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Most of centrin in animal cells is not centrosome-associated and centrosomal centrin is confined to the distal lumen of centrioles

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SUMMARY

Centrin is a member of the calcium-binding EF-hand protein superfamily present in centrosomes of widely divergent species. Investigating the cellular distribution of human centrin by both immunofluorescence and cell fractionation, we report that centrin is biochemically complex in human cells, displaying as much as ten isoforms in 2-D electrophoresis. This suggests that centrin may be subject to multiple regulations. Strikingly, more than 90% of centrin is not associated with the centrosome fraction. The centrosome-associated centrin, however, displays a specific pattern in 2-D electrophoresis and is concentrated within the distal lumen of the centrioles, where a complex structure has been previously described. This precise localization allows the resolution of centrioles at the optical level throughout the cell cycle and provides a valuable tool for monitoring centriole duplication. To get insights on centrin function, we performed injection experiments of recombinant heterologous centrin in two-cell stage frog embryos in an attempt to produce dominant negative effects. We report that green algae and human centrin delay cleavage and promote the formation of abnormal blastomeres in which the distribution of microtubule asters and of nuclei is dramatically impaired. This suggests that centrin could be involved in the centrosome reproduction cycle, in the coordination of cytoplasmic and nuclear division or in cytokinesis.

Key words: Centrin, Centriole, Centrosome, Cell division cycle, Ca^{2+}-binding protein, *Xenopus laevis*

INTRODUCTION

Centrosome biogenesis and activity are ancient and yet poorly understood issues in cell biology. A revival of interest in this organelle has recently been illustrated in several studies which all indicate that Boveri's original view of the centrosome coordinating cytoplasmic and nuclear division is likely to be correct (reviewed by Tournier and Bornens, 1994). Thus, understanding the molecular mechanisms which control the activity and the biogenesis of the centrosome may be critical for the understanding of cell division and cell morphogenesis.

The structural and biochemical complexity of the centrosome in animal cells is considerable and is not easily amenable to genetic analysis. Centrosome biogenesis occurs through a process of duplication which involves structural continuity, a feature holding for all kinds of centrosomes, from the simple spindle pole bodies (SPB) in fungi to the complex centriole-containing centrosomes in animal cells. One can thus expect a comparative study of the different types of centrosomes among divergent species to benefit from the particular features observed in widely divergent systems, and thus to get at general principles of centrosome inheritance and activity.

Among the gene products identified so far as components of the centrosome in widely divergent systems, two families have been characterized and are certainly the most relevant in terms of centrosomal functions. One, γ-tubulin (for a review, see Joshi, 1993), which appears concentrated at the centrosome although the vast majority is not associated with it (Stearns and Kirschner, 1994; Vassilev et al., 1995; Moudjou et al., 1996), has been shown by both genetic and biochemical approaches to be critical for microtubule nucleation (Oakley and Oakley, 1989; Joshi et al., 1992). The other one, the centrin family, is a member of the calcium-binding EF-hand protein superfamily like calmodulin, and is apparently essential to the organization of eukaryotic centrosomes (Salisbury, 1995; Schiebel and Bornens, 1995).

Centrin is an ancient protein thought to have arisen within the ancestor of all eukaryotes via gene duplication (Moncrief et al., 1990; Bhattacharya et al., 1993). It was first discovered in the flagellar apparatus of unicellular green algae where it is directly responsible for the contraction of calcium-sensitive structures (Salisbury et al., 1984; Huang et al., 1988). Such an activity appears important for basal body localization and orientation, but also for segregation of the basal bodies during mitosis. These functions are probably performed by centrin containing structures that connect basal bodies to one another and to the nucleus (Wright et al., 1985, 1989; McFadden et al., 1987; Taillon et al., 1992).

In *Saccharomyces cerevisiae*, the *CDC31* gene product,

which is also a member of this family (Nakayama et al., 1992), was isolated in a genetic screen for cell division cycle mutants and was shown to be necessary for the initiation of SPB duplication (Byers et al., 1981; Schild et al., 1981; Baum et al., 1986). It has been localized at the half bridge of the SPB (Spang et al., 1993).

Antibodies raised against centrin from unicellular green algae has unravelled the presence of centrin homologues in cells from widely divergent origins such as brown algae, ciliate, bryophyte, pteridophyte, arthropod, or mammalian cells (Katsaros et al., 1990; Baron et al., 1992; Wolfrum, 1992; Vaughn et al., 1993; Madeddu et al., 1996). Recently, cDNAs encoding centrin have been cloned in several animal cells including frog (Stearns and Kirschner, 1994), mouse (Ogawa and Shimizu, 1993) and human (Lee and Huang, 1993; Errabolu et al., 1994). In the latter case, two closely related genes have been characterized which are 90% identical and map to chromosomes 18 and X. Indirect immunofluorescence revealed that the gene products appeared localized at the centrosome in human cells (Lee and Huang, 1993; Errabolu et al., 1994).

This report deals with an investigation of the cellular distribution of human centrin by immunofluorescence and by cell fractionation using antibodies raised against centrin from *Chlamydomonas reinhardtii*. We have also carried out the biochemical and ultrastructural characterization of centrosome-associated centrin. Finally, we have attempted to provide experimental evidence of a role for centrin during cleavage cycles in the frog embryo.

MATERIALS AND METHODS

Cell culture

The KE37 cell line of T lymphoblastic origin was grown in RPMI 1640 medium containing 10% foetal calf serum. HeLa cells were maintained in DMEM supplemented with 10% foetal calf serum.

Antibodies

Anti-centrin antibodies have been described elsewhere (Baron et al., 1992; Sanders and Salisbury, 1994).

Two rabbit sera were used for centrosome staining, serum 0013 which reacts with a 350 kDa pericentriolar material (PCM) antigen (Gosti et al., 1986) and an anti-Clip 170 serum (Pierre et al., 1992), a generous gift from T. Kreis, which cross-reacts with the same PCM-antigen (M. Moudjou, unpublished data).

Anti-γ-tubulin polyclonal antibody was raised against the amino-terminal peptide 38-53 from human γ-tubulin (Moudjou et al., 1996).

Anti-α-tubulin mAb was obtained from Amersham International (Little Chalfont, England). Anti-tyrosinated and detyrosinated tubulin rabbit sera were a generous gift from C. Bulinski.

Recombinant centrin production and purification

Chlamydomonas centrin was bacterially expressed and purified as described elsewhere. Recombinant *Homo sapiens* centrin 2 (HsCen2) protein was produced in bacteria, and purified from bacterial lysate after heat treament of the lysate at 80°C and two purification steps by chromatography on TSK DEAE 650 S and TSK Phenyl 5PW columns (Wiech et al., 1996). They were then dialysed against PBS and quantified by BCA assay (Pierce-Rockford, Illinois).

When indicated, HsCen2 was submitted to heat treatment (2 hours 30 minutes at 95°C under mineral oil) and centrifuged 5 minutes at 10,000 ***g***. For proteolysis, centrin was incubated for 1 hour at 37°C in the presence of 2 mM trypsin from bovine pancreas (Fluka-St-Quentin Fallavier, France). Trypsin was heat inactivated at 95°C for 30 minutes. The centrin sample was further incubated with proteinase K from *Tritiracium album* (Boehringer-Mannheim, Germany) at 0.4 mg/ml. Proteinase K was then heat-inactivated and the sample centrifuged at 10,000 ***g***.

Immunofluorescence microscopy

HeLa cells were grown on coverslips, washed in PBS and fixed in methanol –20°C for 7 minutes. Alternatively, after PBS wash, cells were extracted in PHEM buffer (45 mM Pipes, 45 mM Hepes, 10 mM EGTA, 5 mM $MgCl_2$ adjusted to pH 6.9 and 1 mM PMSF) containing 1% Triton X-100 and fixed as described above. Cells were then rinsed in PBS containing 0.1% Tween-20. Primary antibodies diluted in PBS containing 3% BSA were added for 30 minutes at room temperature. Cells were then rinsed in PBS-Tween. The same procedures were used for fluorescein or rhodamine-labelled secondary antibodies (Jackson Immunoresearch Laboratories). Cells were finally dehydrated in ethanol and mounted in Citifluor (City University, London, England).

Cellular fractionation

Soluble and insoluble protein fractions were prepared as follows: KE 37 cells were washed in PBS and lysed in PHEM buffer containing 1% Triton X-100 and protease inhibitors (1 mg/ml leupeptin, 1 mg/ml pepstatin and 1 mg/ml aprotinin). Insoluble proteins were pelleted at 300 ***g*** and the pellet washed with PHEM buffer. Finally, for SDS-PAGE analysis, the insoluble pellet was solubilized in SDS-PAGE sample buffer (SSB) and soluble proteins were precipitated with 9 volumes of methanol at 4°C for 1 hour, pelleted and the pellet solubilized with the same amount of SSB as for the insoluble proteins. Alternatively, for IEF, 1 volume of IEF sample buffer (6.6% NP40, 16.6% 2-mercaptoethanol, 8.3% ampholines 3.5-10; LKB) was added to soluble proteins or to insoluble proteins resuspended in PHEM buffer containing 1% Triton. Finally, 1 g of urea was added per ml of final volume.

Cytosol/particulate and cytoplasm/nucleus fractionation of KE 37 cells were done as described by Bailly et al. (1989).

Centrosomes were isolated from KE 37 cells and from calf thymocytes as previously described (Moudjou and Bornens, 1994; Komesli et al., 1989).

Centrosome subfractionation

For chemical extraction of centrosomes, centrosome-containing gradient fractions were diluted in 10 mM K Pipes, pH 7.2, and centrifuged at 10,000 ***g*** for 15 minutes. Pelleted centrosomes were incubated for 30 minutes at 4°C in extraction buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA) containing either 1 M NaCl, or 0.5% NP40 (1-D buffer), or 0.5% NP40 and 0.5% deoxycholate (DOC; 2-D buffer), or 0.5% NP40, 0.5% DOC and 0.1% SDS (3-D buffer), or else 8 M urea. Alternatively pelleted centrosomes were resuspended in extraction buffer alone and incubated at 45°C for 15 minutes. After treatment, centrosomal proteins were fractionated into pellet and supernatant by centrifugation at 10,000 ***g*** for 15 minutes.

Protein analysis

One-dimensional SDS-PAGE was performed according to the method of Laemmli (1970) using 12% polyacrylamide gels. Two-dimension electophoresis was done according to the method of O'Farrell (1975). Immunoblotting experiments were achieved according to the method of Towbin et al. (1979). For anti-centrin antibodies, however, a modified protocol from Van Eldik and Wolchok (1984) was used. Briefly, proteins were fixed after transfer onto a nitrocellulose filter by incubation with 0.2% glutaraldehyde in TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 15 minutes at room temperature. The nitrocellulose filter was washed in distilled

water and saturated in TBS containing 5% non-fat dry milk for 1 hour at 37°C before incubation with antibodies. Alkaline phosphatase-conjugated secondary antibodies were purchased from Promega. Biotin-conjugated secondary antibodies and alkaline phosphatase-conjugated streptavidin were purchased from Amersham International.

Electron microscopy

Centrosomes were sedimented on glass coverslips at 20,000 ***g*** and processed for ultrastructural analysis as described by Paintrand et al. (1992), or for pre-embedding immunogold staining with mAb 20H5 as described by Moudjou et al. (1991). Alternatively they were processed for post-embedding immunogold staining as described by Moudjou et al. (1996).

Injection in two-cell stage frog embryos

Two-cell stage embryos were obtained by in vitro fertilization of eggs squeezed from *Xenopus laevis* females. Eggs were dejellied 30 minutes after fertilization, and distributed in three batches kept at 16°C, 20°C or 23°C until the two-cell stage was reached. This took about 90 minutes at 23°C and slightly more than 3 hours at 16°C. In this way, a 90 minutes period was available for injection. Injections of 25 nl were performed in one of the two blastomeres, using a nanoject system (Drummond). Embryos were then allowed to develop at 23°C.

Immunofluorescence on *Xenopus* embryos

Control and injected embryos were fixed in methanol at room temperature 10 hours after fecondation and processed for immunofluorescence according to the method of Gard et al. (1990), using an anti-α-tubulin mAb. Embryos were grossly fractured, mounted in thin chambers and observed with a confocal microscope (Leica).

RESULTS

Cellular distribution of centrin

Cell fractionation

Two monoclonal antibodies mAb 20H5 and 11B2 generated against bacterially expressed purified trpE-centrin from *C. reinhardtii* (Sanders and Salisbury, 1994) have been used. Antibodies were probed on three protein fractions from human lymphoblasts: Triton X-100 soluble (S) and insoluble (I) fractions, and a fraction highly enriched in isolated centrosomes (Ctr). A representative experiment is shown in Fig. 1A. Both antibodies gave a specific immunoreactive pattern at about 21 kDa in all three fractions. The signal was specifically enriched in the centrosomal fraction where several bands were resolved including a slowly migrating one at 23 kDa.

Isolated centrosomes can be easily counted by immunofluorescence. As they are single copy organelles, we could normalize in terms of the cell number the semi-quantitative analysis of the signals of S, I and Ctr fractions obtained using an NIH image program: S and I proteins from 2- to 8×10^5 cells were usually loaded together with proteins from 2- to 5×10^7 centrosomes. Since the Ctr signal was only 5 and 3 times higher than the S and I signals, respectively (three independent blots), we could establish that roughly 95% of cellular centrin was not associated with the centrosome.

Centrin was found to partition equally between high speed cytosol and particulate fractions after mechanical cell disruption (Fig. 1B). Centrin was also found in similar amounts in clean demembranated nuclei prepared by the citric acid method

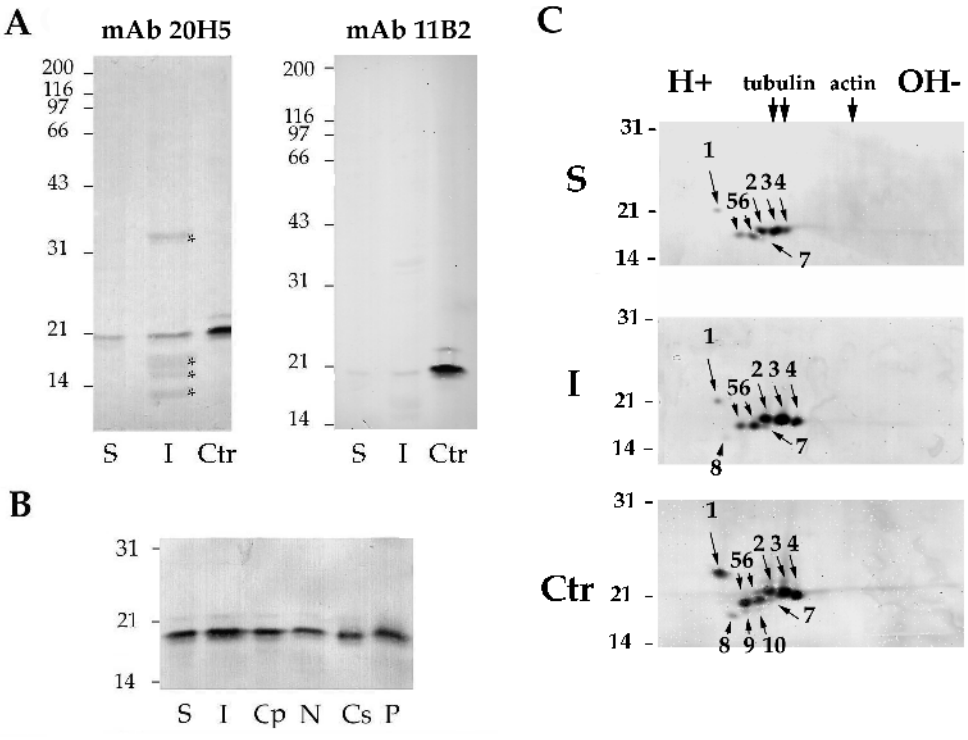

Fig. 1. Biochemical characterization of centrin in human cells. (A) Western blot analysis of low speed Triton X-100-soluble (S) and insoluble (I) fractions and of a fraction highly enriched in isolated centrosomes (Ctr) using two monoclonal anti-centrin antibodies, mAb 20H5 and mAb 11B2. Asterisks mark Ponceau red staining of abundant proteins of I fraction (histones). This staining is maintained due to glutaraldehyde fixation of proteins on the nitrocellulose filter. These bands are absent when the ECL detection kit is used. (B) Centrin distribution after cellular fractionation. Cells were either submitted to Triton X-100 extraction as in A (S,I) or fractionated into cytoplasmic fraction (Cp) and nuclei (N) by the citric acid method, or were mechanically disrupted and separated into cytosol (Cs) and particulate (P) fractions by high speed centrifugation. Signals with mAb 20H5 were enhanced using a biotin-conjugated secondary antibody and alkaline phosphatase-conjugated streptavidin. (C) Analysis of centrin by 2-D electrophoresis. Soluble (S), insoluble (I) and centrosomal (Ctr) proteins were submitted to IEF in the first dimension and to SDS-PAGE in the second dimension. Proteins were then blotted onto nitrocellulose and centrin revealed with mAb 20H5. Note that 7 spots (numbered 1 to 7) can be distinguished in all three fractions. An eighth spot is observed in the insoluble fraction (spot 8) and two more spots (spots 9 and 10) in the centrosomal fraction. Note that spots 1 and 5 seem to be enriched in the centrosomal fraction in comparison to the other isoforms. The positions of α-tubulin, β-tubulin and actin are indicated by arrows at the top.

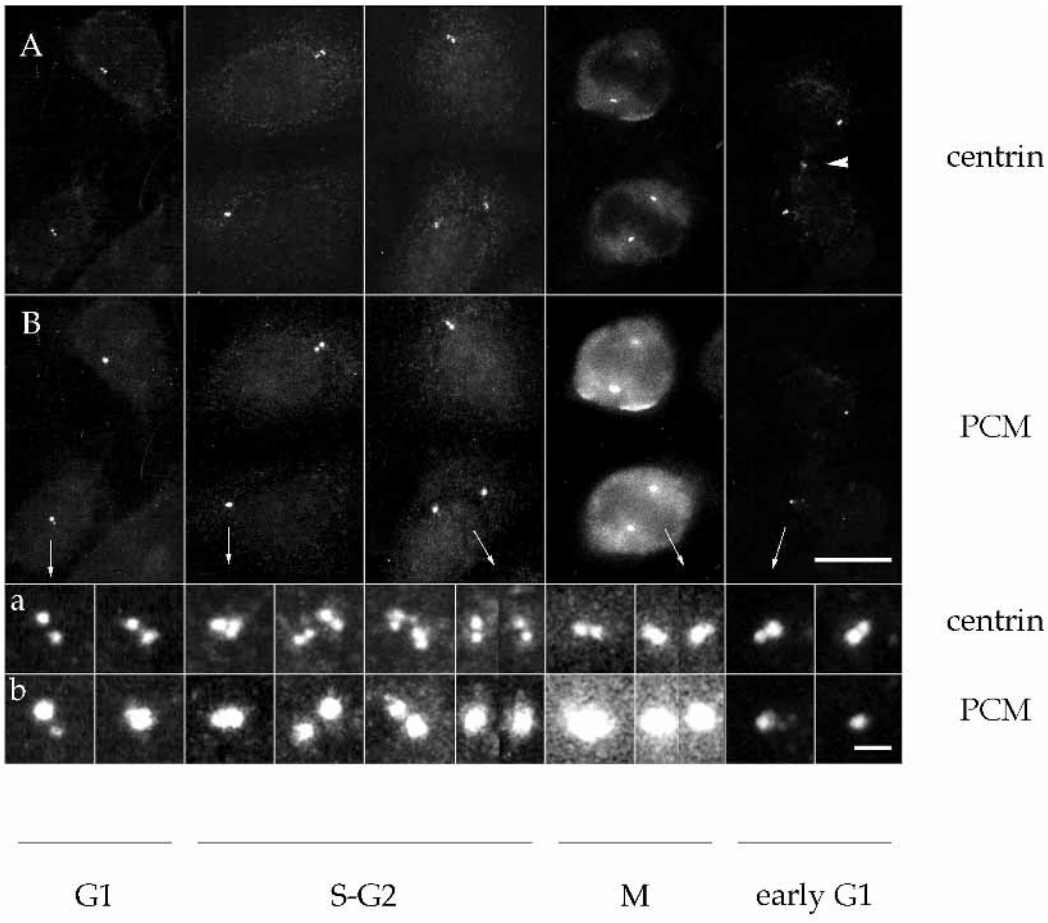

Fig. 2. Cellular distribution of centrin in HeLa cells throughout the cell cycle. Cells were double-labelled for centrin (A,a) and for PCM (B,b) using mAb 20H5 and an anti-350 kDa PCM antigen rabbit serum. Cells have been ranged from left to right according to their position in the cell cycle defined by the size of the PCM staining. A fivefold magnification of individual centrosomes shown in A and B is presented in a and b as indicated by arrows. Note the specificity of centrin decoration for centrioles all along the cell cycle and the faint diffuse background. Note also that the closely associated centrioles of early G_1 centrosomes can be distinguished from the dissociated centrioles of late G_1 centrosomes. In early G_1, the midbody between sister cells is also slightly stained (arrowhead). Bars: 10 μm in B; 1 μm in b.

and in the corresponding supernatant. Together, these data suggest that a vast portion of centrin is not associated with the centrosome.

2-D electrophoresis separation of proteins from S, I and Ctr fractions revealed the presence of at least 10 isoforms of centrin (Fig. 1C). The pattern was qualitatively similar in all three fractions. However, the fainter spots (8, 9, 10) were either not observed or hardly detectable in the S and I fractions and spots 1 and 5 seemed specifically enriched in the Ctr fraction.

Immunofluorescence

Immunofluorescence experiments on HeLa cells with mAb 20H5 revealed, in addition to a general diffuse background both in the cytoplasm and in the nucleus, a rather strict localization of centrin to one or two pairs of dots within a given centrosome during interphase and to one pair of dots at each pole of the spindle in mitosis. Corresponding images with anti-PCM sera (see Materials and Methods) showed, respectively, one or two dots (Fig. 2A,B). Centrin staining was independent of the fixation used (see Materials and Methods) but obviously dependent on the progression of the cell division cycle. PCM staining, which increases with the progression of the cell cycle and culminates at metaphase, provided a rough estimate of the position of individual cells in the cell cycle. Using this criteria, we could establish that two dots likely to correspond to the two centrioles were stained in G_1 phase before the duplication of the centrosome, and that four dots organized in two close pairs were stained in S and G_2 phases after duplication (Fig. 2). In mitosis, two centrioles were visible at each pole of the spindle. The midbody joining sister cells was also slightly stained (Fig. 2, M). Strikingly, the inter-dot distance in a centriole pair was smaller in M and early G_1 cells than in late G_1 cells. We could also observe that after the decrease of the PCM staining at the exit of mitosis, the remaining PCM was mainly associated with one of the centrioles (Fig. 2, early G_1). It was redistributed equally to each centrosome after duplication (Fig. 2, S-G2). Thus, centrosomes decorated with anti-centrin antibody appeared as convenient structural landmarks for the progression of the cell division cycle.

Corroborative observations could be done on synchronized HeLa cells as well as using two polyclonal anti-centrin antibodies, sera 26/14.1 and 26/14.2 (data not shown).

Centrosomal centrin

Immunolocalization in isolated centrosomes

Centrosomes isolated from KE 37 cells were double stained for tubulin, which identifies centrioles, and for centrin (using mAb 20H5). Both stainings were quite similar but centrin staining was often more discrete than tubulin staining (Fig. 3A,B). In some cases, the two dots decorated by mAb 20H5 in a given centrosome appeared farther apart from each other than the corresponding dots decorated by anti-tubulin antibodies (arrows in Fig. 3A,B).

One way to understand this feature was to turn to isolated centrosomes from calf thymocytes which have been shown to possess a peculiar and stereotyped configuration, the two cen-

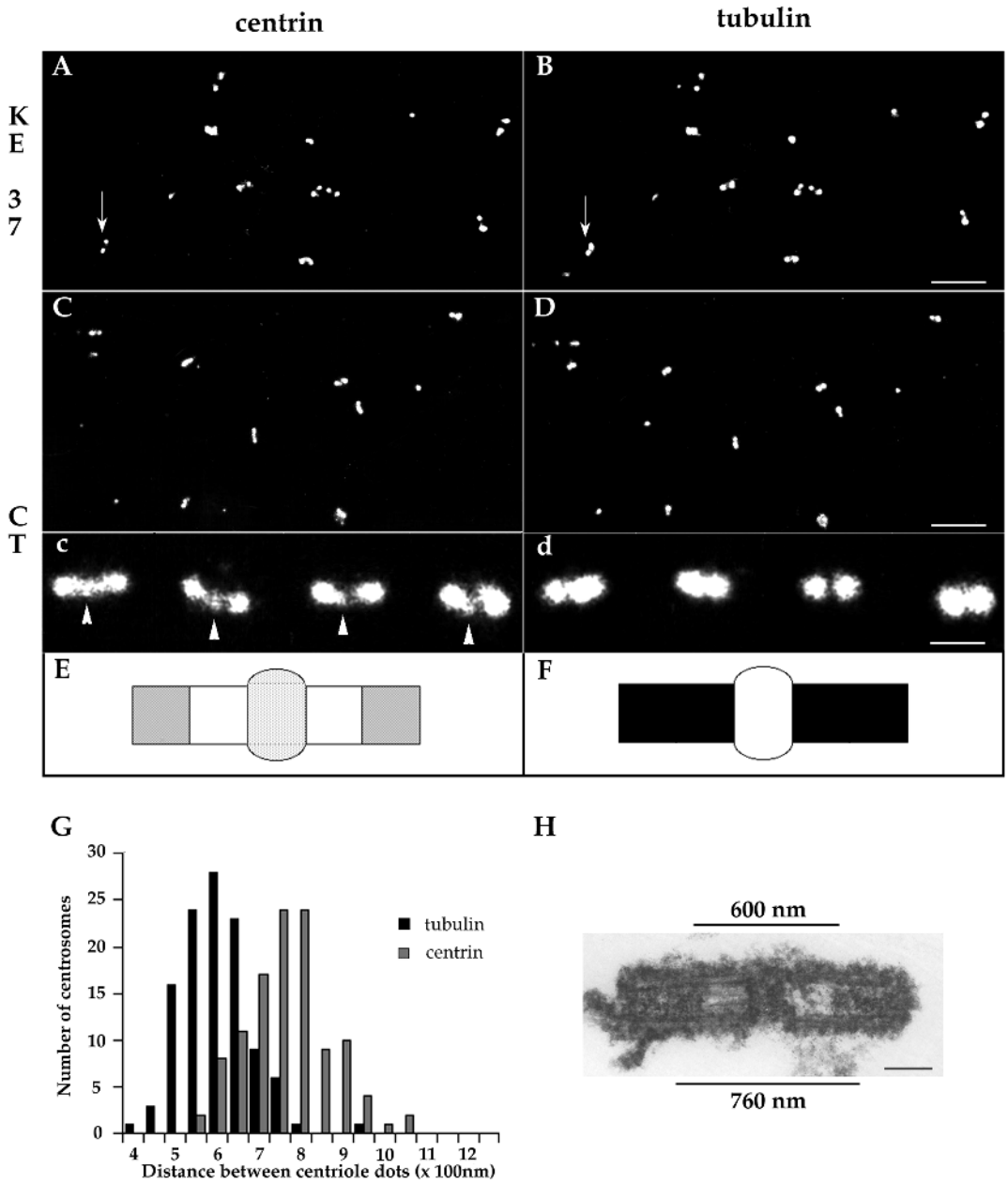

Fig. 3. Centrin localization in centrosomes isolated from KE 37 cells and from calf thymocytes (CT). Centrosomes were double labelled for centrin (A,C,c) and tubulin (B,D,d) with mAb 20H5 and a rabbit serum anti-detyrosylated tubulin. (A,B) Centrosomes isolated from KE 37 cells. Note that centrin decoration of KE 37 centrosomes is quite similar but often more discrete than that observed with anti-tubulin antibodies. In some cases, the two dots decorated by mAb 20H5 seem more distant than the corresponding dots decorated by anti-tubulin antibodies (arrows in A,B). Bar, 5 μm. (C,D,c,d) Centrosomes isolated from calf thymocytes. (C-D) General view. Bar, 5 μm. (c,d) High magnification of four individual centrosomes. Bar, 0.5 μm. Note that each antibody stains centrosomes as a pair of dots but that the inter-dot distance in a pair is different with each antibody. mAb 20H5 also stains a structure between the two dots (arrowheads). (E,F) Schemes representing the putative localization of centrin (in grey) and tubulin (in black) in calf thymocyte centrosomes. Centrioles are represented as rectangles joined by the PCM. (G) Distribution of center to center distances between centriolar dots in 100 calf thymocyte centrosomes double stained for centrin and tubulin. Two distinct modes of distribution are identified with a mean distance value of 600 nm for tubulin staining and of 760 nm for centrin staining. (H) Electron micrograph of an isolated calf thymocyte centrosome. Bar, 0.2 μm. The two black segments at the top and the bottom of the micrograph correspond to the mean inter-dot distances for centrin (bottom) and tubulin staining (top) obtained in G. They have been reported for comparison with centrosome ultrastructure, the middle point of each segment being placed in register with the symmetry center of the centrosome.

trioles being linearly associated by their proximal ends (Komesli et al., 1989). We checked that mAb 20H5 specifically reacted with bovine centrin (data not shown) and then compared the staining of isolated calf thymus centrosomes with that observed with an anti-tubulin antibody. A striking and constant difference was observed between the labelling with both antibodies (Fig. 3C,D,c,d): the modal inter-dot distance in a pair (from center to center) was 600 nm for the tubulin decoration, and 760 nm for the centrin decoration (Fig. 3G). These distances are, respectively, close to the mean distance between the two mid-centrioles and to the mean distance between the middle of the distal parts of the centrioles, as calculated on electron microscopy pictures (Fig. 3H). Thus, the likely interpretation of these observations was that centrin is concentrated at the distal end of centrioles as illustrated in Fig. 3E. An additional concentration of centrin staining was observed half-way between the two distal dots (arrowheads in Fig. 3c) which could correspond to the pericentriolar network concentrated between the proximal ends of the two centrioles (Komesli et al., 1989).

Ultrastructural immunogold staining of centrin on isolated centrosomes was carried out for both human and bovine cen-

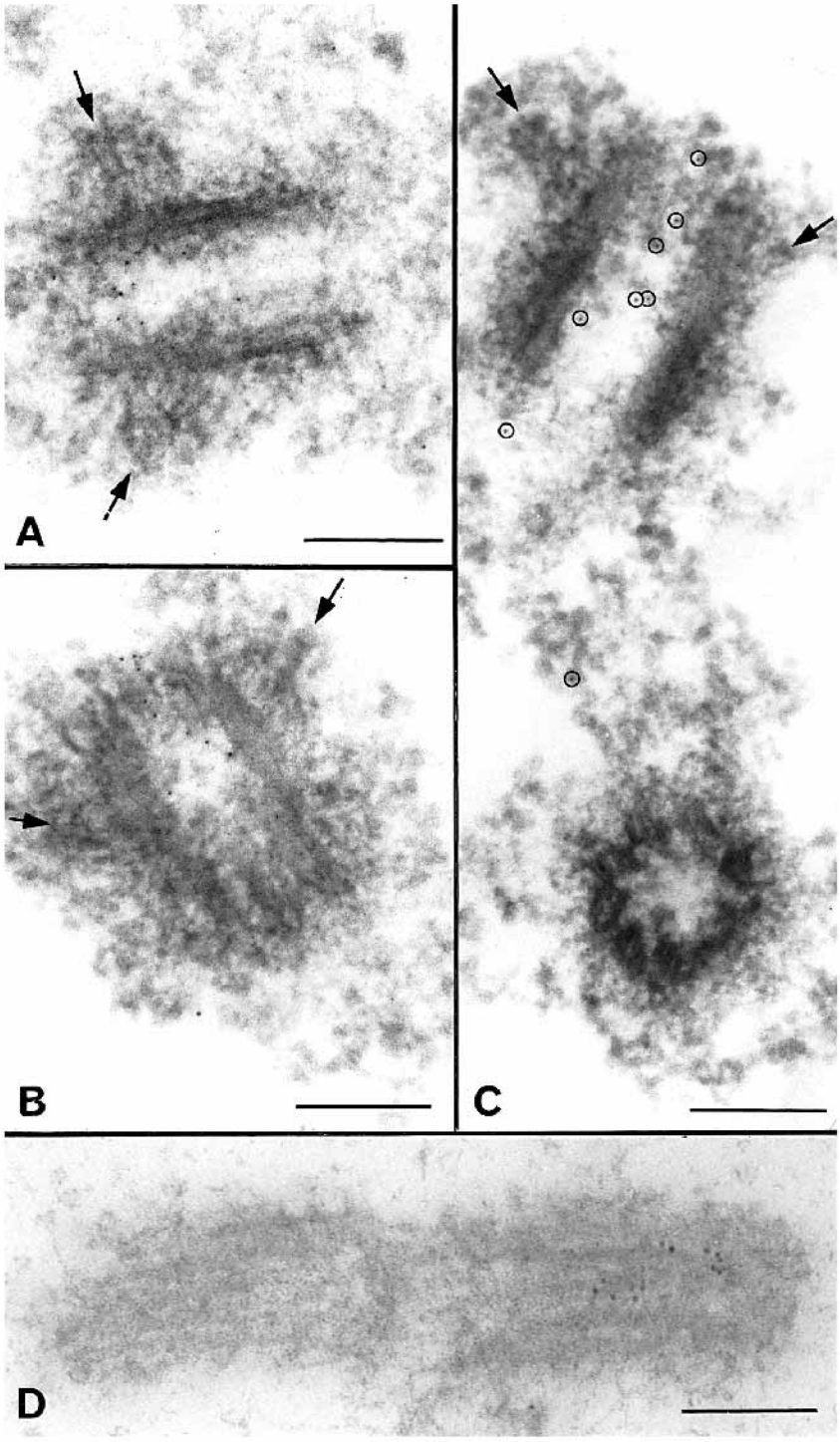

Fig. 4. Ultrastructural localization of centrin in isolated centrosomes. (A-C) Pre-embedding immunogold localization of centrin in KE37 centrosomes. (A-B) Sagital (A) and oblique sections (B) demonstrating an accumulation of 5 nm gold particles in the distal lumen of centrioles (the distal end of mother centrioles is easily identified by the presence of pericentriolar appendages, indicated by arrows). No particles can be seen outside centrioles. (C) Centrosome in which the mother centriole on the top is sagitally sectioned and the daughter centriole transversally sectioned at the proximal end (as judged by the presence of triplet microtubules). Gold particles have been circled. Note that they are clustered in the distal lumen of the mother centriole, and are absent from the proximal lumen of the daughter centriole or from the external side of centrioles. Two particles can be seen in the centrosome matrix linking the two centrioles. (D) Post-embedding immunolocalization of centrins in calf thymocyte centrosomes. The distal end of the right centriole contains numerous gold particles. The left centriole is tangentially sectioned. No gold particle can be observed external to the centriole wall. All experiments were done using mAb 20H5 as the primary antibody. Bars, 0.2 μm.

trosomes using mAb 20H5 (Fig. 4). The same results were obtained using pre and post-embedding techniques. The staining fully confirmed the interpretation drawn from immunofluorescence data and further revealed a luminal localization of centrin in both kinds of centrosomes.

Centrin association with centrosome

We attempted to estimate how centrin is associated with the centrosome using several extracting conditions. Isolated centrosomes were incubated at 4°C for 30 minutes with salt, detergents or urea and then centrifuged for 15 minutes at 10,000 ***g***. Alternatively, they were treated at 45°C for 15 minutes, a treatment which is known to disassemble axonemal microtubules (Stephens et al., 1989) and to extract tubulin from centrosomes (Moudjou, 1992). A representative experiment is shown in Fig. 5A. Neither 1 M NaCl, nor 0.5% NP40 (1-D), or 0.5% NP40 together with 0.5% DOC (2-D) did extract any significant amount of centrin. Only drastic conditions such as the use of a triple detergent-containing medium (3-D: 0.5% NP40, 0.5% DOC and 0.1% SDS), or the use of 8 M urea were

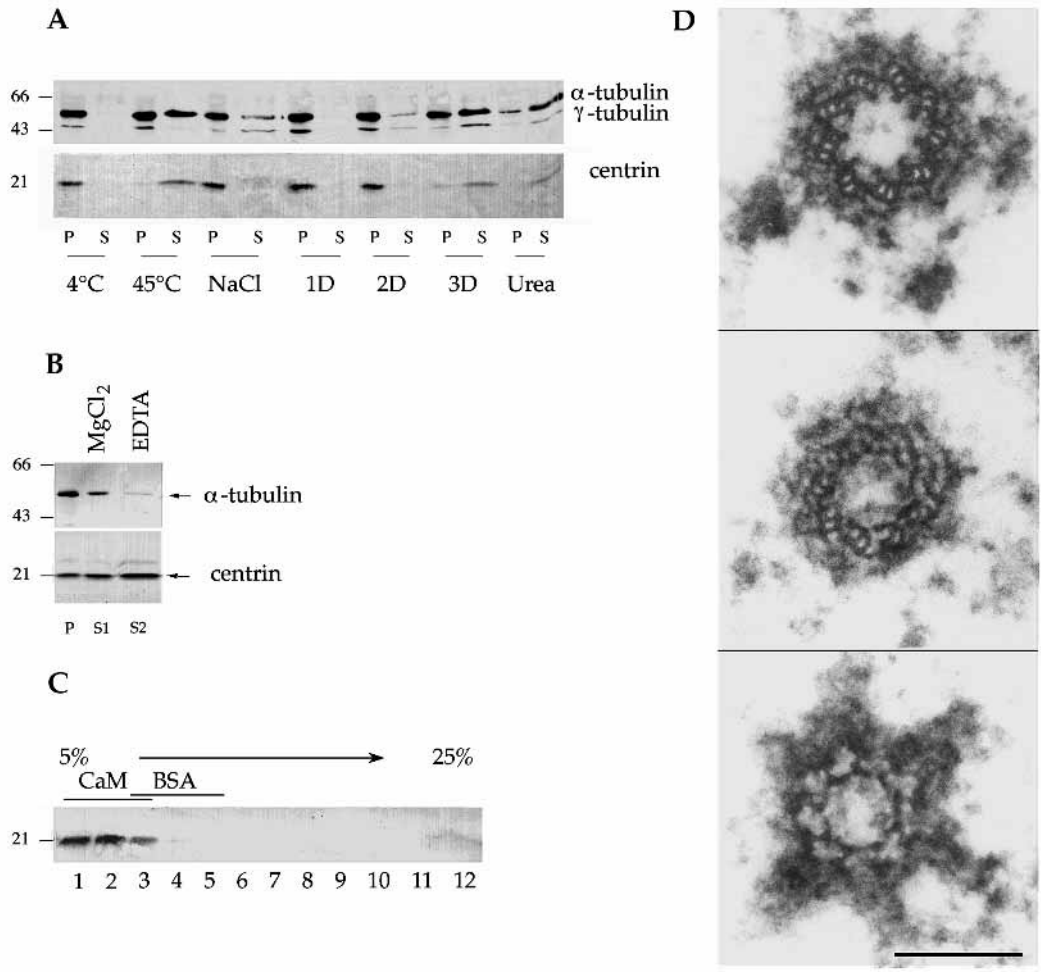

Fig. 5. Centrin association with centrosomes. (A) Isolated centrosomes were incubated at 4°C for 30 minutes with the buffer only (20 mM Tris-HCl, pH 7.4, 2 mM EDTA) (4°C), or with the same buffer containing either 1 M NaCl (NaCl), or 0.5% NP40 (1D), or 0.5% NP40 and 0.5% DOC (2D), or 0.5% NP40, 0.5% DOC and 0.1% SDS (3D), or 8 M urea (Urea), or finally with the buffer only at 45°C for 15 minutes (45°C). After treatment, centrosomes were fractionated into pellet (P) and supernatant (S) by centrifugation at 10,000 ***g***. Pellets (P) and supernatants (S) were analyzed by western blot using mAb 20H5, an anti-α-tubulin mAb and a rabbit anti-γ-tubulin serum. Note that only 45°C, 3D and Urea were capable of extracting the major part of the centrosomal centrin and a significant part of α-tubulin. γ-Tubulin was poorly affected by heat treatment. (B) Effect of Mg^{2+} on centrin extraction. Isolated centrosomes were submitted to two sequential treatments at 45°C for 15 minutes, first in 10 mM K Pipes, pH 7.2, containing 1 mM $MgCl_2$ and second in 10 mM K Pipes, pH 7.2, containing 2 mM EDTA. Final pellet (P), first supernatant (S1) and second supernatant (S2) are shown. Centrin release was reduced in the presence of $MgCl_2$. Additional centrin, but no tubulin, was released in the second incubation. (C) Analysis of the molecular size of extracted centrin. Extracted proteins were laid on a linear 5-25% (w/w) sucrose gradient and centrifuged 16 hours at 100,000 ***g*** to equilibrium. Centrin is found concentrated in the first three fractions corresponding to the top of the gradient. Behaviour of bovine serum albumin (BSA) and calmodulin (CaM) in a similar gradient is shown. (D) Ultrastructural analysis of centrosomes after double heat treatment as in B. Transversal sections through a centriole proximal (top), middle (middle) and distal parts (bottom) are shown. Note that microtubules are specifically removed from the distal part of centrioles and that part of the intraluminal material is apparently also lacking. Bar, 0.2 μm.

capable of extracting the major part of the centrosomal centrin. Remarkably, temperature treatment was quite efficient in releasing centrin. This release paralleled the release of centriolar α-tubulin, whereas most of the centrosomal γ-tubulin was not extracted. In the other extraction conditions, α-tubulin also behaved as centrin. These results suggested that the association of centrin with the centrosome was correlated with the integrity of centriole microtubules. However, unlike α-tubulin, centrin extraction was sensitive to divalent cations (Fig. 5B). Namely, centrin extraction at 45°C was reduced in the presence of 1 mM $MgCl_2$. This effect was reversed after chelation of the remaining Mg^{2+} by 2 mM EDTA: centrin, unlike α-tubulin, was further released in the second extraction at 45°C. Similar results were obtained with Ca^{2+} (not shown).

We analysed the molecular size of centrin extracted at 45°C. Extracted proteins were laid on a linear 5-25% (w/w) sucrose gradient and centrifuged at equilibrium for 16 hours at 100,000 ***g***. As shown in Fig. 5C, centrin was found in the first three fractions of the gradient. In a similar control gradient, soluble calmodulin (molecular mass 19 kDa) was found in the same fractions (1 to 3) and soluble BSA (molecular mass 66 kDa) peaked in fractions 3 to 5 (not shown). This revealed that extracted centrin was likely monomeric and not associated with tubulin or other centrosomal components.

Centrosomes submitted to a double-extraction treatment at 45°C as above (Fig. 5B) were analysed at the ultrastructural level. A specific removal of the microtubule doublets was observed in the distal part of centrioles (Fig. 5D, bottom), whereas microtubule triplets of the proximal part were apparently well preserved (Fig. 5D, top). Part of the distal lumen material was also lacking after the treatment. This revealed that the distal part of centrioles was particularly sensitive to heat treatment and further suggested a correlation between centriole microtubule integrity and centrin association with centrioles.

Injection of heterologous centrins in frog eggs

From the above data, we conclude that centrin is biochemically complex, the major part not associated with the centrosome but

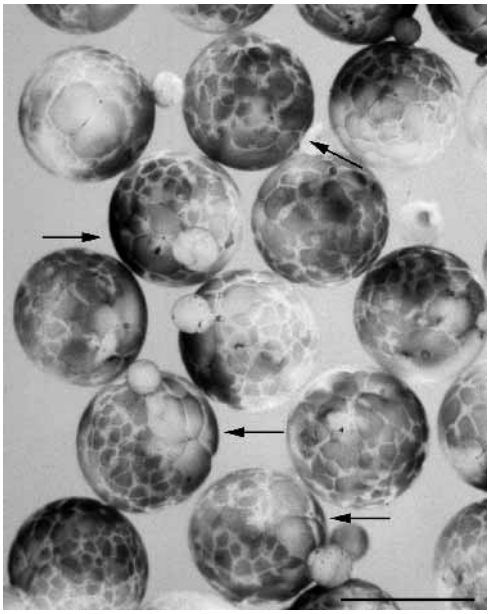

Fig. 6. Effect of recombinant HsCen2 on frog embryo cleavage. Embryos were injected with 25 nl of 12 mg/ml HsCen2 in one blastomere at the two-cell stage. They were photographed 7 hours after fertilization. Arrows point to undercleaved half embryos originating from injected blastomeres. Bar, 1 mm.

distributed both in the cytoplasm and the nucleus. We further conclude that the centrosome-associated centrin, which is even more complex that the non centrosomal form, is concentrated within the distal part of the centriole lumen. The latter observation a priori suggests an involvement of the centrosomal centrin in a centriole-associated function rather than in microtubule nucleating activity which has often been associated with the PCM. No function can be anticipated for the major non-centrosomal centrin as it is not associated with obvious structures. We therefore attempted to provide experimental support for centrin function in animal cells, in centrosome biogenesis or in any other centrosome-dependent activities.

We tested the potential of two heterologous centrins, the centrin from the green algae *C. reinhardtii* and the human centrin HsCen2, to interfere with embryo cleavage after injection into two-cell stage frog embryos. In this assay, the uninjected blastomere served as an inner control for embryo cleavage time-course. We reasoned that modifying the pool of centrin with heterologous centrin could possibly interfere with the regulations of centrin-dependent functions during the cell cycle and thus produce a dominant negative effect. The rapid frog embryonic cell division cycles provided a convenient and potent system for demonstrating cell cycle perturbation, as any effect would be cumulative and thus rapidly observed.

Embryos were observed around mid-blastula transition (MBT, 6-10 hours after fertilization) to analyze the time-course of cleavage (Table 1, Fig. 6 and Fig. 7 left) and 20-30 hours after fertilization during late gastrulation and neurulation (Table 1, and Fig. 7, right). A dose-dependent effect was observed with *Chlamydomonas reinhardtii* centrin (CrCen): with 11 mg/ml or less, only a late effect could be observed, most of the embryos showing exogastrulation, or lysis of the injected side. With 15 mg/ml or more, undercleavage in the half part originating from the injected blastomere was observed. At the neurulation stage, the undercleaved part of the embryo had lysed whereas the uninjected part had kept developing or lysed, depending probably on the moment of injection with respect to the completion of the first cleavage. A similar early effect was observed with 12 mg/ml HsCen2. To ensure the specificity of these effects, we used the thermostability of centrin as a criterium: heat-treated HsCen2 was as effective as the native protein (see Table 1 and Fig. 7). We could also check that the protein was not degraded and had not precipitated after 2 hours 30 minutes at 95°C (Fig. 8, lane 2). On the contrary, proteolysis by trypsin and proteinase K totally degraded centrin (Fig. 8, lane 3) and as expected, the corresponding sample did not show any effect on embryo cleavage (Table 1 and Fig. 7). We also carried out control injections of BSA (Fig. 8, lane 5) at protein concentrations similar to HsCen2 and CrCen concentrations, or of PBS (Table 1 and Fig. 7): the cleavage time-course was normal or minimally affected and the major part of the embryos developed normally. Some variability could be observed from one experiment to the other depending on the quality of the eggs. All the results illustrated in Fig. 7 are from the same experiment.

From the above data we conclude that the injection of het-

Table 1. Short and long-term effects of heterologous recombinant centrins in two-cell stage injected embryos

Injected solution	Embryos showing under-segmentation (%)	Post-MBT development (%)			Number of eggs (number of experiments)
		N	E	L	
Cr Cen (5.5 mg/ml)	0	24	58	18	71 (3)
Cr Cen (11 mg/ml)	0	5	80	15	39 (2)
Cr Cen (15 mg/ml)	76	0	9	91	33 (1)
Cr Cen (22 mg/ml)	86	22.5	8	69.5	59 (2)
Hs Cen 2 (12 mg/ml)	90	4	44	52	117 (4)
Heat treated Hs Cen 2	94	10	14	76	51 (2)
Proteases treated Hs Cen 2	0	72	17	11	18 (1)
BSA (15 mg/ml)	9	74	9	17	89 (3)
BSA (25 mg/ml)	10	38	45	17	29 (1)
PBS	5	86	5	9	129 (5)
Uninjected eggs	0.5	97	3	0	193 (6)

25 nl of CrCen, of native or treated HsCen2, or of control solutions (BSA and PBS) were injected in one blastomere of two-cells stage embryos at the indicated concentration. The number of embryos showing undercleavage in one half was scored 8 to 10 hours after fertilization for short-term effects. Long-term effects were observed 20 to 30 hours after fertilization (post-MBT development). Development of the injected side of the embryo was scored as normal (N), participating in exogastrulation of the whole embryo (E) or lyzed (L).

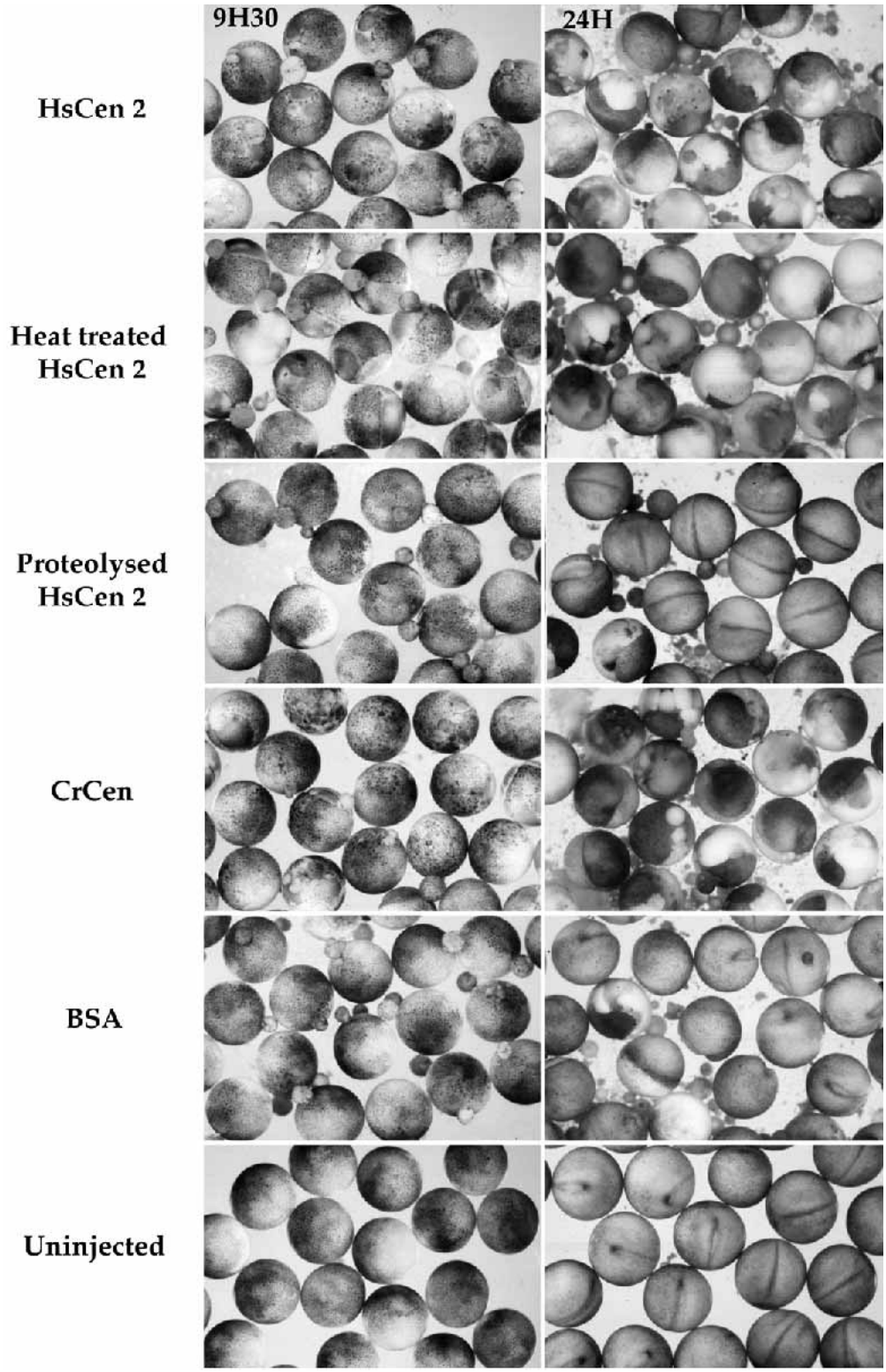

Fig. 7. Effect of heterologous centrin from green algae and human on frog embryo early development. The experiment shown was done with fertilized eggs from a single female. One blastomere of two-cell stage embryos was injected with HsCen2 (12 mg/ml), heat-treated HsCen2, protease-treated HsCen2, CrCen (15 mg/ml), or BSA (15 mg/ml) in a 25 nl volume. Uninjected embryos are also shown. Pictures were taken 9 hours 30 minutes (left) and 24 hours (right) after fertilization. Note that cleavage is slowed down in embryo halves originating from blastomeres injected with Hscen2, heat treated HsCen2 and CrCen. At the neurulation stage, the undercleaved part of the embryos has often lysed whereas the uninjected part can demonstrate some autonomous development. ×11.

erologous centrin dramatically impairs frog embryo cleavage cycles.

Microtubule organization in undercleaved embryos

In a first attempt to analyse the mechanisms underlying the cleavage delays obtained by injecting CrCen and HsCen 2 at the two-cell stage, we analysed microtubules distribution in injected embryos 10 hours after fertilization.

In embryos injected with HsCen 2, whole parts of the embryos displayed huge blastomeres that were very heterogenous in size (Fig. 9). Some blastomeres contained multiple and evenly distributed asters of microtubules (Fig. 9A,C)

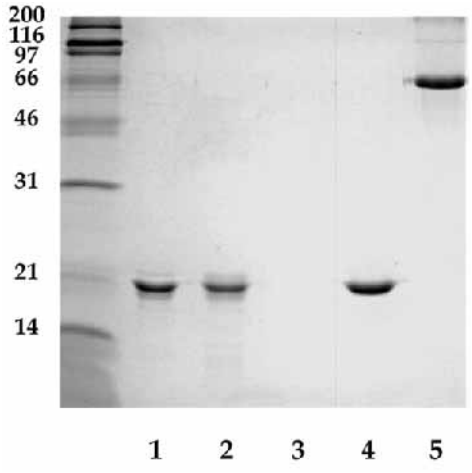

Fig. 8. Analysis by SDS-PAGE of the recombinant centrins used in the injection experiments. Proteins were stained with Coomassie blue. Molecular mass markers are shown on the left side (in kDa). Lane 1, HsCen2 (1 mg); lane 2, heat-treated HsCen2 (95°C for 2 hours 30 minutes; 1 mg); lane 3, protease-treated HsCen2; lane 4, CrCen (2 mg); lane 5, BSA (2 mg).

whereas others lacked any obvious microtubule array (Fig. 9B,D). Some incompletely cleaved furrows could be observed which seemed to maintain continuity between blastomeres (Fig. 9B). Although a high background did not allow us to clearly identify chromosomes or nuclei by specific DNA staining, one could observe from the interphase or mitotic microtubule organization that the size and distribution of nuclei in blastomeres was also completely abnormal, many blastomeres being devoid of nuclei or chromosomes. Many blastomeres appeared to have multiple 'holes' at the periphery or in the center of their cytoplasm, i.e many irregular areas apparently devoid of cytoplasm, giving a lacerated aspect to such blastomeres (Fig. 9A-C). One could also note that the length of microtubules in blastomeres with multiple asters differed from one blastomere to the other (compare Fig. 9A and C), suggesting that the whole cytoplasm in individual undercleaved blastomeres were in different states in terms of mitotic kinase activity (Belmont et al., 1990; Verde et al., 1990). We also often observed pairs of asters, like in Fig. 9C, which could be produced by centrosome duplication.

By contrast, some areas looked like control embryos at the same stage (bottom of Fig. 9B). These areas probably corresponded to the uninjected bastomere or to regions of the injected blastomere in which the protein had not diffused.

Similar observations could be done in embryos injected with CrCen at 15 mg/ml (data not shown).

From these obsevations, we conclude that the undercleavage provoked by the injection of heterologous centrin in early frog embryos is accompanied by severe perturbations of the distribution of centrosomes and nuclei between blastomeres, which eventually lead to embryo lysis.

DISCUSSION

We have used two antibodies raised against *C. reinhardtii* centrin that recognize centrin in broadly divergent species (Katsaros et al., 1990; Wolfrum, 1992; Vaughn et al., 1993; Stearns and Kirschner, 1994; Uzawa et al., 1995). mAb 20H5 epitope could be mapped to a very conserved region at the N-terminal side of the first and third EF-hand domains of *C. reinhardtii* centrin (J. L. Salisbury, unpublished data). In human cells, this mAb is highly specific for centrin: it recognizes both bacterially expressed HsCen1 and HsCen2 (data not shown), does not react with higher molecular mass centrin-related proteins and does not recognize calmodulin (data not shown).

Most of centrin is not associated with the centrosome

Cellular fractionation revealed that a great amount of centrin was not associated with the centrosome. The significance of this cellular distribution is unknown. Non-centrosomal centrin could represent a pool necessary for centrosome biogenesis or for centrosomal centrin turnover. In support of the first possibility, the presence of complexes containing centrin and hsp 70 or 90 has recently been observed in CSF-arrested extracts from frog eggs (Uzawa et al., 1995). However, the reason why somatic cells should need such a large centrin store is unclear. Alternatively, centrin could have specific and as yet unknown functions in the cytoplasm and in the nucleus.

Human cells contain multiple isoforms of centrin

2-D electrophoresis revealed a great complexity in centrin pattern. Several possibilities could account for the numerous isoforms. First, two cDNA clones encoding human centrin, HsCen1 and HsCen2, have been characterized, coding for highly related proteins of respective molecular mass and isoelectric points of 19,500/4.6 and 19,700/4.7 (Lee and Huang, 1993; Errabolu et al., 1994) and the search for centrin genes in human species might not be exhausted. Second, a calcium-induced shift such as the one described for algal centrin (Salisbury et al., 1984) and for the *CDC31* gene product (Spang et al., 1993) could take place. However, we did not detect a calcium-induced shift of human centrin in SDS-PAGE (data not shown). Third, centrin may be subjected to post-translational modifications. Indeed, algal centrin has been shown to be a phosphoprotein (Salisbury et al., 1984) and putative phosphorylation sites for PKA and cdc2 kinase have been found on human centrin sequences (Lee and Huang, 1993; Errabolu et al., 1994). In any case, such a complex pattern suggests that centrin is the object of multiple regulations in human cells. It is also noteworthy that two spots are specifically enriched in the centrosome in comparison to the other isoforms and might in this regard, have functional specificities.

Centrosomal centrin is concentrated in the distal lumen of centrioles

From both optical and electron immuno-microscopy on isolated centrosomes, we could establish that centrin is concentrated within the distal lumen of centrioles where a complex structure has been previously described (Paintrand et al., 1992). Accordingly, we observed a strict centriolar localization of centrin in HeLa cells. Centrin in situ staining in KE 37 cells seemed, however, more complex, an accumulation of centrin being sometimes observed around centrioles in addition to the centriole centrin (data not shown). Some staining could also be observed between the two centrioles in calf thymocytes centrosomes, a region where the pericentriolar material is con-

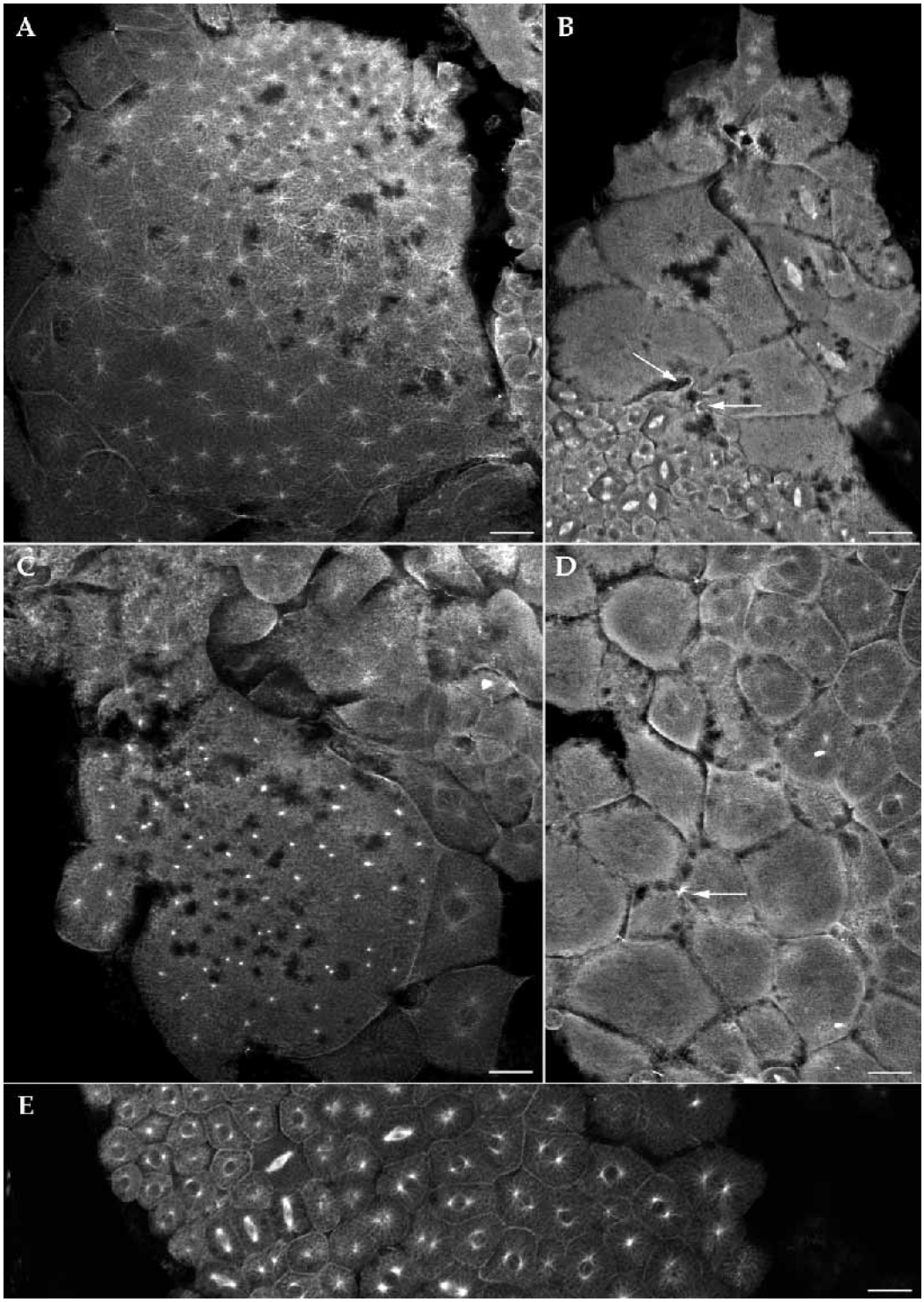

Fig. 9. Microtubule organization in HsCen2-injected embryos. Eggs injected with 12 mg/ml HsCen2 in one blastomere at the two-cell stage (A-D), and control embryos (E), were fixed 10 hours after fertilization. Microtubules were stained using a mAb against α-tubulin and observed by confocal microscopy. Huge blastomeres, surrounded by smaller ones can be observed (A and C). They contain numerous evenly distributed asters of long (A) or short microtubules (C), no obvious nuclei or chromosomes, and many irregular areas apparently devoid of cytoplasm giving a lacerated aspect to such blastomeres. The periphery of abnormal blastomeres can also display 'holes' (D). Midbody-like figures can be observed between abnormal blastomeres (arrows in B and D). Juxtaposition of abnormally and normally cleaved areas is often visible (B and D). Control embryos at the same stage show asynchronous cell division cycles (E). Bars, 50 μm.

centrated (Komesli et al., 1989). Thus, the presence of centrin in the pericentriolar matrix might depend on the cell type.

Centrin staining provides a means to resolve centrioles in the light microscope throughout the cell cycle. In particular, a change in the distance between centrioles has been observed during G_1 that we interpret as reflecting the loss of the orthogonal configuration of the centriole pair before the initiation of centrosome duplication (Kuriyama and Borisy, 1981). Centrin staining also enabled us to visualize the association of PCM with a single centriole at mitosis exit, as described at the ultrastructural level by Rieder and Borisy (1982) and Vorobjev and Chentsov (1982).

Chemical extraction experiments on centrosomes corroborated immunofluorescence and EM data on centrin localization: centrin was co-extracted with centriolar tubulin at 45°C. Furthermore, EM analysis showed that after heat treatment microtubules were specifically removed in the distal part of centrioles where centrin is confined. This revealed a greater

sensitivity of centriole distal end to heat treatment. It also reinforces the idea already discussed by Paintrand et al. (1992), that centrioles are made of two parts: a proximal part built on the specific microtubule triplets of basal bodies, and a distal part, reminiscent of axoneme organization, built on microtubule doublets, heat sensitive, and containing centrin in its luminal structure.

Centrin has previously been found in association with the flagellar apparatus in several systems (Wolfrum, 1995; Katsaros et al., 1993; Le Dizet and Piperno, 1995; Levy et al., 1996). In the amoeboflagellate *Naegleria gruberi*, centrin is associated with the basal bodies in the flagellate form but is absent in the amoeba, which lacks a flagellar apparatus (Levy et al., 1996). In *C. reinhardtii*, centrin is present in the luminal stellate fibers of the transition zone between basal body and axoneme. The Ca^{2+}-dependent contraction of these fibers triggers axoneme excision (Sanders and Salisbury, 1989, 1994). In animal cells, in vitro Ca^{2+}-dependent structural modifications of the centrioles have also been reported (Moudjou and Bornens, 1992; Paintrand et al., 1992). Given centrin localization, an appealing idea would be that these structural modifications could be performed by centrin-containing contractile fibers. However, the physiological occurrence and significance of such a structural change of centrioles remains to be determined. In any case, determining if centrin containing fibers could be found in animal cells is an important issue.

Centrin function in animal cells

Centrin functions in animal cells are still unknown (Schiebel and Bornens, 1995). From genetic studies in unicellular organisms, two types of cell pathways are candidate for being controlled in a centrin-dependent manner, the centrosome duplication mechanism itself, and the mechanisms which couple the centrosome reproduction pathway with cell division at the exit of mitosis: in *S. cerevisiae*, centrin is the product of *CDC 31*, a cell division cycle essential gene, and is directly implicated in an initial event of the SPB duplication pathway (Baum et al., 1986); in *C. reinhardtii*, the mutant strain *vfl 2* bears a point mutation in the centrin gene. The mutant cells are viable but have a variable number of flagella that results from an unequal distribution of the basal bodies at mitosis (Wright et al., 1985). This phenotype is probably linked to the disruption of the nucleus-basal body connector (Taillon et al., 1992). It is worth noticing that in this mutant, all the centrin containing fibers (nucleus-basal body connector, distal striated fiber, stellate fibers) are lacking, but centrin is still present in the basal bodies (Taillon et al., 1992). Only further genetic analysis of centrin or centrin gene disruption could reveal the function of basal body associated centrin (in basal body duplication in particular) and if centrin carries an essential function as in *S. cerevisiae*.

The presence of centrin in centrioles of animal cells, reported in the present work, suggests the implication of centrin in a centriole-associated function. Very little is known about centriole functions except that the centrosome reproductive process and the duplication of centrioles are likely to be closely integrated (Sluder and Rieder, 1985; Sluder et al., 1989). It is also well known that a structural continuity is required in most animal species for centrosome inheritance at fertilization, in which centrioles apparently play a major role (Maller et al., 1976; Klotz et al., 1990). Furthermore, Maniotis and Schliwa (1991) could demonstrate by microsurgical removal of centrosomes that centriole assembly cannot proceed de novo in mammalian somatic cells. On the other hand, we also demonstrated that a vast portion of centrin is not associated with centrosomes in human cells, which could reflect the existence of other centrin-dependent processes in the cell. In this regard, centrin might, like calmodulin, regulate several independent enzymatic activities in a Ca^{2+}-dependent manner.

Centrin function was assessed by injecting recombinant heterologous centrin in frog embryos. We have shown that both HsCen2 and CrCen specifically perturbed frog early development, suggesting a dominant negative effect of injected centrin on centrin-dependent activities. One explanation could be that heterologous centrin would compete for frog centrin targets in the egg but would not be able to carry properly its functions. Immunofluorescence analysis of microtubule organization in injected embryos further revealed that in addition to cleavage perturbation, the distribution of microtubule asters and nuclei in blastomeres was grossly affected. This is reminiscent of the phenotype of the *C. reinhardtii vfl 2* mutant.

The rapid succession of embryonic cycles requires a tight coordination between mitotic spindle formation and cytokinesis. According to Rappaport (1986), the location of cytokinesis is determined by the mitotic spindle. Depending on the cell system under study, both spindle poles and spindle midzone have been proposed to play a critical role (Rappaport, 1961; Margolis and Andreassen, 1993; Cao and Wang, 1996). In both cases, the orientation of the mitotic apparatus is critical. It is defined in turn by the pattern of migration of centrosomes at prophase which itself depends on the interaction of centrosomes with both the chromosomes and the cell cortex (for reviews see Fishkind and Wang, 1995; White and Strome, 1996). Any perturbation in the centrosome duplication itself or in its timing, in the separation of the daughter centrosomes, in their pattern of migration or in the signaling between centrosomes and cell cortex would thus produce cleavage pattern perturbation. Moreover, since embryonic cell division cycles lack feedback controls by downstream events (Murray, 1992) and centrosome duplication can proceed independently of the mitotic clock (Gard et al., 1990; Sluder et al., 1990) perturbations occuring during early division cycles should be rapidly amplified in the successive cycles and have a dramatic outcome.

Immunofluorescence analysis of injected embryos suggests that centrin is not involved in centrosome duplication itself although this point has to be ascertained by electron microscopy. Centrin could participate in one of the events of the centrosome reproduction pathway necessary for the coordination of cytoplasmic and nuclear division, or could participate directly in cell cleavage as we observed a faint centrin staining on midbodies. In any case, centrin seems to be essential for the proper execution of cell division.

In conclusion, this work provides evidence for an unexpected complexity of the centrin profile in human cells and for the association of centrin with the centrosome as luminal components of centrioles. This work also provides evidence of an important role for centrin in early cleavage of frog embryos. Clarifying centrin function in higher eukaryotes and identifying its molecular partners will be an important issue to understand the centrosome reproduction cycle and the mechanisms

by which the centrosome coordinates cytoplasmic and nuclear division.

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