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Pericentrin, a Highly Conserved Centrosome Protein Involved in Microtubule Organization

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Summary

Antisera from scleroderma patients that react widely with centrosomes in plants and animals were used to isolate cDNAs encoding a novel centrosomal protein. The nucleotide sequence is consistent with a 7 kb mRNA and contains an open reading frame encoding a protein with a putative large coiled-coil domain flanked by noncoiled ends. Antisera recognize a 220 kd protein and stain centrosomes and acentriolar microtubule-organizing centers, where the protein is localized to the pericentriolar material (hence, the name pericentrin). Anti-pericentrin antibodies disrupt mitotic and meiotic divisions in vivo and block microtubule aster formation in Xenopus extracts, but do not block γ-tubulin assembly or microtubule nucleation from mature centrosomes. These results suggest that pericentrin is a conserved integral component of the filamentous matrix of the centrosome involved in the initial establishment of organized microtubule arrays.

Introduction

The microtubule cytoskeleton is anchored to the centrosome or some other form of microtubule-organizing center (MTOC) (see Brinkley, 1985), which is thought to serve as a site of microtubule nucleation. In most cells, the centrosome is comprised of a pair of centrioles that lie at the center of a dense, partially filamentous matrix, the pericentriolar material (PCM) (see Brinkley, 1985). The centrioles are similar, if not identical, to basal bodies, which in many species form the template for the flagellum of the sperm and subsequently serve as the centrioles following fertilization (see Wheatley, 1982). Some cells lack centrioles altogether and accomplish mitotic (Peterson and Ris, 1990; Clayton et al., 1985) and meiotic (Calarco et al., 1983) divisions using an MTOC that is structurally and immunologically related to the PCM of centrosomes. Thus, it is believed that the PCM alone is fully capable of organizing microtubule spindles (Gould and Borisy, 1977).

Little is known about the structure of the PCM and the process by which microtubules are nucleated. There are paradoxes in the functional and structural properties of centrosomes. For example, though the PCM is amorphous when viewed in the electron microsocope, the number of microtubules nucleated from centrosomes is saturable, the nucleated microtubules have a uniform polarity, and the number of protofilaments in the microtubule lattice, distal to the centrosome, is constrained by the nucleating material to 13 (Evans et al., 1985).

Crucial to our understanding of the centrosome is the identification and characterization of molecular components that are involved in nucleation and in organization of the nucleating material. A limited number of proteins and antigens associated with centrosomes have been described (reviewed by Kuriyama, 1992; Kalt and Schliwa, 1993), but none has been ascribed a function. It has been speculated that γ-tubulin (Oakley et al., 1990; Stearns et al., 1991), which is specific to centrosomes, might participate in microtubule nucleation (Joshi et al., 1992).

Several autoimmune sera (Tuffanelli et al., 1983) stain the centrosome specifically and have been useful markers of centrosome dynamics in a wide variety of organisms. Using sera from patients with scleroderma that recognize highly conserved centrosome antigens (Tuffanelli et al., 1983; Calarco et al., 1983; Clayton et al., 1985), we have isolated cDNAs encoding a centrosome protein that we call pericentrin. We demonstrate here that pericentrin is a highly conserved coiled-coil protein probably present in all centrosomes and MTOCs. It is exclusively localized to the MTOC, and its levels change with the nucleating capacity of centrosomes during the cell cycle. Its function was tested by injection of specific antibodies into mouse oocytes and Xenopus embryos, where they blocked mitotic and meiotic divisions and disrupted spindle structures. In cytoplasmic extracts prepared from Xenopus eggs, the antibodies blocked the assembly of the microtubule aster around centrioles but did not prevent accumulation of γ-tubulin. These results suggest that pericentrin is required for the initial organization of spindles in all cells, but may not be involved in the final steps of microtubule nucleation.

Results

Isolation of a cDNA Encoding Pericentrin

An autoimmune serum from a patient with scleroderma, designated 5051, has long been used as an immunofluorescence marker for centrosomes and other MTOCs (Tuffanelli et al., 1983; Calarco et al., 1983; Clayton et al., 1985). Since the antigen(s) recognized by the serum is conserved from plants (Clayton et al., 1985) to humans (Tuffanelli et al., 1983), we felt that it might play an important functional role in the centrosome. To identify genes encoding the 5051 antigen, a mouse λgt11 cDNA expression library was screened with 5051 and two other autoimmune sera with similar properties. Of the 5×10^6 clones screened, only one cDNA of 1.5 kb was identified (λpc1.1; see Experimental Procedures), indicating that the

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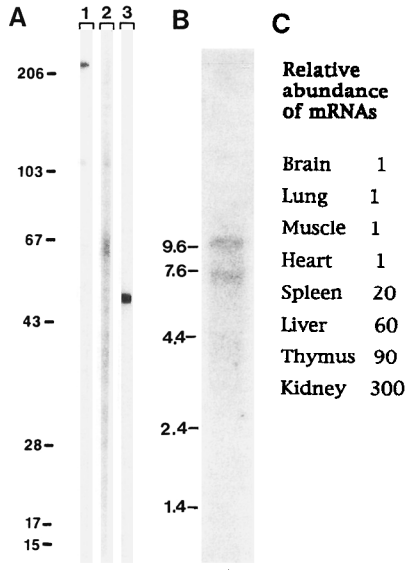

Figure 1. Pericentrin Protein and mRNAs

(A) Western blots of centrosome fractions isolated from mouse NIH 3T3 cells (Mitchison and Kirschner, 1984) using affinity-purified anti-pericentrin antibody (lane 1), preimmune serum (lane 2), or anti-tubulin antibody (lane 3). The equivalents of 5 × 10^6 (lanes 1 and 2) and 2.5 × 10^5 (lane 3) centrosomes were loaded per lane. Markers are in kilodaltons.

(B) Northern blot of polyadenylated RNA (10 μg per lane) isolated from day 16 mouse embryos and probed with the λpc 1.1 cDNA fragment. Markers are in kilobases.

(C) Relative amount of pericentrin mRNA found in mouse tissues as determined by PCR. Similar results were obtained in duplicate from two independent PCRs at several different amplification cycles.

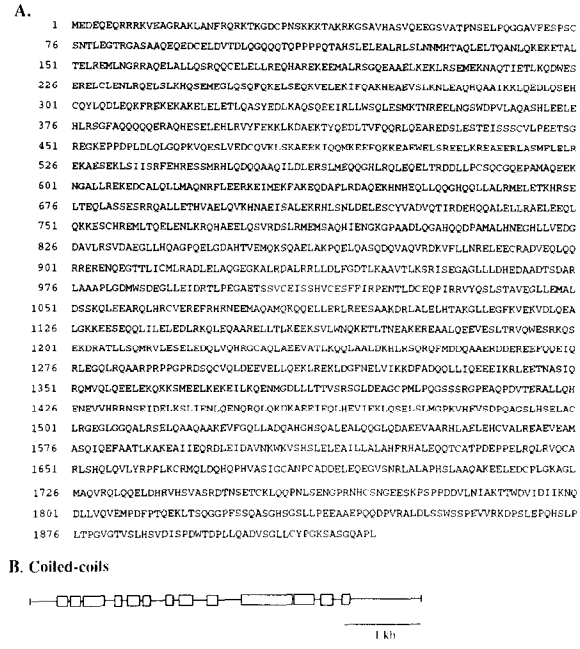

Figure 2. Amino Acid Sequence of Pericentrin

(A) The predicted amino acid sequence of the pericentrin coding region. (B) Coiled-coil regions of pericentrin, predicted by the program of Lupas et al. (1991), are represented by boxed regions, while noncoiled regions are shown as single lines.

mRNA for this molecule was most likely rare. The protein encoded by the cDNA was called pericentrin (see below).

Pericentrin Is a 220 kd Protein of the Centrosome

The 1.5 kb insert was cloned into an Escherichia coli expression vector (Rosenberg et al., 1987). The resulting fusion protein of 90 kd was overexpressed, gel purified, and used to generate rabbit polyclonal antiserum. Western blots demonstrated that the anti-pericentrin and the original 5051 antisera both recognized the 90 kd fusion protein (data not shown). Affinity-purified anti-pericentrin antibodies, but not preimmune sera, recognized a protein of 220 kd in cellular fractions enriched in centrosomes (Figure 1A). Pericentrin was not detectable in whole-cell lysates (data not shown), suggesting that the protein was rare. In addition, two other antisera that were raised against different parts of pericentrin and gave identical immunofluorescence staining did not detect the protein in whole-cell lysates (data not shown; see Experimental Procedures). In fact, from the concentration of pericentrin (normalized to the immunoreactivity of the fusion protein), we estimate that there were only 540 molecules in each isolated centrosome compared with our estimate of 2.7 × 10^4 molecules of tubulin. The original 5051 autoimmune serum recognized several high molecular mass bands in centrosome fractions, including one of 220 kd (Snyder and Davis, 1988; S. J. D., unpublished data), suggesting that pericentrin was one of the original 5051 antigens.

By Northern blotting techniques, two mRNAs of 7 kb and 9.5 kb were identified (Figure 1B) in mouse embryos. Neither message was specific to embryos, since a similar pattern was obtained using NIH 3T3 cells (data not shown). Pericentrin mRNAs were barely detectable on gels overloaded with polyadenylated RNA, and their concentration, estimated from blotting intensity, was 0.01% that of actin mRNA (data not shown). The rarity of the message, like the protein, was consistent with its poor representation in each of seven different cDNA libraries screened in this study. The levels of pericentrin mRNA varied in different tissues. Using polymerase chain reaction (PCR) techniques (see Experimental Procedures; Chelly et al., 1988), we found that kidney, thymus, and liver had the highest levels of pericentrin mRNA (Figure 1C), while brain, muscle, lung, and heart had ~200-fold less.

Pericentrin Is Predicted to Be a Large Coiled-Coil Protein with Noncoiled Ends

The full sequence of the pericentrin coding region was determined using a combination of cDNA and genomic library screening and PCR methods (see Figure 9 in Experimental Procedures). Several features of the sequence together with the low abundance of the mRNA posed difficulties in cloning. The nucleotide sequence is available from GenBank; the predicted amino acid sequence is shown in Figure 2A. The putative initiation ATG is preceded by three in-frame stop codons and begins an open reading frame of 5760 nt. The stop codon, determined by

the size of in vitro translation products (see Experimental Procedures), is followed by three additional in-frame stop codons and a polyadenylation consensus sequence.

The pericentrin coding sequence predicts a molecular mass of 218,425 daltons, in good agreement with that determined by electrophoresis and Western blotting. The polypeptide sequence was compared with known translated sequences in several data bases (see Experimental Procedures). The central region of pericentrin, extending from amino acid 110 to 1600, was weakly homologous to coiled-coil domains of several other proteins, such as myosin and keratin (Cohen and Parry, 1990; Lupas et al., 1991), while the N-terminal 110 amino acids and C-terminal 320 amino acids showed no significant homologies.

Further analysis indicated that pericentrin was a three-domain molecule comprised of a very long central α-helical coiled-coil segment (~1500 amino acids) flanked by non-coiled domains (Figure 2B). Several coiled-coil segments are found in the α-helical domain of pericentrin, the longest being 250 amino acids in length (Figure 2B), interrupted by short segments that lack heptad periodicity that are similar to those found in some other coiled-coil proteins such as laminin (Beck et al., 1990). These segments often contain proline residues, which usually disrupt α helices. The N- and C-termini of pericentrin have ~110 and 320 amino acids stretches, respectively, that are nonhelical and noncoiled (Figure 2B). Though noncoiled domains of other coiled-coil proteins are often involved in interactions with other proteins, no consensus sequences for microtubule binding (Butner and Kirschner, 1991) or nucleotide binding, as seen in motor proteins (see Vale and Kreis, 1993), were found. A number of protein kinase recognition sequences were identified, including 6 sites for the cell cycle–specific cdc2 kinase (Shenoy et al., 1989) that are clustered mainly at the N- and C-termini of the molecule. Other sites include 1 for tyrosine kinase at amino acid 429 (Ward and Kirschner, 1990), 5 for cAMP-dependent protein kinase, 29 for protein kinase C (Kemp and Pearson, 1990), and 41 for casein kinase 2.

Pericentrin Is a Highly Conserved, Integral Protein of the Centrosome

Antibodies raised against the original fusion protein were tested for their ability to stain centrosomes by immunofluorescence. In Rat-1 cells, the antisera gave the characteristic centrosome staining pattern of one or two foci in the perinuclear region of interphase cells (Figure 3A) and foci at each pole of mitotic spindles (Figures 3B and 3C). The staining pattern ranged in morphology from a single tight focus to diffusely focused structures with variable numbers of projections in different cell types. Multiple small dots were present in addition to the larger, dominant ones in NIH 3T3 cells (Figure 3D). No staining was seen with preimmune serum (Figure 3E). Pericentrin staining intensity changed with the nucleating capacity of the centrosome during the cell cycle, being highest at metaphase and lowest at telophase (data not shown).

Since it was formally possible that the antisera recognized an epitope other than pericentrin, antibodies were raised against two different parts of the molecule, which were located outside, that were originally recognized by the 5051 serum (see Experimental Procedures). Both antisera recognized centrosomes by immunofluorescence, and the 5051 antiserum did not react with either of the fusion proteins (data not shown), demonstrating that pericentrin was a component of the centrosome and not a different molecule with a shared epitope.

The species specificity of anti-pericentrin antibodies was tested. The original antibody recognized centrosomes in all mammalian cell types tested, including rat, hamster, mouse (Figure 3), cow (D. Albertini, personal communication), human, monkey, dog, kangaroo rat (data not shown), and Xenopus embryos (see below). Focal staining was observed in centrosomes of Drosophila (J. B. Tucker et al., unpublished data) and Naegleria gruberi (C. Walsh, personal communication), as well as in the oral apparatus in Tetrahymena (Stearns and Kirschner, 1994 [this issue

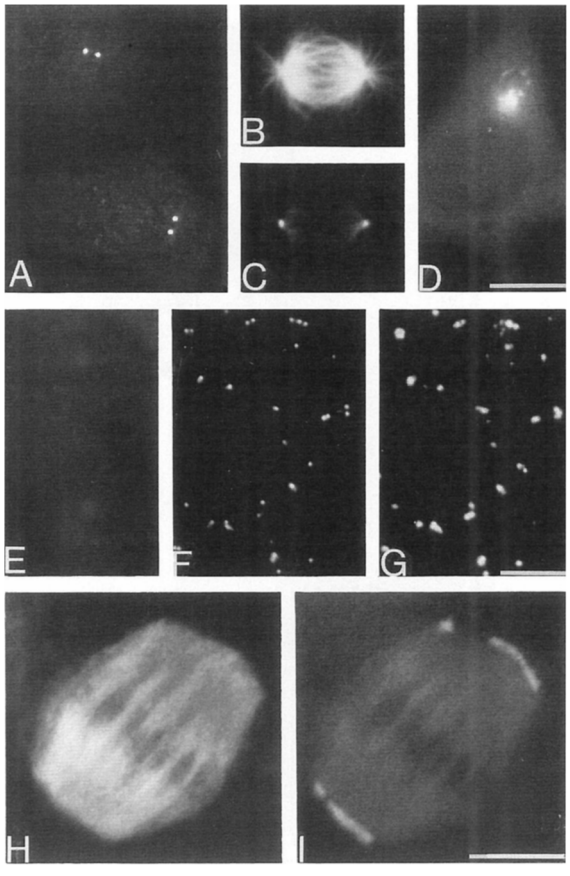

Figure 3. Immunoflourescence Localization of Pericentrin to MTOCs in a Variety of Organisms

Rat-1 cells show prominent focal staining with anti-pericentrin antibody (A). A mitotic Rat-1 cell costained with anti-tubulin (B) and anti-pericentrin (C) antibodies. A mouse NIH 3T3 cell stained with anti-pericentrin antibody (D) showing numerous small foci that are sometimes seen in addition to the larger foci. No staining is seen with preimmune serum (E). Centrosomes isolated from CHO cells co-stained with anti-α-tubulin (F) and anti-pericentrin (G) antibodies. A mouse meiotic spindle, which lacks centrioles, stained for tubulin (H) and pericentrin (I). Scale bars, (A–E) 10 μm; (F and G) 10 μm; (H and I) 40 μm.

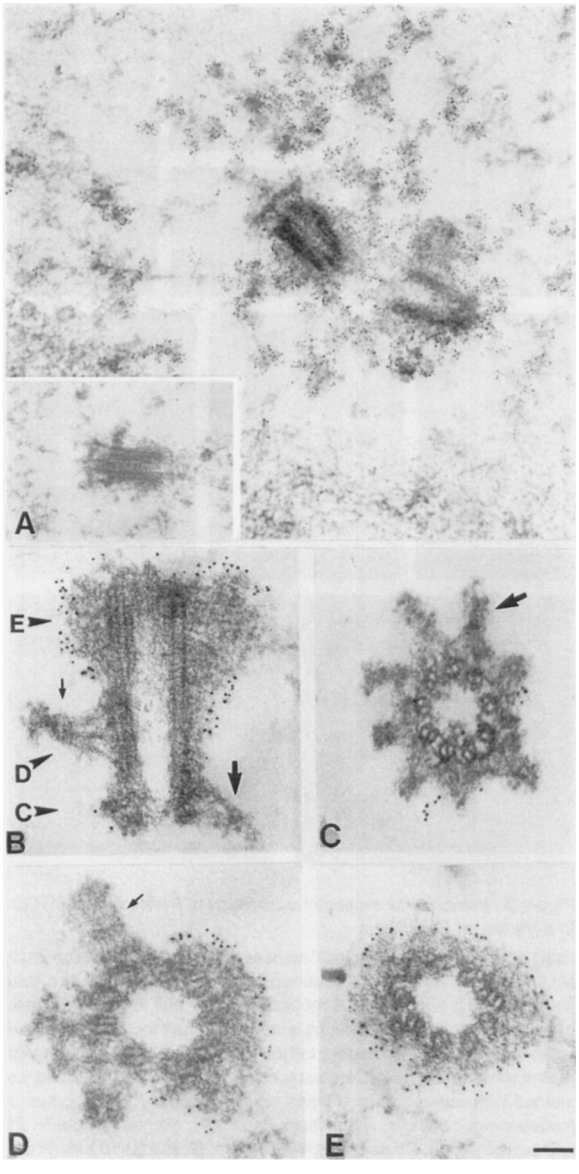

Figure 4. Ultrastructural Localization of Pericentrin

Rat-1 cells processed for immunogold electron microscopy using anti-pericentrin antibodies (A) show numerous gold particles on the PCMs associated with both centrioles and on PCM-like bodies (satellites) surrounding the centrioles. Preimmune serum gave no specific centrosome staining (inset). Centrosomes isolated from Rat-1 cells (B–E) also showed staining of the PCM with anti-pericentrin antibodies, which was absent from from centriolar microtubules and two sets of appendages (large and small arrows). (C–E) define representative areas and sections designated by the arrowheads in the longitudinal section in (B). (C) includes one set of appendages (large arrow), (D) includes the other (small arrow), and (E) has only PCM. Scale bars, (A) 0.22 μm; (inset) 0.26 μm; (B–E) 0.1 μm.

of *Cell*]). A second antibody against a different part of the pericentrin protein showed similar reactivity (data not shown). The strong centrosome staining in mammals, amphibians, insects, ciliates, and amoeba flagellates demonstrated that pericentrin was a highly conserved protein.

Pericentrin appears to be an integral component of the centrosome and not simply material that is transported there along microtubules. Complete depolymerization of microtubules did not change the staining pattern of pericentrin observed in untreated cells (data not shown). Similar results were obtained with cytochalasin, which depolymerizes actin filaments. When both drugs were used to isolate centrosomes from cultured Chinese hamster ovary (CHO) cells, pericentrin staining colocalized with the anti-α-tubulin antibody (Figures 3F and 3G), which presumably stains the centriole cylinders (Mitchison and Kirschner, 1984). Treatment of cells with nonionic detergents did not alter the staining pattern. When isolated centrosomes were treated with high salt, the tubulin epitope was lost or greatly diminished and the microtubule structure was lost in the electron microscope, while the pericentrin staining remained unchanged (data not shown). Thus, pericentrin is a robust and integral part of the centrosome that persists even in the absence of centrioles.

Localization of Pericentrin to MTOCs Other Than Centrosomes

To determine whether pericentrin was a component of MTOCs or whether it had an obligatory association with centrioles, we examined acentriolar MTOCs. Mouse oocytes, arrested in metaphase of meiosis II, possess PCM-like material at the spindle poles but do not contain centrioles (Szollosi et al., 1972). Anti-pericentrin antiserum and not preimmune serum stains this material (Figures 3H and 3I) as well as similar material found in other acentriolar MTOCs of a variety of morphologies, including mammalian muscle cells (E. Ralston, personal communication), dual-action organizing centers of Drosophila flight muscle (Tucker et al., 1986; J. B. Tucker et al., unpublished data), the oral apparatus of Tetrahymena (Stearns and Kirschner, 1994), and spindle poles of Naegleria (C. Walsh, personal communication). In addition, a low level signal is observed around centrioles of Xenopus sperm and Tetrahymena (Stearns and Kirschner, 1994). Electron microscopy revealed that the staining is once again confined to a small amount of electron dense material surrounding the centriole cylinders and is not present on the microtubules that comprise the centrioles (data not shown). Kinetochores of chromosomes from CHO cells and HeLa cells did not stain for pericentrin (data not shown).

Organization of Pericentrin in the Centrosome

We examined Rat-1 cells and isolated centrosomes by electron microscopy following immunogold labeling. Gold particles in intact cells were found on the PCM surrounding the centrioles and on pericentriolar satellites (Figure 4A), which are believed to be extensions of the PCM (see Brinkley, 1985; Baron and Salisbury, 1988; Paintrand et al., 1992). The satellite staining may correspond to the smaller dots sometimes seen by immunofluorescence (see Figure 3D). No cytoplasmic structures other than satellites in the vicinity of the centrosome were stained with anti-pericentrin antibody. No specific staining was observed with preimmune serum (inset in Figure 4A).

Isolated centrosomes provided better resolution of the gold-labeled structures (Figures 4B–4E; see Figure 5). Particles were again found on the PCM and on PCM-like

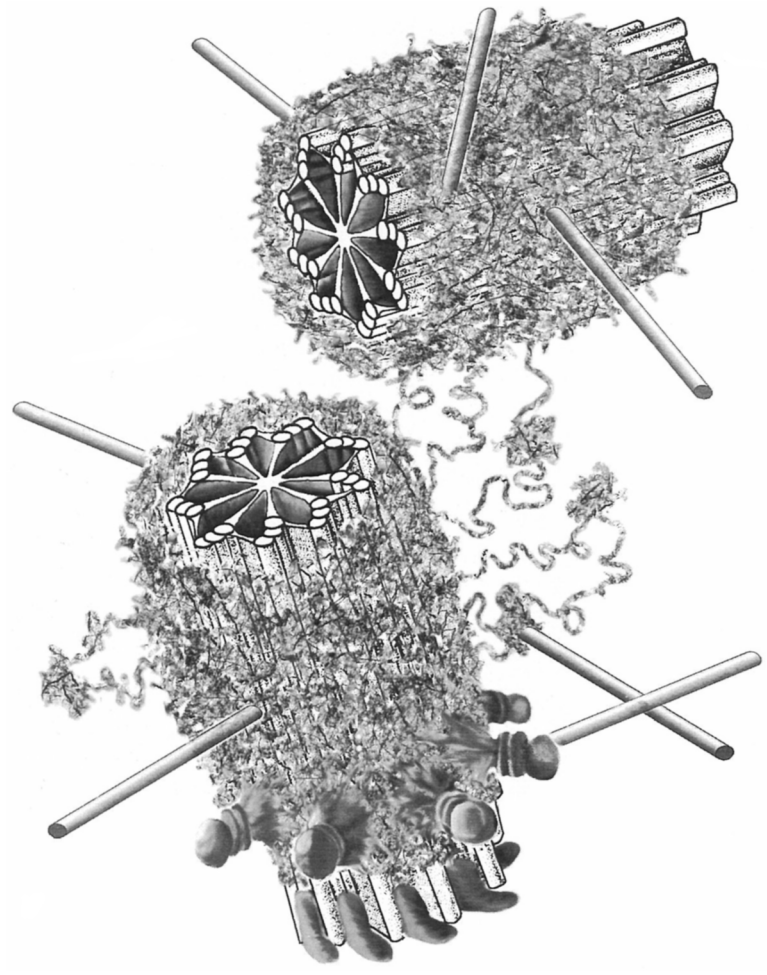

Figure 5. Schematic Representation of the Centrosome Based on Ultrastructural Reconstruction

Details are shown of centrosome structure, based on electron microscopic observations of centrosomes in situ and after isolation from two cultured cell lines. See text for details. Microtubules are shown as truncated polymers. Centrioles were modified from a drawing by Krstic (1976).

densities near centrosomes that probably represented the satellites stained in intact cells (Figure 4A). No other material in the preparation stained over background. The centrioles and two different sets of appendages that radiate from the centriole wall (arrows in Figure 4B; Figures 4C and 4D; Bornens et al., 1987; Baron and Salisbury, 1988; Paintrand et al., 1992) did not label with gold. As evidence of good penetration of regents, we found that gold was present in large quantities in the lumen of the centrioles, when gold-labeled secondary antibody was not washed out prior to fixation. However, when washed before fixation, virtually no gold remained, indicating that there was no specific binding on the lumenal aspect of centriolar microtubules (data not shown). In addition, neither the microtubules of basal bodies (centrioles) of ciliated epithelium nor those of cilia themselves stained with anti-pericentrin antibodies (data not shown). Figure 5 is a schematic representation of a centrosome that shows the arrangement of the various structures described above, including those that stain for pericentrin. These data suggest a specialization of certain centriole satellites and appendages for microtubule nucleation, while others presumably have some other function.

In Vivo Assays of Pericentrin Function Using Anti-Pericentrin Antibodies

As a test of pericentrin function, we examined the effects of affinity-purified anti-pericentrin antibodies following injection into developing mouse oocytes. Mouse oocytes were chosen for these experiments because the microtubule-nucleating material is known to exist in dispersed form that should be accessible to injected antibodies (Calarco et al., 1983; S. J. D., unpublished data). The native form of pericentrin was recognized by pericentrin antibodies in unfixed, permeabilized cells (data not shown; see Experimental Procedures).

In cells injected with control antibodies or in uninjected cells, round polar bodies of equal size appeared after the first and second meiotic divisions (Table 1; Figures 6A and 6B), while in cells injected with anti-pericentrin antibodies, polar bodies did not form or appeared grossly abnormal and contained variable amounts of DNA (Table 1; Figures 6C–6F). In controls, pericentrin staining was present at the poles and DNA was aligned on the metaphase plate (see Figures 3H and 3I). In contrast, 80% of meiotic spindles in cells injected with anti-pericentrin antibodies lacked polar pericentrin staining and the spindles were disrupted

Table 1. Anti-Pericentrin Antibodies Interfere with Polar Body Formation and Meiotic Spindle Organization in Mouse Oocytes

	Polar Bodies		Microtubule Spindles		
Antibody	Normal	Abnormal	Normal	Abnormal	Absent
Anti-pericentrin	36	64	20	71	9
Control	89	11	92	8	0

Oocytes were injected with antibodies and examined for polar body formation by phase–contrast microscopy and for spindle morphology by immunofluorescence labeling of tubulin and pericentrin. A total of 105 anti-pericentrin-injected cells and 78 control-injected cells were analyzed in a total of nine experiments using two different affinity-purified anti-pericentrin antibody fractions. Values are expressed as a percentage of surviving cells, were similar in control- and anti-pericentrin-injected cells, and never exceeded 66%. See Results and Experimental Procedures for details.

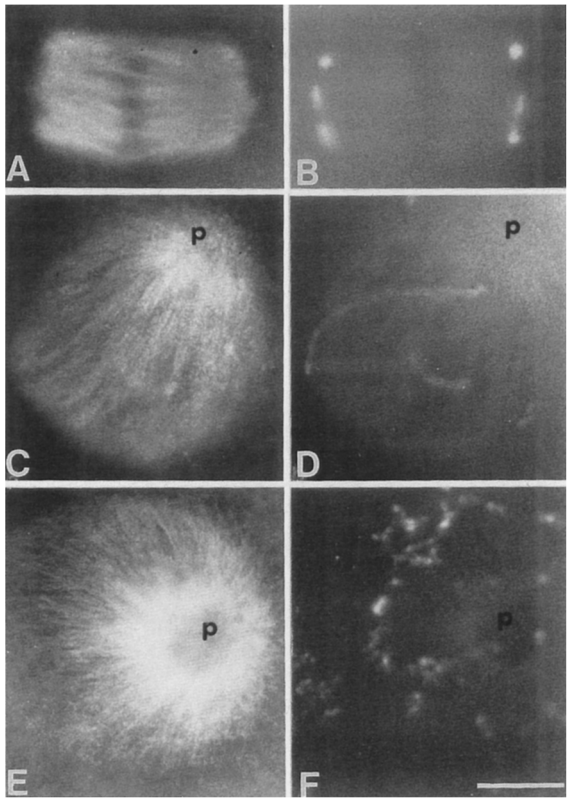

Figure 6. Effects of Anti-Pericentrin Antibody Injection on Meiotic Spindle Organization in Mouse Oocytes

Mouse oocytes injected with control (A and B) or affinity-purified anti-pericentrin (C–F) antibodies and processed 20 hr later for immunofluorescence using anti-tubulin (A, C, and E) or anti-pericentrin (B, D, and F) antibodies. In cells injected with anti-pericentrin antibodies, the bipolar arrangement of the spindle and polar localization of pericentrin staining seen in controls are lost. Instead, microtubules form a loose focus at the cortex and course randomly toward the center of the oocyte (C and E), while patches of pericentrin staining are distributed randomly throughout the area of the spindle (D and F). Scale bar, 10 μm.

or absent (Figures 6C–6F; Table 1). In most cases (71%), disrupted microtubule arrays formed a loose focus near the cell cortex and coursed randomly toward the center of the oocyte (Figures 6C and 6E). Pericentrin-staining structures were usually dispersed throughout the disorganized microtubule array (Figures 6D and 6F; see Table 1). In some cases (9%), spindles were replaced by a cytoplasmic network of interwoven microtubules with pericentrin-staining structures and chromosomes randomly distributed throughout the cytoplasm (Table 1). Normal spindles that were observed in 20% of the cases may have resulted from insufficient antibody delivery (see Experimental Procedures).

Anti-pericentrin antibodies had the same effect on mitotic divisions in Xenopus embryos as they did on meiotic divisions in mouse oocytes. The antibodies, which react with the native Xenopus protein (data not shown; see Experimental Procedures), were injected into one cell of a 2-cell embryo. Uninjected cells divided normally, while cells injected with anti-pericentrin antibodies divided one to three times and then arrested (Figure 7; Table 2). Similar disruptive effects were observed throughout a range of final antibody concentrations in the cytoplasm of 0.5–50 μg/ml. Antibody injection did not affect surface contraction waves in eggs that reflect the inherent cyclin–cdc2 oscillator (Murray and Kirschner, 1989), thus demonstrating that the effect on cell division was not a nonspecific effect on the cell cycle or general physiology of the cells. Embryos injected with control antibodies divided and developed normally.

In control embryos, microtubule organization appeared normal (Table 2; Figures 7B–7D and 7G), with pericentrin staining appearing as bright foci at spindle poles in mitotic cells (Figure 7B). DNA was either aligned on the metaphase plate (Figures 7C and 7G) or within interphase nuclei (Figure 7G). In contrast, in embryos injected with anti-pericentrin antibodies (Figures 7E, 7F, 7H, and 7I; Table 2), 79% of the cells had undetectable levels of pericentrin and 49% lacked microtubule structures. The lack of pericentrin staining correlated with the absence of microtubule structures. Anti-pericentrin antibodies sometimes produced spindles reduced in size (Figure 7E) and lacking the striking astral microtubules seen in control cells (Figure 7D), but the DNA was aligned on the metaphase plate (Figure 7H). These spindles could not progress through mitosis. In some cases (12%), the spindles were multipolar (Figures 7F and 7I) or completely disorganized (Table 2), as seen in other systems when pericentrin organization is disrupted (Vidair et al., 1993).

In Vitro Assays of Pericentrin Function

Although anti-pericentrin antibodies disrupted microtubule organization, it was not clear whether they inhibited the assembly of the centrosome, microtubule nucleation, or microtubule elongation. To distinguish among these, we studied the effects of anti-pericentrin antibodies on aster formation in Xenopus extracts and on microtubule growth from centrosomes with purified tubulin. When basal bodies (or centrioles) are added to extracts, they acquire nucleating capacity by the accretion of nucleating components (Murray and Kirschner, 1989; Sawin and Mit-

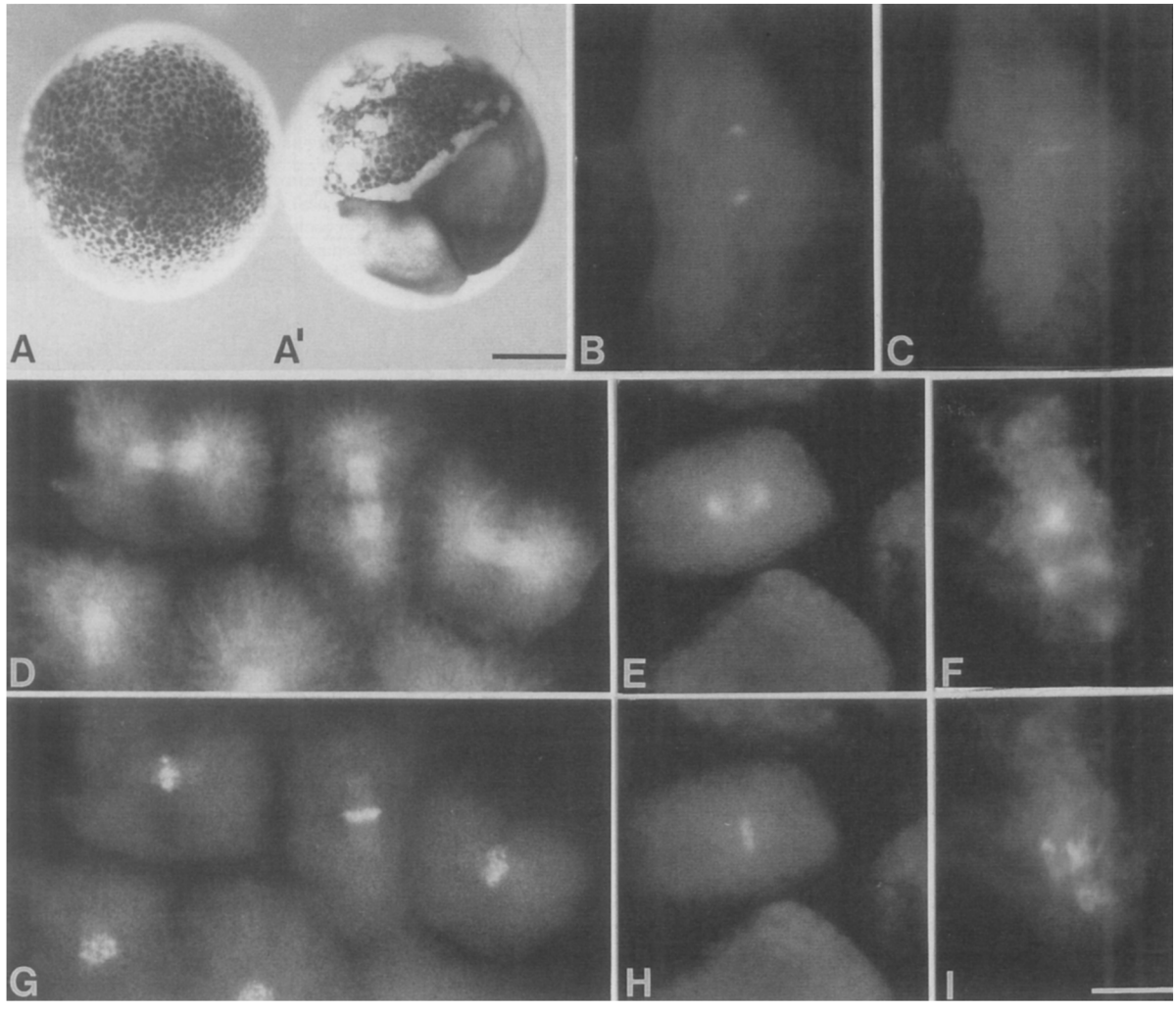

Figure 7. Effects of Anti-Pericentrin Antibodies on Cell Division and Microtubule Organization in Xenopus Embryos

Two-cell Xenopus embryos injected into one cell with control (A) or anti-pericentrin (A') antibodies and incubated for 3 hr. Cell division is arrested in the cell injected with anti-pericentrin antibodies (A', lower right) while the uninjected cell (A', upper left) and the cell injected with control antibodies (A) divided normally. In embryos injected with control antibodies and processed for immunofluorescence microscopy (B, C, D, and G), cells display bright pericentrin staining (B), extensive microtubule arrays in both interphase and mitosis (D), and normal DNA organization (C and G). In the presence of anti-pericentrin antibodies, over half the cells have no detectable microtubule- or pericentrin-staining structures. When observed, the microtubule arrays are most often in the form of small spindles lacking astral microtubules (E) with DNA aligned on the metaphase plate (H). Multipolar spindles are also observed (tubulin in [F]; Hoechst in [D]). Scale bars, (A) 0.3 μm; (B–I) 50 μm.

chison, 1991). To test the role of pericentrin in microtubule aster assembly, antibodies were added to extracts along with the addition of centrioles, and the number of microtubules arising from the newly assembled centrosomes was measured. In extracts containing control antibodies, microtubules grew from the centrosome after a 7–10 min lag period (Figure 8A). In the presence of anti-pericentrin antibodies, the number of microtubules per aster was reduced by ~50%. Furthermore, preincubation of extracts with antibodies prior to the reaction reduced the number of microtubules per aster by 91% (Figures 8B and 8C). Pretreating the nuclei with anti-pericentrin antibodies had no effect. Microtubule length was similar to controls, indicating that there was no effect on microtubule elongation rate.

When sperm nuclei are incubated in Xenopus extracts, the centrioles mature into centrosomes and progressively increase their nucleation capacity. When these centrosomes were incubated in extracts with anti-pericentrin antibodies, microtubule nucleation was blocked. In the presence of high concentrations of dimethyl sulfonate (DMSO), the inhibitory effect of anti-pericentrin antibodies on aster formation from Xenopus centrosomes was overcome. However, the resulting asters were disorganized and had many fewer microtubules (data not shown). These results suggest that anti-pericentrin antibodies inhibit microtubule nucleation on both immature centrosomes and mature centrosomes when incubated in cytoplasmic extracts.

To determine directly whether anti-pericentrin antibodies inhibit mircrotubule nucleation or elongation, we exam-

Table 2. Anti-Pericentrin Antibodies Interfere with Microtubule Organization in Xenopus Embryos

Antibody	Arrested Embryos[a]	Microtubule Spindles				
		Absent	Multipolar	Reduced	Normal	Disrupted
Anti-pericentrin	97	51	8	34	3	4
Control	0	4	0	2	94	0

[a] Two-cell embryos were injected with antibodies into both cells, incubated for 3 hr, and examined for division arrest. Numbers shown are expressed as a percentage of surviving embryos, which was >89% in all cases. For immunofluorescence studies, all cells of 4- to 8-cell embryos were injected and examined 2.5 hr later. A total of 438 cells from anti-pericentrin-injected embryos and 302 cells from control antibody–injected embryos were analyzed in four experiments using two anti-pericentrin antibody preparations (see Experimental Procedures).

ined assembly from axonemes (Gard and Kirschner, 1987). We observed no difference in microtubule length in the presence of control (mean length is 6.2 ± 0.55 μm; n = 56) or anti-pericentrin antibodies (6.0 ± 0.3 μm; n = 67). Spontaneous assembly of microtubules in extracts driven by DMSO (see Stearns and Kirschner, 1994) occurred in the presence of anti-pericentrin antibodies, although at slightly higher concentrations of DMSO and to a lesser degree than in controls (data not shown). It should be noted that "spontaneous" assembly of microtubules in extracts may involve the accumulation of nucleating components (Buendia et al., 1992).

When centrosomes isolated from CHO cells were incubated with pure tubulin in vitro, the number of nucleated microtubules were similar in the presence of anti-pericentrin and control antibodies or buffer (Figures 8D–8F). Similar results were obtained in this in vitro assay using Xenopus centrosomes from activated Xenopus sperm. Thus, the anti-pericentrin antibody seemed to exert its effect in cytoplasm on aster formation from immature centrosomes but not in vitro with purified tubulin. In addition, although anti-pericentrin antibodies blocked nucleation in extracts, they did not block the assembly of γ-tubulin on immature sperm centrosomes, an event thought to be important for microtubule assembly (Stearns and Kirschner, 1994).

Discussion

It is known that the PCM of centrosomes and similar material in other MTOCs are required for microtubule nucleation and organization, but little is known about the components involved and how they accomplish these functions. To address this, we have identified and characterized pericentrin, a highly conserved protein of mammalian centrosomes and perhaps all other MTOCs. Pericentrin is found together with γ-tubulin at sites that have been implicated in microtubule nucleation and is also found in low amounts in PCM-like material that surrounds centrioles, which is not capable of nucleating microtubules. The fact that the localization of pericentrin is not dependent on the presence of centrioles or microtubules and is resistant to high salt extraction suggests that it is an integral component of the microtubule-nucleating material of these structures.

There appear to be only about 500 molecules of pericentrin in each centrosome isolated from Rat-1 cells, weighing 0.2 fg per cell and representing less than 10^{-5}% of the protein of the cell. This may be an underestimate since some pericentrin may be lost during the preparation. Similar estimates of the protein composition of isolated centrosomes showed about 27,000 molecules of tubulin, weighing about 5 fg; this is about 70% of what is expected from the expected mass of the centriolar cylinder (Mandelkow et al., 1986). Given the accuracy of the methods, this is consistent with tubulin being of centriolar origin and pericentrin being present at low stoichiometry.

Pericentrin appears to have a central α-helical region flanked by nonhelical domains. The central region is pre-

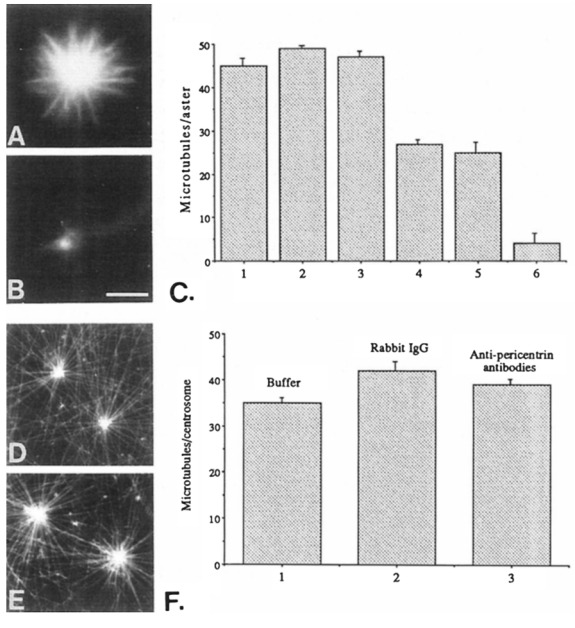

Figure 8. Effects of Anti-Pericentrin Antibodies on Aster Formation and Microtubule Nucleation in Xenopus Extracts

(A–C) Microtubule aster formation was assayed in the presence of control (A) and anti-pericentrin (B) antibodies. The number of microtubules per aster was quantitated in the presence of rabbit immunoglobulin G (bar 1 in [C]), anti–gene 10 antibodies (bar 2 in [C]), buffer (bar 3 in [C]), or anti-pericentrin antibody (bar 4 in [C]) or after preincubation of anti-pericentrin antibody with nuclei (bar 5 in [C]) or extracts (bar 6 in [C]).

(D–F) Microtubule nucleation from isolated CHO centrosomes (Mitchison and Kirschner, 1984) was assayed in the presence of control (D) and pericentrin (E) antibodies, and microtubule number was determined under different conditions (F).

Scale bar, 6 μm.

dicted to form a coiled coil that is among the largest known (Yang et al., 1992). Pericentrin could dimerize to form a coiled-coil rod with a similar pitch to paramyosin or intermediate filaments; in that configuration, the coil would be about 202 nm long (Aebi et al., 1986; Cohen et al., 1987; Mirzayan et al., 1992). In this form, about four molecules placed end to end would wrap once around the barrel of a centriole and 63 times if all of the pericentrin present in the centrosome was in this arrangement. Although the volume taken up by this amount pericentrin in the PCM would be less than 1% of the protein of the centrosome, pericentrin could be providing a scaffold for binding other proteins of the structure.

Pericentrin seems to be required for proper microtubule organization in both meiotic and mitotic spindles. Anti-pericentrin antibodies disrupt microtubule organization from meiotic and mitotic centrosomes. The mitotic spindles, which have centrioles, are seemingly well organized, although dramatically reduced in bulk. This may be due to residual nucleating activity that is unaffected by antibodies. On the other hand, the acentriolar meiotic spindles from eggs and oocytes are more severely disrupted, as well as being functionally deficient. The nucleating material may cycle between assembled and soluble forms and may be more accessible to antibodies and thus more susceptible to disruption. The dynamics and turnover of pericentrin may explain the discrepancy between the lack of effect of anti-pericentrin antibodies on mature centrosomes in vitro and the potent effect of anti-pericentrin antibodies on mature centrosomes incubated in cell extracts. Although pericentrin seems to be a structural component of the centrosome upon which other components are assembled, it does not seem to be required for the localization of γ-tubulin. Anti-pericentrin antibodies, while strongly inhibiting nucleation of microtubules, do not inhibit γ-tubulin mobilization during the 5 min lag phase before microtubule assembly.

Pericentrin thus seems to contribute to the capacity of the centrosome to nucleate microtubule assembly in a manner separate from the pathway required to accumulate γ-tubulin. In the cell or in the complex cellular environment of the extracts, pericentrin either directly or indirectly is required for efficient microtubule assembly. This suggests that the conditions in vivo may be very stringent and require contributions from both γ-tubulin and components regulated by pericentrin. Alternatively, pericentrin may act to prevent inhibitory activities in the cell from blocking microtubule nucleation. Even in pathways in which the spontaneous assembly of microtubule asters is induced by taxol or DMSO, the nucleating centers accumulate pericentrin as well as γ-tubulin.

Pericentrin and γ-tubulin therefore seem to represent separate requirements for microtubule nucleation in vivo. Both are present in the same microtubule-nucleating sites, both are highly conserved in metazoans, and both seem to be essential. Of the two, pericentrin seems the more intrinsic to the centrosome structure as it is localized to centrioles in sites in the cortex of Tetrahymena. γ-Tubulin seems more highly regulated, as shown by the assembly of γ-tubulin on the sperm centrosome, which already contains pericentrin. How each of these contributes to the assembly and function of the microtubule aster and how these or other components contribute to the kinetic and structural properties of microtubule nucleation in the centrosome are important remaining questions.

Experimental Procedures

Cloning Strategy

The initial screen was performed on a mouse B cell λgt11 cDNA library essentially as described by Sambrook et al. (1989). Autoimmune sera (see below) was diluted 1:500 in PBS and 5% BSA and incubated with filters together with bacterial lysate to reduce background. Of 5×10^6 plaques screened, one 1.5 kb cDNA was identified and subcloned into Bluescript vector (Stratagene, La Jolla, California). The cDNA was sequenced by the dideoxy chain termination method using Sequenase DNA polymerase (U. S. Biochemical Corporation, Cleveland, Ohio) and contained one continuous open reading frame.

To isolate cDNAs spanning the entire protein coding region, a combination of library screening (cDNA and genomic) and PCR technologies was used (Sambrook et al., 1989; Mount, 1982). A restriction map of overlapping pericentrin cDNAs, PCR fragments, and genomic clones and the areas of the coding region used to make fusion proteins is shown in Figure 9.

Sequence analysis was done by searching the GenBank data base using the TFASTA programs (Pearson and Lipman, 1988), the NBRF-

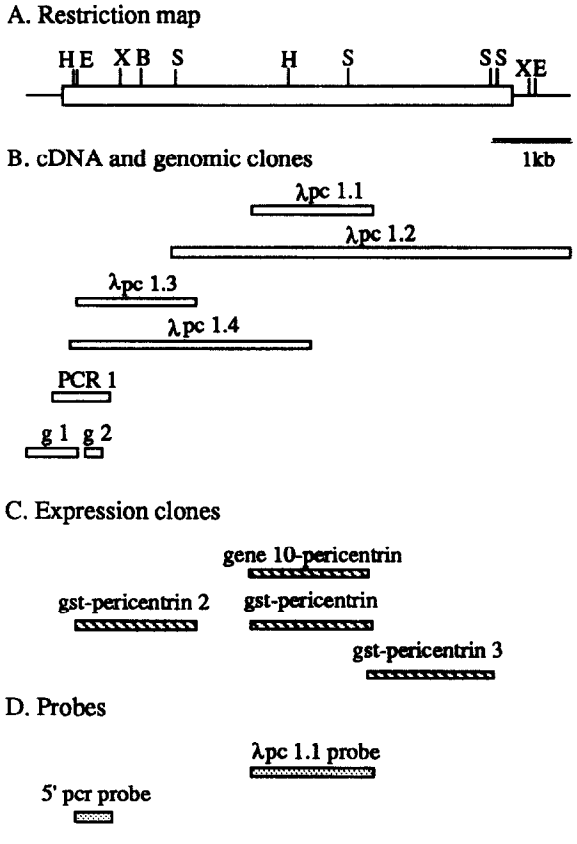

Figure 9. Pericentrin Restriction Map and cDNA, Genomic, and Expression Clones

(A) Restriction map of the pericentrin coding region (B, BamHI; E, EcoRI; H, HindIII; S, SmaI; X, XbaI). (B) Relative positions of the overlapping pericentrin cDNAs, PCR fragments, and genomic clones. (C) The portions of pericentrin overexpressed as fusion proteins in E. coli (see below). (D) Probes used for screening libraries and for Northern blots.

PIR data base using the FASTA programs, and the NCBI and Swissall data bases using the GENINFO (R) BLAST network (Altschul et al., 1990). The PROSITE.LIS program (A. Bairoch) was used to identify sequence motifs, secondary structure analysis was done using the program of Garnier et al. (1978), and coiled-coil analysis was done using the program of Lupas and Stock (1991).

Fusion Protein Production

The original pericentrin cDNA of 1.5 kb (λpc1.1) was subcloned into pET-3x (Rosenberg et al., 1987) and pGEX-1 (Amrad Corporation, Abbotsford, Victoria, Australia). The resulting fusion proteins were composed of ~60 kd of pericentrin and either 29 kd of the gene 10 product (pET-3x; Rosenberg et al., 1987) or 26 kd of glutathione S-transferase (GST) (Smith and Johnson, 1988). Both gene 10::pericentrin and GST::pericentrin fusion proteins were insoluble and were purified as inclusion bodies (Sambrook et al., 1989). Unfused gene 10 and GST proteins were also produced, purified, and used for control purposes. Using similar technology, two other GST::pericentrin fusions were constructed (Figure 9) and the soluble proteins purified as described (Amrad Corporation, Abbotsford, Victoria, Australia).

Antibodies

A gene 10::pericentrin fusion (90 kd) was gel purified, electroeluted, and used to raise antisera in rabbits (Babco Incorporated, Emeryville, California). Antibodies were affinity purified using fusion proteins and unfused vector proteins (GST and gene 10) essentially as described (Harlow and Lane, 1988). Anti-pericentrin antiserum was passed over a column of gene 10 protein to remove anti–gene 10 antibodies and then over a gene 10::pericentrin protein to isolate pericentrin-specific antibodies. Alternatively, anti-pericentrin antiserum was passed directly over a column of GST::pericentrin fusion protein. Affinity-purified antibody fractions contained 95% IgG and reacted with centrosomes by immunofluorescence and with the fusion protein on Western blots (data not shown). The antibody fractions purified against the two different fusion proteins gave indistinguishable results in all assays (see below).

Several controls were used, including affinity-purified anti–gene 10 antibodies, anti-GST antibodies, an IgG fraction purified from the preimmune serum using protein A beads (Sigma Chemical Corporation, St. Louis, Missouri) and eluted under the same conditions used for anti-pericentrin antibodies, and a rabbit IgG fraction (Sigma Chemical Corporation, St. Louis, Missouri). In all experiments, anti-pericentrin and control antibodies were concentrated to 2 mg/ml and dialysed against the appropriate buffer. Antibodies were also made against GST::pericentrin 2 and 3 proteins (see above). They reacted specifically with centrosomes by immunofluorescence (data not shown) and did not cross-react with the gene 10::pericentrin fusion protein on Western blots (see below).

Autoimmune sera for the initial λgt11 screen were obtained from three scleroderma patients (Tuffanelli et al., 1983), a monoclonal antibody to tubulin (DM1α) from Sigma, and human autoimmune serum with kinetochore reactivity from L. Wordeman (University of California, San Francisco).

mRNA Quantitation

Quantitative PCR amplification (see Chelly et al., 1988) was used to determine the amount of mRNA in different tissues. Equal amounts of total RNA isolated from different tissues were used to prepare cDNA. A 300 nt fragment (423–819) was amplified from the cDNA in the presence of radiolabeled dCTP and dATP (Amersham, Arlington Heights, Illinois). An aliquot of the reaction was taken at the end of cycles 18–25, and the PCR products were separated by SDS–PAGE and quantified (PhosphorImager, Molecular Dynamics, Sunnyvale, California).

Antibody Injections

Mouse oviductal oocytes were collected from mice (Sprague Dawley, Indianapolis, Indiana) as described (Calarco et al., 1983) and either placed in TE medium for use immediately or incubated in dibutyryl cAMP (100 μg/ml; Sigma Chemical Company, St. Louis, Missouri) to arrest spontaneous development temporarily. Oocytes were injected with 2 mg/ml antibody in PBS using a Brinkmann microinjection apparatus and transferred to fresh medium. Polar body formation and spindle morphology were assessed by immunofluourescence staining 20 hr after injection.

Xenopus eggs and embryos were injected with 25–50 nl of antibody solution (0.2–2 mg/ml) at the 2-cell stage as described (Amaya et al., 1991). For immunofluorescence studies, thinner needles were used to inject all cells of 4- to 8-cell embryos with appropriate (5–10 nl) volumes. Eggs and embryos were incubated for 2.5–3 hr at room temperature before processing.

Immunofluorescence

Cultured cells, isolated centrosomes, and chromosomes were processed for immunofluorescence as described previously (Mitchison and Kirschner, 1984; Vidair et al., 1993) using MeOH (−20°C) as the fixative. Affinity-purified anti-pericentrin antibody was used at 5 μg/ml and all other sera at 1:500.

Mouse oocytes and Xenopus embryos were processed for double-labeled immunofluorescence to detect pericentrin and tubulin as described (Simerly et al., 1990; Gard et al., 1990). To reduce background levels of fluorescence, Xenopus embryos were dissociated into single cells or small groups of cells by passage through a pipette tip after processing (>95% recovery). The native antigen was localized by permeabilizing live embryos (Xenopus and mouse) in the presence of antibody (50 μg/ml) before processing.

Immunoelectron Microscopy

Rat-1 cells were fixed in 3% formaldehyde in 100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$ (pH 6.8) for 30 min and permeabilized in 0.5% Triton X-100 in the above buffer for 5 min. Cells were washed in PBS with BSA (0.5 mg/ml) and Triton X-100, incubated in affinity-purified anti-pericentrin antibodies (20 μg/ml) for 4 hr, and processed for immunogold electron microscopy as described (Mitchison et al., 1986) using 5 nm gold-conjugated goat anti-rabbit IgG (Amersham, Arlington Heights, Illinois). Centrosomes isolated from Rat-1 cells or N115 cells as described (Mitchison and Kirschner, 1984) were spun onto coverslips and processed for immunogold electron microscopy as above. Thin sections (500–700 nm) of cells and centrosomes were cut on a Sorvall ultramicrotome, stained with uranyl acetate and lead citrate (Ted Pella Incorporated, Redding, California), and viewed in a JOEL electron microscope.

In Vitro Assay for Microtubule Aster Formation

Previously described methods for preparing extracts from Xenopus eggs (Murray and Kirschner, 1989), Xenopus sperm nuclei from testes (Gurdon, 1976), and rhodamine-labeled tubulin from bovine brain tubulin (Hyman et al., 1992) were used to examine aster formation. Iced Eppendorf tubes containing 8 μl of extract, 1 μl of nuclei (~5000), 1 μl of antibody (final concentration, 20–200 μg/ml) or buffer, and 1 μl of rhodamine-labeled tubulin (final concentration, ~1 mg/ml) were warmed to 22°C for various times and fixed as described (Murray and Kirschner, 1989). In some cases, the antibody was incubated with the extract alone or with nuclei alone for 30–45 min on ice prior to the addition of other components.

Microtubules emanating from the ends of nuclei were counted, photographed, or both. In controls, 15 min incubations yielded microtubules that were long enough to be clearly separated from one another. Microtubules from 45 to 67 asters were counted for each condition shown in Figure 8 in three separate experiments using anti-pericentrin antibodies that were affinity purified by two different methods (see above). Asters that had large bundles of microtubules, which constituted 5%–8% of the fractions, were not included in the quantitation.

Assay for Microtubule Nucleation

The effects of anti-pericentrin antibodies on microtubule nucleation from centrosomes isolated from CHO cells were tested by using the assay described by Mitchison and Kirschner (1984). Antibodies were added to the tubulin solution as for aster formation above. A total of 75–100 randomly chosen asters were counted for each condition. Bundled microtubules (5%–10%; see above) were not included in the quantitation.

Assay for Microtubule Elongation, DMSO-Induced Assembly of Microtubules, and Assembly of γ-Tubulin on Centrioles

Growth of microtubules from axonemes was as described in Gard and

Kirschner (1987). Assays for DMSO-induced assembly of microtubules and assembly of γ-tubulin were done as described in the accompanying paper (Stearns and Kirschner, 1994). Antibodies were used at 1/5 dilution in extracts as described above.

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GenBank Accession Number

The accession number for the nucleotide and amino acid sequence of pericentrin cDNA is U05823.