotic apparatus microtubules inducing the entry into anaphase. The 62/64-kD human centrosomal protein, in contrast to the sea urchin 62-kD protein, is exclusively associated with centrosomes during all cell cycle phases of the studied human cultured cells. Oakley et al. (1990) have identified a new member of the tubulin family, a γ tubulin which is associated with the spindle pole body in *Aspergillus nidulans*. In this work the authors propose that the γ tubulin protein attaches to the spindle pole body and plays some role in the nucleation of microtubules and the establishment of microtubule polarity in vivo. Recently, Tousson et al. (1991) have reported that a mAb raised against kinetochore-enriched chromosome extract from HeLa cells recognize two polypeptides of 180 and 210 kD called centrophilin. It is relocated from the centromeres to the centrosomes to the midbody depending on the mitotic phase of the cell and is nuclear during interphase. The microtubule-nucleating protein assignment to the centrophilin is however based on indirect arguments and a direct biochemical demonstration that the antigen is specifically enriched in microtubule organizing centers is lacking.

Recently, Centonze and Borisy (1990) have reported that preincubation of mitotic CHO centrosomes with MPM2 antibody or their treatment with alkaline phosphatase prevent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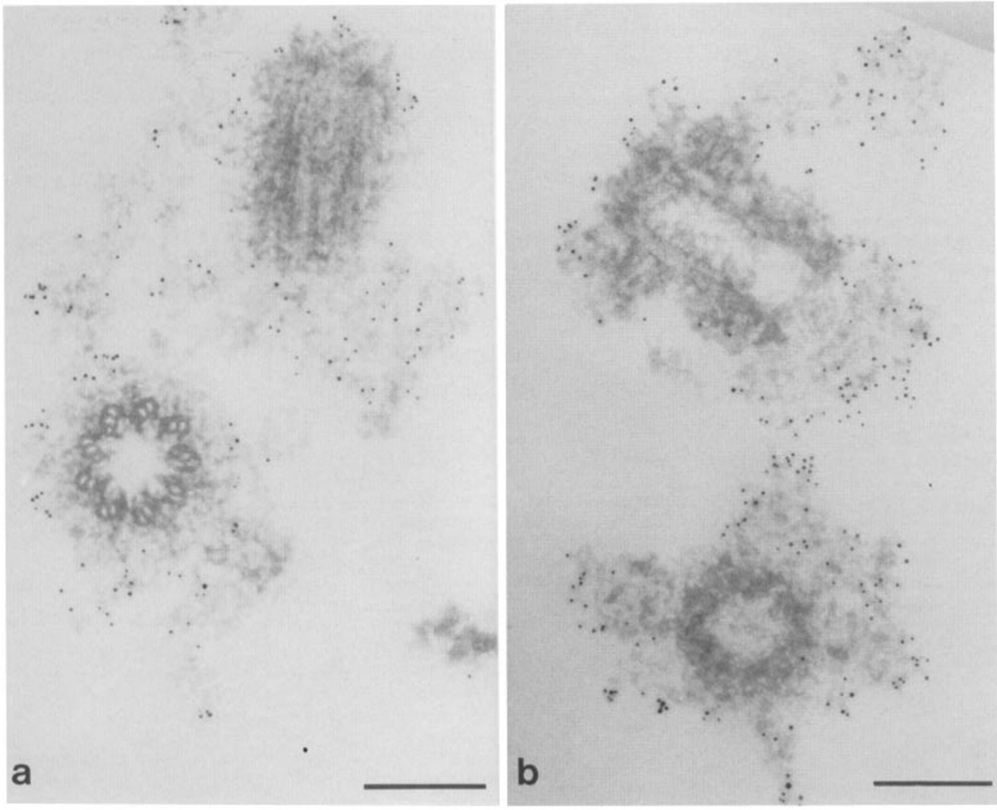

Figure 8. Immunogold labeling with the anti-230-kD antibody of untreated centrosomes (*a*) and centrosomes first treated with 0.5 M KI for 30 min and then incubated with the anti-centrin antibody 26/14-1 (*b*). Note the complete extraction of the centriolar microtubules. The gold particles are still associated with the unextracted PCM and with the intercentriolar link (*b*). Bar, 0.2 μm.

their in vitro nucleating activity. The possibility that regulation of microtubule nucleating activity of centrosomes by modulation of the phosphorylation level of centrosomal antigens has also been discussed by several authors (Bailly et al., 1989; Kuriyama and Borisy, 1981; Kuriyama, 1989; Vandre et al., 1986). The complexity of the bands reacting with the anti-centrin and the anti-230-kD antibodies in human centrosomes could correspond to posttranslational modifications such as phosphorylation, possibly associated with Ca^{2+}-binding activity. Centrin is known to exist as two isoforms which differ by the presence of a phosphate group, Ca^{2+} being able to induce the dephosphorylation of centrin in vivo (Salisbury et al., 1984). The 62/64-kD centrosomal protein is particularly insoluble even in chaotropic agents, a property which precluded the determination of the number of isoforms, and renders direct functional studies difficult. Molecular characterization of this novel 62/64-kD centrosomal protein will hopefully lead to a better understanding of the mechanism by which centrosomal structure and microtubule nucleation activity could be controlled.

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Figure 7. Immunolocalization of the 62/64-kD proteins at ultrastructural level on human isolated centrosomes. (*a*) Centrosome incubated with the second antibody alone (GAR. G5). Centrosomal structure was well preserved after their isolation: note the paired configuration, the link between the two centrioles (*black arrow*), the distal (*black arrowheads*), and the subdistal arms (*open arrows*) on the mother centriole, the pericentriolar network surrounding the daughter and mother centrioles. No labeling was observed in this condition. (*b*) Centrosomes incubated with the anti-230-kD antibody after glutaraldehyde fixation. The gold labeling is associated with the PCM at the proximal end of the daughter and the mother centrioles and along the intercentriolar link. (*c*) Centrosomes incubated with anti-centrin antibody 26/14-1. Similar results were obtained with the anti-centrin antibody 08/28 (not shown). Note the strikingly similar labeling in comparison to the anti-230-kD staining. No specific staining was observed directly in the subdistal pericentriolar arms (*open arrows*) of the mother centrioles with anti-centrin (*d*) nor with anti-230 kD (*e*) when observed in transverse sections. Bar, 0.2 μm.

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