Kendrin/pericentrin-B, a centrosome protein with homology to pericentrin that complexes with PCM-1

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SUMMARY

The centrosome is responsible for nucleating microtubules and performing other cellular roles. To define the organization of the centrosome more completely, a human anti-centrosome serum was used to screen a human cDNA library, and a cDNA encoding a >350 kDa centrosome protein was identified. Sequence analyses revealed that this novel centrosome protein contains two coiled-coil domains bounded by non-coiled regions. The N-terminal region of the protein, named pericentrin-B, shares 61% identity (75% similarity) with pericentrin, suggesting an evolutionary relationship between these proteins. Antibodies against pericentrin-B stain centrosomes at all stages of the cell cycle, and pericentrin-B remains associated with centrosomes following microtubule depolymerization. Immunodepletion of neither pericentrin-B nor PCM-1 from cellular extracts inhibited the ability of salt-stripped centrosomes to recover microtubule nucleation potential, demonstrating that neither protein plays a key role in microtubule nucleation processes. Moreover, the binding of both PCM-1 and pericentrin-B with salt-stripped centrosomes required intact microtubules, demonstrating that the association of PCM-1 and pericentrin-B with centrosomes is a late event in the centrosome maturation process. Finally, pericentrin-B and PCM-1 coimmunoprecipitate, suggesting that PCM-1 and pericentrin-B form a functional complex in cells. This observation may help to explain the generation of anti-centrosome autoantibodies in certain autoimmune patients and may be important for centrosome function.

Key words: Centrosome, PCM-1, Pericentrin, Autoantigen, Autoantibody, Centromatrix

INTRODUCTION

The centrosome is the principal microtubule organizing center in mammalian cells (Balczon, 1996; Brinkley and Goepfert, 1998). Morphologically, the centrosome is composed of a pair of centrioles surrounded by an osmiophilic substance termed pericentriolar material (PCM). Functional studies have demonstrated that the microtubule nucleating capacity of the centrosome is localized to the PCM (Gould and Borisy, 1977), while recent data suggest that centrioles may have a role in organization of the PCM (Bobinnec et al., 1998; Schnackenberg and Palazzo, 1999).

In addition to nucleating microtubules, various other processes have been attributed to centrosomes. For example, the microtubule nucleating ability of the centrosome can fluctuate (Verde et al., 1990), centrosomes undergo replication during each cell cycle (Balczon et al., 1995; Hinchcliffe et al., 1999), centrosome function is required for the completion of interphase (Maniotis and Schliwa, 1991) and mitosis (Tugendreich et al., 1995), and aberrant centrosome activity has been associated with tumor progression (Boveri, 1914; Fukasawa et al., 1996; Lingle et al., 1998; Zhou et al., 1998; Brinkley and Goepfert, 1998). Collectively, these observations demonstrate the complexity of the organelle.

The molecular composition of the centrosome has only recently begun to be defined (Balczon, 1996; Brinkley and Goepfert, 1998). Experimental strategies including biochemical purification, mutant analyses and hybridoma production have resulted in the identification of several centrosome proteins, including γ tubulin, a PCM component whose activity is critical for microtubule nucleation (Oakley and Oakley, 1989; Joshi et al., 1992; Joshi and Baas, 1993). However, the majority of other centrosome proteins that have been identified have not been characterized in detail.

An additional strategy for identifying centrosome proteins is to use centrosome autoantisera as probes for defining centrosome components (Connolly and Kalnins; 1978; Balczon et al., 1994; Doxsey et al., 1994; Mack et al., 1998). The best characterized centrosome autoantigen is NuMA, a protein whose activity is critical for the organization of mitotic and meiotic spindle poles (Compton, 1998). Other centrosome autoantigens include pericentrin (Doxsey et al., 1994), PCM-1 (Balczon et al., 1994), and Cep 250 (Mack et al., 1998). No definitive function has been determined for any of these other PCM autoantigens, and present studies are aimed at determining the roles of the centrosome autoantigens in microtubule nucleation, PCM organization and cell cycle regulation.

The pathological processes that result in the production of anti-centrosome autoantibodies in certain autoimmune patients

have not been elucidated. Based on observations that autoimmune patients often produce antibodies against several protein components of an organelle and reports that autoantibodies generally inhibit the function of a target autoantigen, Tan (Tan, 1989) proposed that autoantigens are multiprotein aggregates, and autoantibodies are generated against either functionally important or catalytic centers of the autoantigenic complex. If true, this would explain the reactivity of autoantisera towards several autoantigens and the capacity of autoantisera to inhibit cellular processes. Concerning the centrosome, the hypothesis put forth by Tan (Tan, 1989) suggests that the PCM autoantigens may form a functional complex in cells, and the reported studies tested that possibility.

To define the centrosome more completely, and to establish relationships between PCM autoantigens, an expression library was screened with anti-centrosome autoantiserum. A cDNA encoding a centrosome protein that shares considerable homology to pericentrin, termed kendrin/pericentrin-B, was discovered. Characterization of this new centrosome protein revealed an interaction between PCM-1 and pericentrin-B. A preliminary description of this protein, based on our deposited sequence, has been reported by others (Flory et al., 2000).

MATERIALS AND METHODS

Library screening and cDNA cloning and sequencing

A human fetal liver cDNA expression library (Clontech Laboratories, Palo Alto, CA, USA) was screened with a previously characterized human anti-centrosome serum (Osborn et al., 1982; Balczon and West, 1991) using published procedures (Balczon et al., 1994). A phage clone, termed 13K1, was obtained that encoded a portion of the kendrin/pericentrin-B cDNA. To obtain the full-length cDNA, a HeLa cell ZAP cDNA expression library (a generous gift of Dr D.W. Cleveland, University of California at San Diego, USA) and H1262 cDNA library (generously provided by Drs M.-L. Chu, Thomas Jefferson University, Philadelphia, PA, USA and W.-H. Lee, The University of Texas Health Science Center, San Antonio, TX, USA) were screened with the 13K1 cDNA. Screens were performed using standard procedures (Balczon et al., 1994; Meng et al., 1997). Positive phage were identified and purified to homogeneity.

DNA sequencing was performed using previously published methods (Balczon et al., 1994). DNA sequence was determined using the Prism Kit (Perkin Elmer), and DNA sequences were aligned and analyzed using programs available through the Wisconsin GCG package.

5′ RACE

5′ RACE System for Rapid Amplification of cDNA Ends (Gibco-BRL, Gaithersburg, MD, USA) was used to obtain a portion of the cDNA sequence that could not be obtained using standard plaque hybridization techniques. The sequence-specific primer 5′ GCT TCC CGC TGT AGC TCC TCC 3′ was used to convert HeLa mRNA for pericentrin-B into first strand cDNA. The cDNA then was amplified using an anchor primer (5′ CUA CUA CUA CUA GGA CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG 3′, Gibco-BRL) and the nested gene specific primer 5′ CTT TGG AAT TCC TGT TTC ATC 3′. The longest PCR product was subcloned into TA cloning vector pCR II (Invitrogen, Carlsbad, CA, USA). The 5′ RACE product sequence was determined as described earlier.

Expression and purification of recombinant proteins

A fragment of the 13K1 cDNA (nucleotides 5905-6732) was subcloned into pGEX-3X vector (Pharmacia, Piscataway, NJ, USA). Fusion protein was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) (to a final concentration of 0.1 mM) to a liquid culture of DH5 cells containing the pGEX construct. Following a 2 hour incubation at 37°C with shaking, the cells were pelleted and then resuspended in 20 ml of 50 mM Tris-HCl, pH 7.5, supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, PMSF; 10 μg/ml Nα-p-tosyl-L-arginine methyl ester, TAME; 5 μg/ml pepstatin A; 6 μg/ml leupeptin). The cells were lysed by passing three times through a French press at a setting of 3,000-4,000 psi, and then an additional portion of protease inhibitors and 1 ml 20% Triton X-100 were added. The lysate was clarified by centrifugation at 15,000 rpm in an SS34 rotor for 15 minutes at 4°C, the supernatant was retained, and the fusion protein was purified by addition of glutathione-agarose beads (Sigma, St Louis, MO, USA) that were pre-equilibrated with 50 mM Tris-HCl, pH 7.5 (binding buffer). Following a 30 minute incubation at 4°C, the beads were washed extensively with binding buffer and the bound fusion protein was eluted by the addition of 10 mM glutathione in binding buffer. The eluted protein was collected and assayed for purity by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined using the Bio-Rad Protein Assay (Burlingame, CA, USA).

Antibody production and purification

Immunization of rabbits was performed by Rockland Immunochemicals (Gilbertsville, PA, USA). Primary injection was performed using 500 μg of GST-pericentrin-B fusion protein emulsified in complete Freund's adjuvant. Subsequent booster immunizations were performed on days 7, 14 and 21 using 250 μg of fusion protein emulsified in Freund's incomplete adjuvant. A test bleed demonstrated anti-centrosome antibodies on day 39. Pre-immune serum was also collected and used for some control studies.

For affinity column preparation, either GST or GST-fusion protein was suspended in 0.1 mM Mops, pH 7.0, and then incubated with water-washed Affi-Gel 15 at room temperature for 60 minutes. The gel matrices were quenched by incubating for 60 minutes at room temperature with 0.1 gel volume of 1 M ethanolamine-HCl, pH 8.0. The resulting affinity columns were washed sequentially with 10 volumes of 10 mM Tris-HCl, pH 7.5, 10 volumes of 50 mM glycine, pH 2.5, and 10 volumes of phosphate-buffered saline (PBS). The crude serum was diluted 1:10 in PBS and then passed through the GST column to deplete GST antibodies. The flow-through was applied to the GST-pericentrin-B fusion protein column, the column washed with 20 volumes of PBS followed by 20 volumes of PBS containing 0.5 M NaCl, and the bound antibody eluted using 10 volumes of 50 mM glycine, pH 2.5. The eluted antibodies were neutralized by the addition of one volume of 1 M Tris-HCl, pH 8.0, and the antibody was dialyzed against PBS and concentrated for long term storage.

For some studies, anti-pericentrin-B antibodies were purified directly from the human anti-centrosome serum. For these experiments, *E. coli* strain Y1090 cells were infected with 13 Kl phage, plated, and then incubated at 42°C until plaques appeared. The plates were overlain with a nitrocellulose filter presoaked in 10 mM IPTG, and the plate was incubated at 37°C for 2-3 hours. The filter was placed in 3% powdered milk in PBS for 2 hours at room temperature, and then incubated overnight at 4°C in a 1:1000 dilution of autoantiserum in the milk solution. The following morning, the filter was rinsed six times in Tris-buffered saline (TBS) (5 minutes each), then three times in TBS containing 0.5 M NaCl (2 minutes each), and the bound antibodies were eluted by addition of 0.5 ml 50 mM glycine, pH 2.5, for 2 minutes. The eluted antibodies were neutralized immediately by addition of 50 μl 0.5 M Na_2HPO_4, and then desalted into PBS and concentrated using a Centricon 30 tube (Amicon, Beverly, MA, USA). The affinity-purified human anti-pericentrin-B antibodies were used immediately for immunofluorescence labeling of cells.

Cell culture

HeLa and CHO cells were maintained using previously reported procedures (Balczon et al., 1994; Balczon et al., 1995). To induce microtubule depolymerization, cells either were treated with 5 μg/ml colcemid or were maintained at 4°C for 30 minutes. To stabilize microtubules, cells were incubated with 10 μg/ml taxol for 90-120 minutes.

For some studies, PCM protein overproduction was induced by culturing CHO cells in 2 mM hydroxyurea (HU) and 5 μg/ml nocodazole for 40 hours (Balczon et al., 1999).

Immunoblot analysis

SDS-PAGE and immunoblotting were performed using previously reported procedures (Balczon et al., 1994). Blots were developed using chemiluminescence procedures (Bao et al., 1998).

Immunofluorescence microscopy

Cultured cells were processed for immunofluorescence microscopy using standard procedures (Balczon and West, 1991; Balczon et al., 1994). Purified anti-pericentrin-B rabbit antibody was used at a 1:200 dilution and anti-tubulin antibody (Sigma Chemical Co.) was used at a 1:100 dilution. Affinity-purified human anti-pericentrin-B autoantibody was used undiluted, the complete anti-centrosome autoantiserum was used at a 1:250 dilution, anti-γ tubulin (BAbCO, Richmond, CA, USA) was used at 1:100, and anti-pericentrin (BAbCO) was used at 1:100. Fluorochrome-labeled secondary antibodies were purchased commercially (Boehringer-Mannheim, Inc., Indianapolis, IN, USA).

Isolation of centrosomes, preparation of salt-stripped centrosomes, and reconstitution of microtubule nucleation capacity

Collection of oocytes from *Spisula solidissima* females, oocyte activation and centrosome isolation were performed using previously published procedures (Palazzo et al., 1988; Vogel et al., 1997; Palazzo and Vogel, 1999). Centrosome scaffolds (centromatrices) were produced by treatment of isolated centrosomes with KI (final concentration of 1 M) for 15 minutes, and the KI-treated centrosome remnants (KICRs) were centrifuged onto coverslips as described previously (Schnackenberg et al., 1998). The microtubule nucleating capacity of KICRs was reconstituted by incubating KICRs with an extract prepared using a slight modification of the previously outlined methods. Briefly, CHO cells that were arrested for 40 hours with HU and nocodazole were collected and then rinsed extensively with ice-cold 20 mM Pipes buffer, pH 7.2, 100 mM KCl, and 5 mM $MgSO_4$ plus protease inhibitors (Palazzo et al., 1988). The supernatant was removed completely, and the cells were lysed by repeated passage through a 27-gauge needle. The lysate was centrifuged at 4°C at 14,000 ***g*** for 30 minutes, the supernatant was collected, an equal volume of 40% sucrose in lysis buffer was added, and the extracts either were used immediately or were frozen in portions at −80°C. For some studies, randomly cycling CHO cells were used for extract production. Incubation of KICRs with extracts was performed as detailed previously (Schnackenberg et al., 1998), the reconstituted remnants were rinsed, and then incubated with 0.3 mg/ml sea urchin tubulin to induce microtubule assembly. Fixation and processing for immunofluorescence microscopy were performed as detailed previously (Schnackenberg et al., 1998). For some studies, extracts were immunodepleted of either γ tubulin, PCM-1 or pericentrin-B prior to addition to KICRs. For immunodepletions, protein A-agarose beads were first incubated with either anti-γ tubulin, anti-PCM-1 or anti-pericentrin-B for 60 minutes at room temperature, rinsed with lysis buffer, and then added to extracts for 90 minutes on ice. The beads were removed by centrifugation, the depletion step was repeated three times for 30 minutes each, and the immunodepleted extracts were added to KICRs. In addition, a portion of each depleted extract was saved and assayed by immunoblot analysis to verify that each protein had been immunodepleted completely. The amount of protein remaining in the extracts was quantified using a Zieneh laser densitometer (Balczon et al., 1995).

Density gradient centrifugation

Extracts from HU/nocodazole-treated CHO cells were overlain on a 5%-40% sucrose gradient prepared in 80 mM Pipes, pH 6.8, 1 mM $MgCl_2$, and 1 mM EGTA, and the preparation was centrifuged at 100,000 ***g*** for 16 hours at 4°C. Fractions (0.75 ml) were collected from the bottom of the tube, and portions of each fraction were analyzed for centrosome proteins by immunoblot analysis (Balczon et al., 1994).

Immunoprecipitation

CHO cells were cultured in either the presence or absence of ^{35}S-methionine in medium containing HU and nocodazole for 40 hours (Balczon et al., 1999). Either anti-PCM-1 or anti-pericentrin-B was added to the extract for 2 hours at 4°C, and immune complexes were precipitated by addition of Protein A agarose beads (Pierce Chemical Co., Rockford, IL, USA). The Protein A beads were collected by brief centrifugation, rinsed extensively, suspended in sample buffer, the bound immunocomplexes resolved by SDS-PAGE, and then the resolved proteins were either visualized by autoradiography or transferred to nitrocellulose. The presence of coimmunoprecipitating PCM-1, pericentrin-B and γ tubulin was then assayed by immunoblotting.

RESULTS

Isolation of cDNA clones encoding the centrosome autoantigen pericentrin-B

A human fetal liver cDNA expression library was screened with a human anti-centrosome serum and a positive clone, 13K1, was obtained. To verify that 13K1 encoded portion of a centrosome protein, antibody specific for the protein encoded by 13K1 was affinity-purified from the human serum using fusion protein expressed by the 13K1 phage. The resulting monospecific anti-13K1 antibody stained centrosomes in both HeLa and CHO cells (Fig. 1). Subsequently, rabbits were immunized to generate anti-13K1 antibodies. The rabbit anti-13K1 antibodies reacted with centrosomes when cultured mammalian cells were processed for immunofluorescence microscopy (Fig. 1), and the antibodies recognized a protein of >350 kDa when HeLa cell proteins were probed by immunoblotting (Fig. 2).

Human cDNA libraries were rescreened repetitively using either the 13K1 cDNA or additional partial cDNAs obtained during these subsequent screenings until virtually the entire cDNA encoding the centrosome autoantigen was obtained (Fig. 3). Sequence analysis (see below) determined that a portion of the 5′ region of the cDNA remained to be identified, and repeated efforts to obtain this cDNA region by plaque hybridization using three different human cDNA libraries failed. Therefore, 5′ RACE was performed using HeLa cell mRNA to obtain the region of cDNA (nt 1846-2292) that was lacking. Further sequence analysis determined that the RACE product still lacked the initiation methionine, and the RACE cDNA product was subsequently used to screen human cDNA library H1262. The 5′ region of cDNA containing the initiation methionine, as well as a short stretch of 5′ untranslated region (UTR), was obtained (Fig. 3).

Sequence analysis of pericentrin-B

Sequence analysis of the cDNA encoding the high molecular mass centrosome protein revealed an open reading frame of 3,321 amino acids (complete sequence available on GenBank accession number U52962). The cDNA contained 52 bp of 5′ untranslated sequence and contained two concensus polyadenylation sequences, beginning at nucleotides 10,029 and 10,413, respectively. Secondary structure predictions identified one large coiled-coil domain (approx. 1,500 amino acids) at the N terminus and a smaller coiled-coil domain (approx. 500 amino acids) at the C terminus. Both regions were flanked by non-coiled ends. From the deduced amino acid sequence, the molecular mass of the centrosome autoantigen was determined to be >350 kDa.

Initially, we named this protein kendrin. However, database searches established a strong homology between the 350 kDa centrosome autoantigen and pericentrin (Fig. 4), a previously defined centrosome autoantigen (Doxsey et al., 1994). Based on the homology between these two proteins and the presumed familial relationship, the preferred name for the new centrosome autoantigen is now pericentrin-B. Pairwise alignment revealed that the N-terminal two thirds of pericentrin-B shared 61% identity (75% similarity) with mouse pericentrin, while the C-terminal one-third of pericentrin-B is unique (Fig. 4). Noticeable gaps of varying lengths were apparent when comparing the sequences of pericentrin and pericentrin-B. Together, these results suggest that pericentrin and kendrin/pericentrin-B are members of a newly defined superfamily of PCM proteins.

Pericentrin-B is an integral component of the PCM

Affinity-purified rabbit anti-pericentrin-B recognized the centrosome throughout the cell cycle (Fig. 5). Furthermore, the cellular localization of pericentrin-B was examined after depolymerization of microtubules by incubation of cells at either a cold temperature or with colcemid. As shown in Fig. 6, pericentrin-B remained associated with the centrosome in the absence of microtubules demonstrating that pericentrin-B is an integral PCM component.

The distribution of pericentrin-B was also investigated in cells following incubation with taxol. As demonstrated in Fig. 6G, pericentrin-B remained associated with the centrosome following taxol treatment, even though cytoplasmic microtubules and microtubule asters were observed in interphase and mitotic taxol-treated cells, respectively. These data indicate that kendrin/pericentrin-B is not associated with non-centrosomal microtubules.

Analysis of pericentrin-B and PCM-1 function using reconstituted salt-extracted centrosomes

Schnackenberg et al. (Schnackenberg et al., 1998) demonstrated that the microtubule nucleation capacity of centrosomes isolated from *Spisula solidissima* oocytes could be depleted by extracting the centrosomes with KI, and that functional centrosomes could be reconstituted by incubation of the KI-insoluble centrosome remnants (KICRs) with oocyte cytoplasmic extract. This system was used to investigate whether pericentrin-B and a previously identified centrosome autoantigen, termed PCM-1 (Balczon et al., 1994), are essential for centrosome-dependent microtubule nucleation. For these studies, extracts were prepared from either randomly cycling CHO cells or from CHO cells that were arrested for 40 hours with HU in the presence of nocodazole, and then the CHO extracts were added to KICRs. As shown in Fig. 7, CHO cytosolic extracts reconstituted only slight microtubule nucleating capacity of KICRs, whereas extracts prepared from

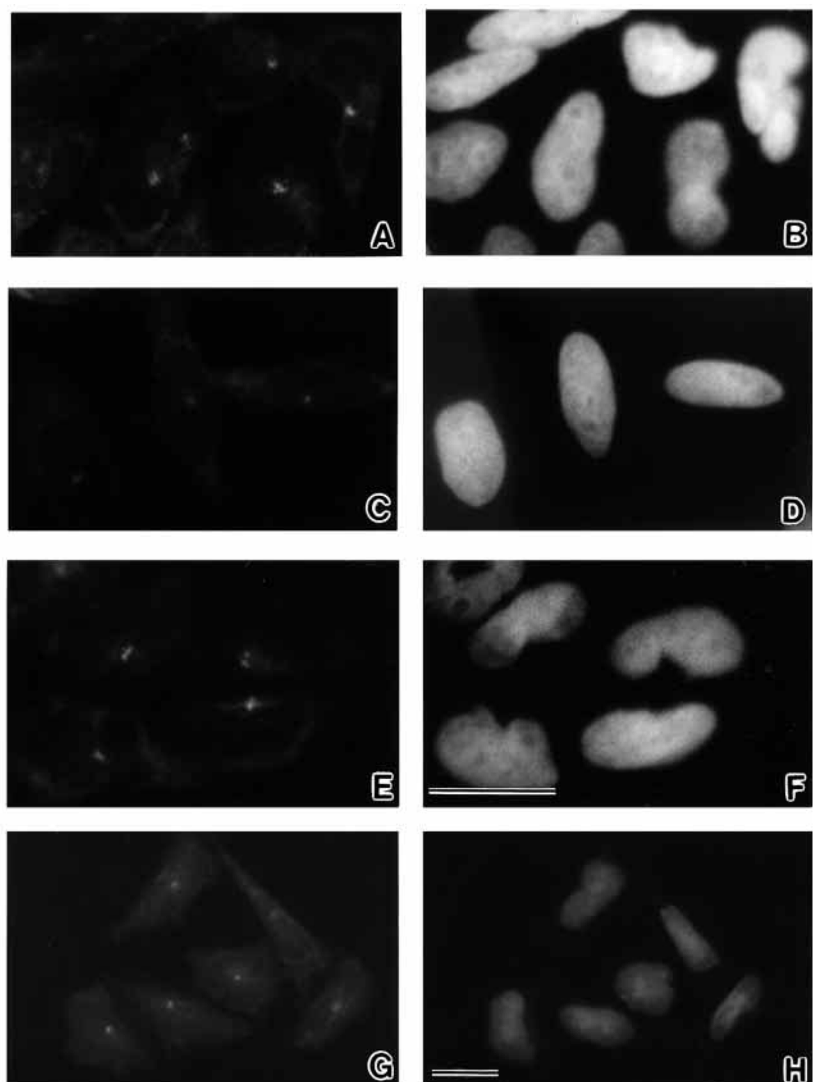


Fig. 1. Antibodies raised against the 13K1 fusion protein recognize centrosomes. HeLa cells (A-F) and CHO cells (G,H) were labeled with either human autoimmune anti-centrosome serum (A), antibodies purified from the human serum using fusion protein generated using the 13K1 cDNA (C), or antibodies generated by immunization of rabbits using a fusion protein produced by coupling a 13K1 polypeptide fragment to GST (E,G). The corresponding DAPI fields are shown in B, D, F, and H. Bars, 10 μm.

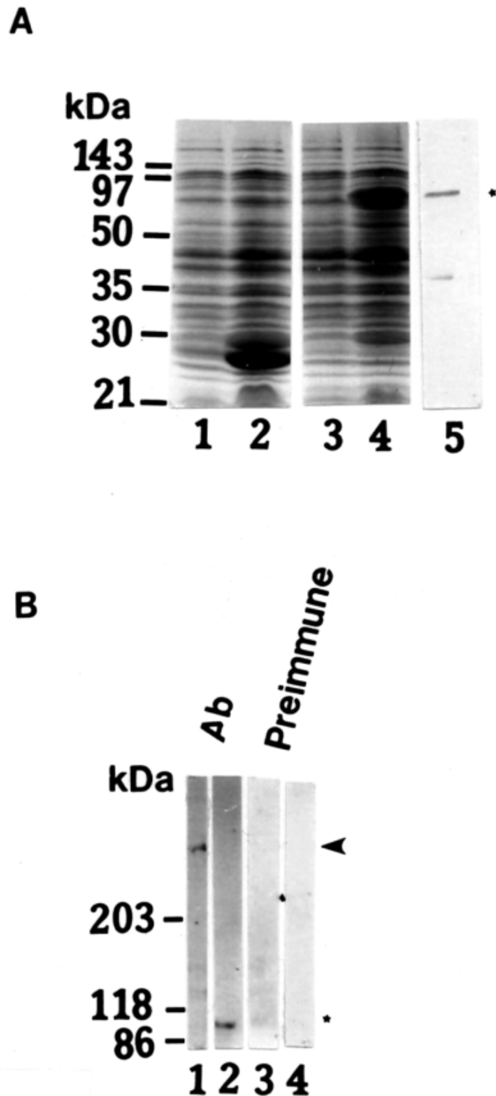
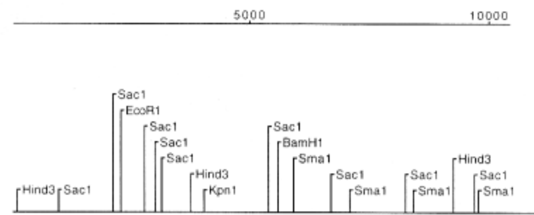


Fig. 2. Affinity-purified anti-13K1 antibody recognizes a protein of >350 kDa in Hela cells. (A) Purification of GST-13K1 fusion protein. A Coomassie Blue-stained gel showing bacterial extracts from cells carrying either the plasmid vector without insert (lanes 1 and 2) or the plasmid with a cDNA fragment obtained from the 13K1 cDNA (lanes 3 and 4). Uninduced (lanes 1 and 3) and IPTG-induced cells (lanes 2 and 4) are shown. The purified GST-13K1 fusion protein (lane 5) is also shown. The fusion protein shows a slower migration on SDS gels than expected for the predicted size of the fusion protein (predicted approx. 60 kDa). Two additional fusion proteins that were generated using this protein fragment also exhibited anomalous migration on SDS gels (not shown). (B) Immunoblot analysis of HeLa cells following extraction with 0.1% Triton X-100 (lanes 1 and 3) and purified GST-13K1 fusion protein (lanes 2 and 4) using either affinity-purified anti-pericentrin-B antibodies (lanes 1 and 2) or pre-immune serum (lanes 3 and 4). The reactive high molecular mass protein is shown (arrowhead). The positions of marker proteins (kDa) are shown.

A. Restriction Pattern of the Pericentrin-B cDNA



B. Molecular Cloning of the Pericentrin-B cDNA

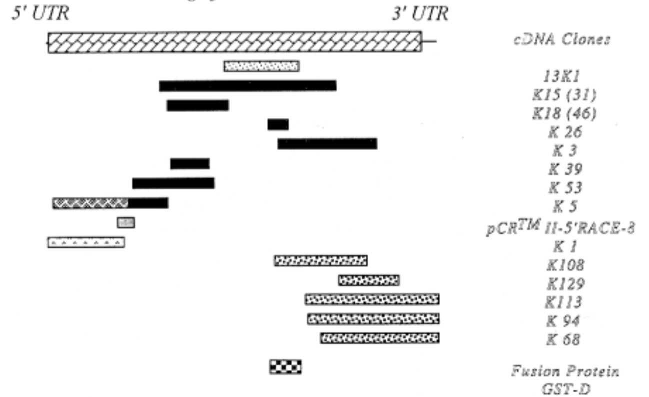


Fig. 3. Molecular cloning of kendrin/pericentrin-B. (A) Restriction map of the pericentrin-B cDNA. (B) The various cDNA clones that were obtained from either library screening or 5′ RACE are shown aligned with the full-length pericentrin-B cDNA. The 5′UTR, coding region and 3′UTR are shown.

HU-arrested CHO cells allowed the formation of robust microtubule asters from the KICRs (Fig. 7). Subsequently, extracts prepared from HU-arrested CHO cells were first immunodepleted of either γ tubulin, pericentrin-B or PCM-1, and then added to KICRs. Immunodepletion of γ tubulin abolished the ability of CHO cell extracts to reconstitute the microtubule nucleating potential of KICRs, while immunodepletion of PCM-1 and pericentrin-B had no effect (Fig. 8). To quantify the results, the number of asters in microscope fields were counted directly, and it was determined that preparations of untreated centrosomes contained on average 4.4±1.6 asters per field, while KI-treated fields contained none. KICRs that were incubated with either control extract, γ tubulin-depleted extract, PCM-1-depleted extract, or pericentrin-B-immunodepleted extract contained on average 4.5±1.1, 0.1±0.3, 4.3±1.2 or 4.3±1.6 asters per field. As a control for the immunodepletions, immunoblots of depleted extracts demonstrated that antibodies directed against either PCM-1 or pericentrin-B successfully immunodepleted over 95% of the respective proteins from the extracts (Fig. 8).

Experiments then were performed to determine whether PCM-1 and pericentrin-B associate with KICRs before or after microtubule assembly occurs. For these studies, incubation of KICRs with CHO extract was performed either in the presence or absence of nocodazole. As shown in Fig. 9, neither PCM-1 nor pericentrin-B could be detected on centrosomes when reconstitution was performed in the presence of nocodazole, while γ tubulin binding to KICRs occurred. In contrast, when centrosome reconstitution was performed in the absence of nocodazole, pericentrin-B and PCM-1 could be detected in the PCM of the reconstituted centrosomes. Moreover, the asters that formed from centrosomes that were reconstituted in the absence of nocodazole appeared more robust and contained more microtubules than asters nucleated by centrosomes that were allowed to reform in the absence of nocodazole.

B

0500 1000 1500 2000 2500 3000 3500

Fig. 4. (A) Sequence alignment between pericentrin (bottom) and pericentrin-B (top). Identical amino acids are indicated by a vertical line (|), similar amino acids are indicated by a colon (:) and less similar amino acids are indicated by a single dot (.). Dissimilar amino acids have no indicating mark between them. (B) A diagram showing the conservation between pericentrin (bottom) and kendrin/pericentrin-B (top). Conserved regions are shown by the overlapping rectangles (unshaded and hatched rectangles are used to reinforce that these regions are not identical). Gaps in pericentrin are indicated by the thin lines (gaps less than 10 amino acids are not shown), while unique areas in kendrin/pericentrin-B are indicated by the black rectangles. The unique C-terminal domain of pericentrin-B is indicated by the cross-hatched rectangle.

Pericentrin-B and PCM-1 comigrate during density gradient centrifugation

Extracts prepared from CHO cells arrested in the presence of HU and nocodazole for 40 hours (Balczon et al., 1999) were subjected to density gradient centrifugation. As shown in Fig. 10, immunoblot analysis of samples from each of the gradient fractions using antibodies against PCM-1, pericentrin-B, and γ tubulin determined that both PCM-1 and pericentrin-B separated into two peaks on the density gradients (Fig. 10A), one peak near the 25%-30% sucrose range (fractions 3-5) and another near the top of the gradient (fractions 8-10). In contrast, γ tubulin levels peaked in fractions 2-6 under the conditions used for fractionation.

The centrosome autoantigens copellet from cytoplasmic extracts

The comigration of pericentrin-B and PCM-1 during density gradient centrifugation suggested that the two proteins might be complexed. To test this possibility, two separate assays were performed. Initially, anti-PCM-1 immunoprecipitations were performed using extracts prepared from ^{35}S-labeled HU-arrested CHO cells. Autoradiograms of the precipitated materials determined that proteins were precipitated whose sizes corresponded to PCM-1, pericentrin-B and several additional polypeptides (Fig. 10B). Subsequently, PCM-1 and pericentrin-B were immunoprecipitated from CHO extracts, and the presence of coimmunoprecipitating proteins was assayed by immunoblot analysis using antibodies against known centrosome proteins. As shown in Fig. 10C, pericentrin-B and PCM-1 copellet from cellular extracts, while γ-tubulin was not detected in the complexes.

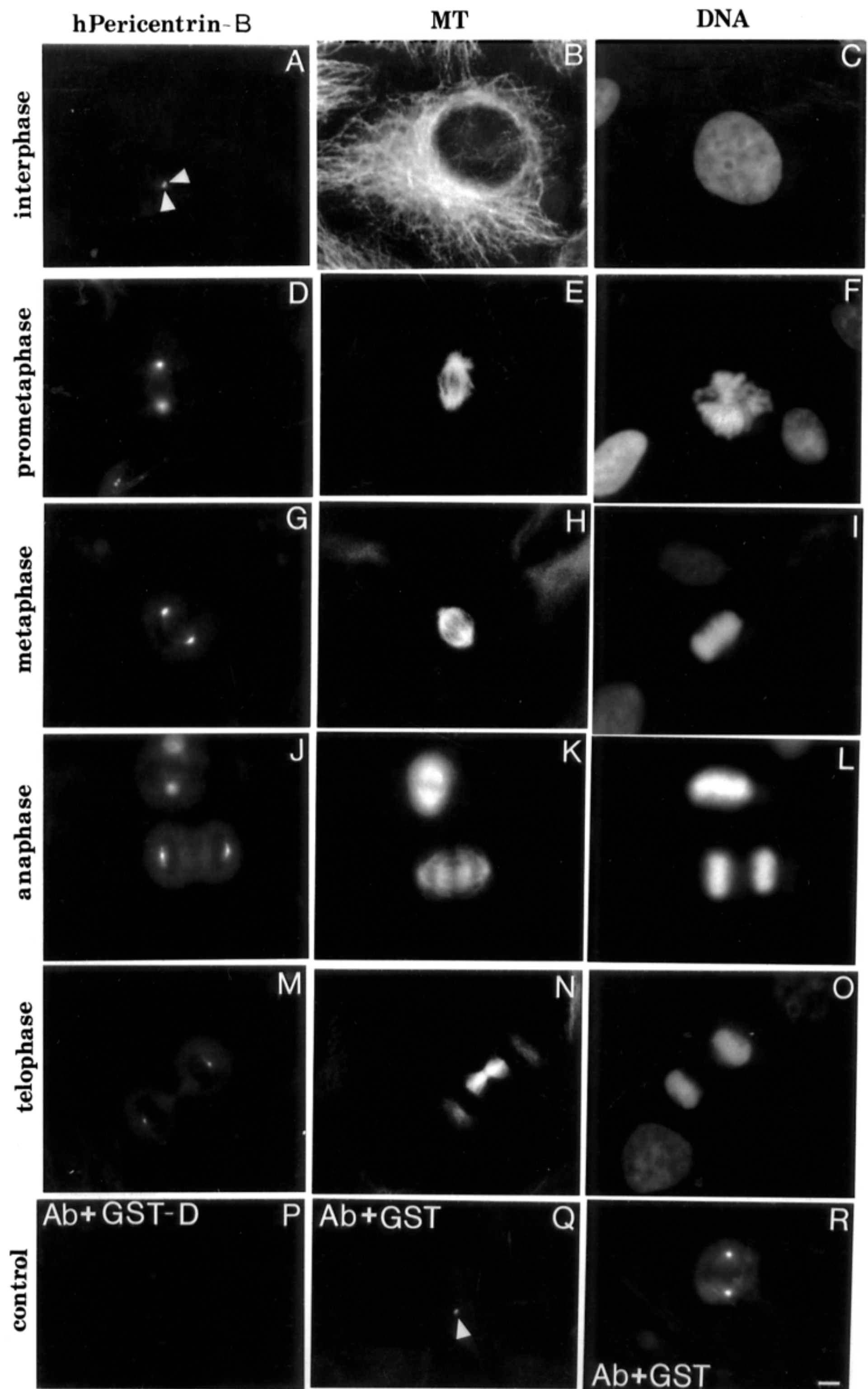


Fig. 5. Immunofluorescence localization of pericentrin-B in HeLa cells. HeLa cells were stained with affinity-purified anti-pericentrin-B (A,D,G,J, M), anti-γ-tubulin antibody (B,E,H,K,N) and DAPI (C,F,I,L,O), respectively. Cells are shown in interphase (A-C), prometaphase (D-F), metaphase (G-I), anaphase (J-L) and telophase (M-O). The centrosomes are indicated by arrowheads. (P-R) Immunofluorescence staining following preincubation of the antibody with either GST-13K1 (P) or GST alone (Q, interphase; R, mitosis). Bar, 4 μm.

DISCUSSION

The centrosome is responsible for nucleating microtubules, organizing the spindle, and regulating cell cycle progression (Balczon, 1996), and recent studies suggest that aberrant centrosome replication may be linked to tumor progression (Brinkley and Geopfert, 1998). To define this intriguing

organelle, sera obtained from human autoimmune patients are being used to identify and characterize components of the centrosome. A high titer human serum has been used to identify a small family of centrosome proteins, including PCM-1 (Balczon and West, 1991; Balczon et al., 1994). To characterize the centrosome autoantigens more completely, a human cDNA library was screened with the anti-centrosome autoantiserum. As demonstrated, a novel centrosome autoantigen, pericentrin-B, was identified, and its relationship to PCM-1 was investigated.

Pericentrin-B and pericentrin may define a family of centrosome proteins

Kendrin/pericentrin-B is a large (>350 kDa) centrosome protein that, like PCM-1 (Balczon et al., 1999), is an integral component of the centrosome complex (Fig. 6). Although we initially termed this protein kendrin, sequence analysis identified a high degree of homology between the centrosome protein described in this report and a previously defined centrosome autoantigen, pericentrin (Doxsey et al., 1994). Therefore, the name pericentrin-B might be more appropriate for this protein because it builds upon the apparent familial relationships between these two proteins. As demonstrated by sequence comparisons (Fig. 4), these two proteins share considerable identity (61%) and similarity (75%) in their amino-terminal regions, suggesting an evolutionary relationship. However, significant differences were identified between these two proteins. For example, pericentrin contains a single large coiled-coil domain flanked by non-coiled ends, whereas pericentrin-B contains two coil-coil domains flanked by non-coiled ends. In addition, pericentrin-B has a unique C terminus region (approx. 1000 amino acids), suggesting the presence of an additional functional domain(s) unique to pericentrin-B. In support of this possibility, a calmodulin-binding domain has recently been localized to C-terminal region of kendrin/pericentrin-B (Flory et al., 2000).

The function of kendrin/pericentrin-B is not known at present. However, predictions concerning its potential function

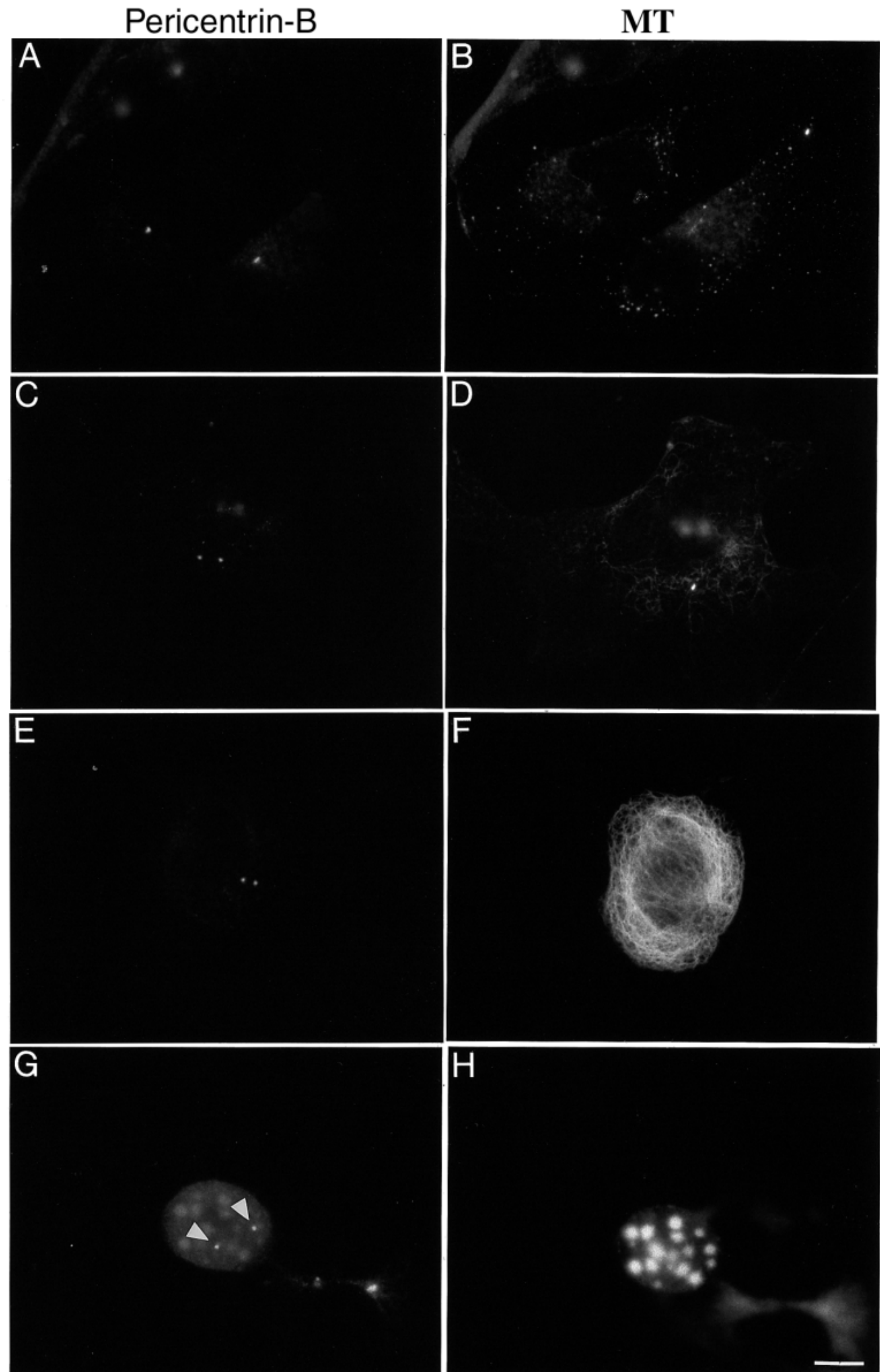


Fig. 6. Immunolocalization of pericentrin-B in either drug- or cold-treated HeLa cells. HeLa cells were treated with either cold (A,B), colcemid (C,D) or taxol (E-H) prior to double-labelling using anti-pericentrin-B (A,C,E,G) and anti-tubulin antibodies (B,D,F,H). Images of both interphase (E,F) and mitotic taxol-treated (G,H) cells are shown. Bar, 5 µm. Arrowheads in G indicate pericentrin-B.

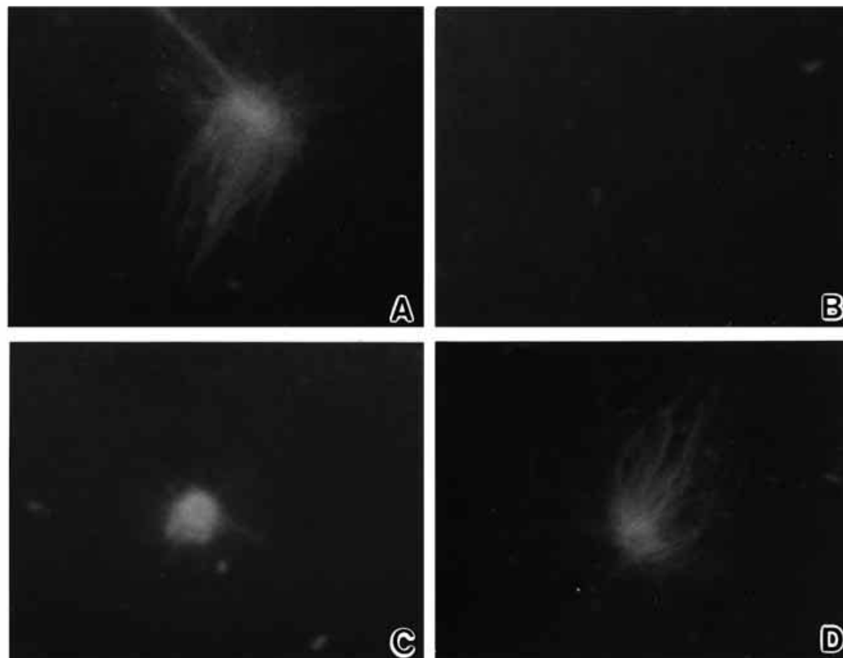


Fig. 7. CHO extracts can reconstitute the microtubule-nucleating potential of isolated *Spisula* centrosomes. An untreated (A) and KI-extracted (B) *Spisula* centrosome following incubation with sea urchin tubulin. KI treatment of centrosomes abolishes microtubule nucleation potential. KICRs were then incubated with CHO extract prepared from either randomly cycling CHO cells (C) or HU-arrested CHO cells (D). Tubulin was then added, the preparations fixed, and then processed for anti-tubulin immunofluorescence microscopy.

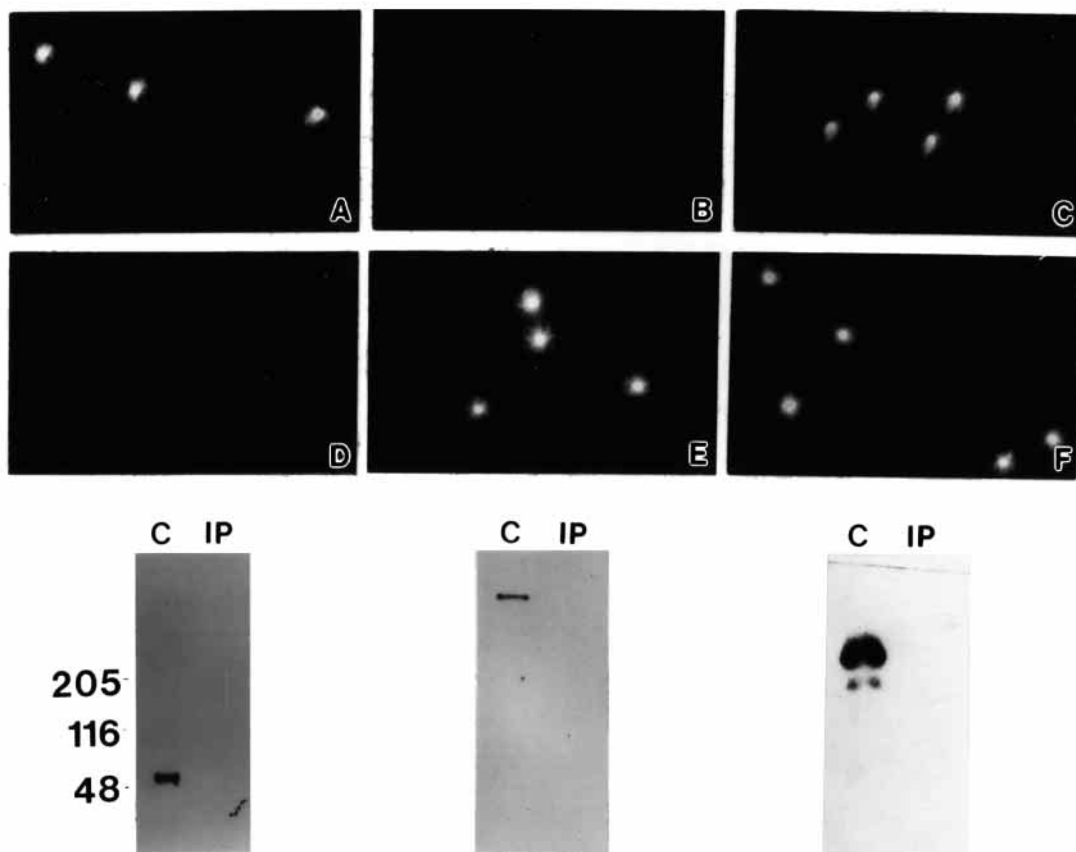


Fig. 8. Pericentrin-B and PCM-1 are not required for microtubule assembly from isolated centrosomes. *Spisula* centrosomes were extracted with KI (B-F) and then incubated with either buffer alone (B), complete CHO extract (C), or extract immunodepleted using either anti-γ tubulin (D), anti-pericentrin-B (E) or anti-PCM-1 (F). Sea urchin tubulin was added, the preparations fixed, and then processed for immunofluorescence microscopy using anti-tubulin. (A) The microtubule nucleating potential of *Spisula* centrosomes prior to KI treatment. To verify that the immunoprecipitation procedure effectively depleted γ tubulin, pericentrin-B and PCM-1, respectively, immunoblot analyses of untreated control (C) and the appropriate immunodepleted (IP) extracts were performed using either anti-γ tubulin, anti-pericentrin-B or anti-PCM-1. The respective blots are shown directly beneath the γ tubulin, pericentrin-B and PCM-1 panels. The positions of molecular mass markers (kDa) are shown.

can be made based on what is known about pericentrin. Specifically, it has been proposed that pericentrin forms a portion of the centrosome scaffold to which other proteins, such as microtubule nucleating complexes, associate (Dictenberg et al., 1998). Whether pericentrin-B might also be involved in establishing this centrosome scaffold through an interaction with pericentrin remains to be determined, although our inability to identify a role for pericentrin-B in microtubule nucleation processes using an in vitro assay (Fig. 8) argues against a role for pericentrin-B in the attachment of microtubule nucleating complexes to the PCM. However, if the proposal that the coiled-coil protein pericentrin serves as a binding site for other proteins during the assembly of the PCM is correct (Dictenberg et al., 1998), then a reasonable extension would be that its homolog, kendrin/pericentrin-B, would serve a similar function within the PCM and might be involved in coupling other centrosome components to the PCM.

Pericentrin-B and PCM-1 are not required for microtubule nucleation

An in vitro system was established that allows rapid screening of whether a PCM protein has a role in microtubule nucleation processes. This system involves incubating salt-extracted centromatrix scaffolds with extracts prepared from CHO cells. Importantly, the proteins that are required for reconstituting the microtubule nucleating potential of the KICRs are apparently conserved, as CHO proteins are capable of binding to *Spisula* KICRs, resulting in the recovery of microtubule nucleation potential (Fig. 7). This assay system was used to demonstrate that pericentrin-B and PCM-1 activities are not essential for microtubule nucleation processes. This result is in agreement with previous studies demonstrating that incubation of extracted cells with autoimmune centrosome serum failed to block microtubule nucleation from centrosomes (Balczon and West, 1991). Although these previous results suggested that the PCM autoantigens are not essential for microtubule nucleation, other interpretations are plausible. For example, important functional epitopes may not be available to the autoantibodies once the PCM assembles.

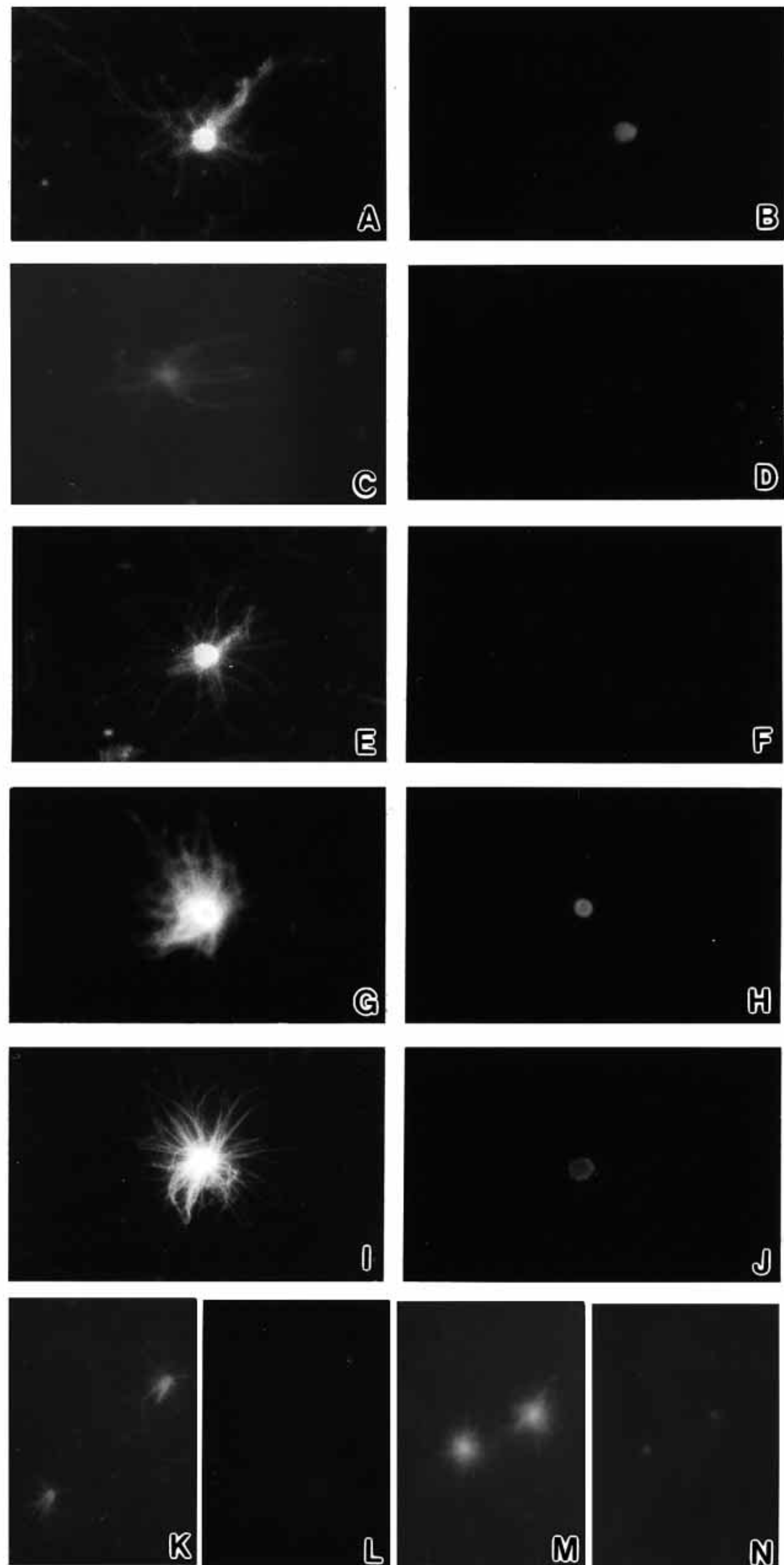


Fig. 9. Pericentrin-B and PCM-1 require assembled microtubules prior to association with KICRs. KICRs were treated with CHO extract that either contained (A-F) or lacked (G-J) nocodazole, then rinsed, tubulin added, and the preparations processed for double-label immunofluorescence microscopy using anti-alpha tubulin (A,C,E,G,I) and either anti-γ tubulin (B), anti-pericentrin-B (D,H) or anti-PCM-1 (F,J). (K-N) Double-label immunofluorescence images of untreated *Spisula* centrosomes that were incubated with sea urchin tubulin and then stained with anti-tubulin (K,M) and either human autoimmune anti-centrosome serum, which contains antibodies that recognize PCM-1 and pericentrin-B (L), or anti-γ tubulin (N). Note that *Spisula* centrosomes do not contain proteins closely related to either PCM-1 or pericentrin-B (L), and immunoreactivity in H and J is due to centrosome proteins acquired from the CHO extract.

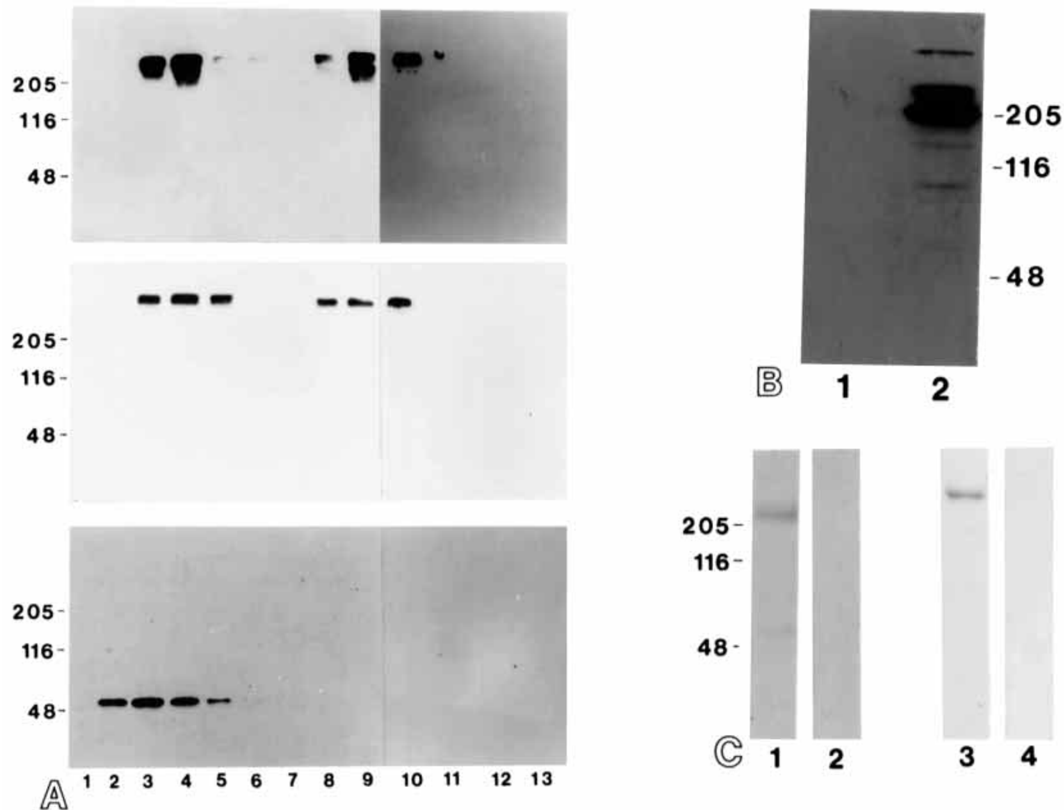


Fig. 10. (A) Immunoblot analysis of fractions collected following sucrose gradient centrifugation of an extract prepared from CHO cells that were arrested with HU and nocodazole. Fraction number 1 indicates the bottom of the tube. The blots were probed with either anti-PCM-1 (top), anti-pericentrin-B (middle) or anti-γ tubulin (bottom). (B) Extracts were prepared from HU-arrested CHO cells maintained in medium containing ^{35}S-methionine. The extracts then were incubated with Protein A-Sepharose beads that either contained bound anti-PCM-1 (lane 2) or were devoid of antibody (lane 1); the bound proteins then were resolved by SDS-PAGE, the gel dried, and apposed to X-ray film. (C) CHO extracts were treated with either anti-pericentrin-B (lanes 1 and 2) or anti-PCM-1 (lanes 3 and 4), and the immunocomplexes were precipitated using protein A-agarose. The immunocomplexes then were probed for coprecipitating proteins by immunoblotting using either anti-PCM-1 (lane 1), anti-pericentrin-B (lane 3) or anti-γ-tubulin (lanes 2 and 4).

The in vitro assay reported here addresses this possibility, and the demonstration that PCM-1 and pericentrin-B depleted extracts still reconstitute microtubule nucleation capacity of KICRs strongly supports the conclusion that neither PCM-1 nor pericentrin-B are critical components of the microtubule nucleating centers in the PCM. A possibility to consider is that *Spisula* forms of pericentrin-B and PCM-1 exist as components of KICRs, and that these *Spisula* forms are not recognized by antibodies directed against mammalian forms of these proteins. If correct, then the reported immunodepletion experiments would not be a true indicator that PCM-1 and pericentrin-B activities are not critical for microtubule nucleation. However, recent antibody microinjection studies using mammalian oocytes (R. Balczon et al., unpublished) further support the conclusion that PCM-1 and pericentrin-B are not essential for microtubule nucleation.

An important observation from the in vitro studies utilizing KICRs and CHO extracts is that neither pericentrin-B nor PCM-1 associated with KICRs when extracts contained nocodazole, while γ tubulin binding to KICRs occurred even when microtubule formation was inhibited (Fig. 9). This observation, together with the data shown in Fig. 8, indicates that the association of PCM-1 and pericentrin-B with PCM is a late event in the centrosome maturation process. These results further suggest that a small number of microtubules assemble during the incubation of KICRs with extracts lacking nocodazole, and the binding of PCM-1 and pericentrin-B to KICRs depends on the presence of intact microtubules. This observation appears to contradict the demonstration that pericentrin-B (Fig. 5) and PCM-1 (Balczon et al., 1999) are integral components of the PCM. A potential explanation for this inconsistency is that microtubules may be required for transport of PCM autoantigens to the centromatrix during centrosome assembly (Balczon et al., 1999), but once the PCM autoantigens are incorporated into the centrosome they no longer require microtubules to retain association with the PCM.

An additional observation is that the microtubule asters formed following incubation of KICRs with extract lacking nocodazole apparently contained more microtubules than those formed following incubation with extracts containing nocodazole (Fig. 9). There are two potential explanations for these results. First, it is possible that a small number of microtubules assemble from reconstituted centrosomes during the incubation in extracts lacking nocodazole, and these microtubules allow the association of additional microtubule

nucleating proteins present in the extract with the KICRs, resulting in more robust microtubule formation. Alternatively, a small number of non-centrosomal microtubules may form in extracts lacking nocodazole, and these tubules may be transported to the reconstituted centrosomes by microtubule motors present in the extracts. Additional studies will be required to address each of these possibilities.

Pericentrin-B forms a complex with PCM-1

PCM-1 and kendrin/pericentrin-B coimmunoprecipitate, suggesting that PCM-1 and pericentrin-B form a complex in cells, although the significance of that association remains to be established. In addition, immunoprecipitations using extracts obtained from ^{35}S-labeled cells determined that additional proteins coprecipitated with PCM-1 and pericentrin-B (Fig. 10), suggesting that PCM-1 and pericentrin-B may actually be subunits of a much larger complex. The identities of the other proteins that coprecipitate with PCM-1 and pericentrin-B remain to be established, but likely candidates are additional centrosome proteins, including other centrosome autoantigens (Balczon and West, 1991; Mack et al., 1998).

In addition to suggesting a functional relationship, the binding of PCM-1 to pericentrin-B may provide important information concerning the nature of the autoimmune response in patients producing anticentrosome antibodies. Autoimmune responses generated against intracellular structures are often directed against several different protein components of an organelle (Tan, 1989; Balczon, 1993). For example, patients producing autoantibodies against snRNPs generally undergo a polyclonal response resulting in the production of antibodies reactive towards the U1-U6 components of the splicing machinery (Tan, 1989; Zieve and Sauterer, 1990; Balczon, 1993), while scleroderma CREST patients generate autoantibodies against three centromere/kinetochore proteins, CENP-A, -B and -C (Tan, 1989; Balczon, 1993; Craig et al., 1999). To explain this phenomenon, Tan (Tan, 1989) proposed that the immunogen driving autoimmune reactions is a multiprotein complex. The demonstration that PCM-1 and pericentrin-B form a complex may help to explain the nature of the immunogen driving the production of anticentrosome antibodies in autoimmune patients.

In summary, a novel centrosome component, kendrin/pericentrin-B, has been identified. Pericentrin-B is a large protein, and our studies demonstrate that pericentrin-B is an integral component of the PCM. Sequence analyses have shown that kendrin/pericentrin-B shares considerable identity with another centrosome autoantigen, pericentrin, suggesting that the two proteins are evolutionarily related. Pericentrin-B copellets with PCM-1, demonstrating that these two proteins may form a functional complex inside cells, and additional results suggested that neither of these proteins is critical for microtubule nucleation events. Studies in progress are aimed at defining the significance of the relationship between PCM-1, pericentrin-B and the other PCM autoantigens.

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