PCM-1, A 228-kD Centrosome Autoantigen with a Distinct Cell Cycle Distribution

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Abstract. We report the identification and primary sequence of PCM-1, a 228-kD centrosomal protein that exhibits a distinct cell cycle–dependent association with the centrosome complex. Immunofluorescence microscopy using antibodies against recombinant PCM-1 demonstrated that PCM-1 is tightly associated with the centrosome complex through G_1, S, and a portion of G_2. However, late in G_2, as cells prepare for mitosis, PCM-1 dissociates from the centrosome and then remains dispersed throughout the cell during mitosis before re-associating with the centrosomes in the G_1 phase progeny cells. These results demonstrate that the pericentriolar material is a dynamic substance whose composition can fluctuate during the cell cycle.

THE centrosome is responsible for regulating and organizing the microtubule cytoskeleton. In most animal cells, the centrosome consists of a pair of centrioles embedded in an osmiophilic cloud of electron-dense pericentriolar material (Brinkley, 1985; Rose et al., 1993; Kalt and Schliwa, 1993). Although not well characterized at the molecular level, it has been well established that the pericentriolar material (PCM)[1] is essential for centrosome function. Studies in which PCM was experimentally dissociated from the centrioles demonstrated that it is the PCM, and not the centriole proper, that is responsible for nucleating cytoplasmic microtubules (Gould and Borisy, 1977). Moreover, some animal cells, such as mouse oocytes, have centrosomes composed only of PCM, with centrioles being undetectable (Szöllösi et al., 1972; Schatten et al., 1986). Despite a lack of centrioles, mouse oocytes still are able to maintain an elaborate microtubule cytoskeleton. Together, these observations demonstrate that one of the keys to understanding the regulation of the microtubule cytoskeleton lies in determining the molecular nature of the PCM.

An additional complexity to understanding cellular regulation of the microtubule cytoskeleton is the observation of cell cycle–specific changes in the centrosome. Although gross morphological alterations in centrosome structure have been observed as the centrosome is replicated during each cell cycle (Robbins et al., 1968), the replication of the centrosome does not appear to influence microtubule kinetics directly. Instead, subtler biochemical changes in the PCM appear to be responsible for the dramatic increase in the microtubule nucleating capacity of the centrosome that occurs at the G_2/M transition. Specifically, changes in the phosphorylation state of unknown PCM proteins that probably occur due to the activity of mitosis promoting factor appear to be responsible for the heightened microtubule nucleating capabilities of the mitotic centrosome (Vandré et al., 1984, 1989; Verde et al., 1990). It is not clear how phosphorylation of PCM proteins up-regulates the microtubule nucleating capacity of the centrosome complex at the G_2/M transition, but several possible explanations can be proposed. For example, phosphorylation may simply activate additional microtubule-nucleating sites in the PCM. Alternatively, phosphorylation of proteins within the PCM may cause proteins that inhibit microtubule nucleation to dissociate from the centrosome thereby exposing additional microtubule nucleation sites. Another possibility is that phosphorylation of the centrosome causes additional proteins to be recruited to the PCM to create additional microtubule nucleation sites. Finally, the increased microtubule nucleating capabilities of the PCM that result from phosphorylation may be due to a combination of the above proposals. Until the molecular composition of the PCM is clearly defined it will not be possible to address each of these proposals experimentally.

In previous studies, a high titer serum from a patient with systemic sclerosis and Raynaud's phenomenon was identified that contained autoantibodies that specifically recognized centrosomes when mammalian cells were processed for immunofluorescence microscopy (Osborn et al., 1982; Balczon and West, 1991). On immunoblots of mammalian cells, centrosomal proteins of 39, 185, and 220 kD were identified (Balczon and West, 1991). In this paper, an extension of those original studies is reported. The human anticentrosome antiserum has been used to screen a human fetal liver λgt11 cDNA expression library and the cDNA encoding the high relative molecular mass centrosomal autoantigen has

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1. *Abbreviations used in this paper:* BrDU, bromodeoxyuridine; IPTG, isopropyl-β-D-thiogalactoside; PCM, pericentriolar material.

been cloned and sequenced. We propose the name PCM-1 for this centrosome autoantigen.

Materials and Methods

Cell Culture

Human HeLa and CHO cells were used for all studies. HeLa cells were cultured in DME supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, and 0.1 mM minimal essential amino acids. HeLa cells were maintained in a 10% CO_2 environment. CHO cells were cultured in McCoy's medium supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, and 0.1 mM minimal essential amino acids. CHO cells were maintained in a 5% CO_2 environment.

Isolation of cDNA Clones

A λgt11 human fetal liver cDNA expression library (Clontech, Palo Alto, CA) was screened using SPJ human anticentrosome antiserum. Approximately 5×10^5 plaque forming units of phage grown on a lawn of Escherichia coli were assayed during the initial screening using the procedures outlined by Young and Davis (1983). Production of fusion proteins was induced by overlying the plates with filters soaked in 10 mM isopropyl-β-D-thiogalactoside (IPTG). The plates then were incubated for 2 h at 37°C, and then the nitrocellulose filters were removed and transferred to a solution of 3% powdered milk in PBS. After a 60-min incubation at room temperature in the 3% milk solution the filters were transferred to a fresh incubation dish containing 3% milk–PBS supplemented with SPJ antiserum diluted 1:1,000. The nitrocellulose filters were incubated overnight at 4°C in the SPJ antiserum and then rinsed four times, 10-min each, with 3% powdered milk solution. The filters then were transferred to an incubation vessel containing peroxidase-labeled goat anti–human IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN) diluted 1:1,000 in 3% powdered milk. After a 2-h incubation at room temperature, the filters were rinsed four times with PBS (10-min each) and then developed with 3, 3'-diaminobenzidine and H_2O_2. Phage plaques giving a positive reaction were purified to homogeneity by secondary and tertiary screenings. From this original round of screenings, two positive plaques, 5A1 and 17A1, were obtained. Before being used for immunoscreening the SPJ antiserum was pre-absorbed by incubating the serum with a plate containing Y1090 infected with control λgt11 phage.

The 1.6-kb insert in clone 17A1 was used to re-screen the same library by plaque hybridization using the procedures outlined by Zimmer et al. (1988). Positive clones were amplified by PCR, digested with EcoR1 and then the cDNA inserts were ligated into pBluescript (Stratagene, La Jolla, CA). The cDNA's from the second round of screening, and the cDNA's obtained by subsequent screenings, were used to re-screen the library until the entire coding sequence of the 228-kD centrosome protein was obtained.

DNA Sequencing

A series of deletions for both strands of each cDNA was generated using the Erase-a-Base System (Promega Biotec, Madison, WI). Single-stranded DNA's were prepared using M13 helper phage and the templates were used for sequencing according to the procedures outlined by Sanger et al. (1977) using the Sequenase System (U.S. Biochemical Corp., Cleveland, OH) with [^{35}S]dATP. Sequence reactions were resolved on 6% polyacrylamide/8 M urea gels and the gels then were dried and exposed to Kodak XAR-5 film overnight at −70°C. Data generated from the multiple sequencing analyses were aligned and analyzed using the Wisconsin GCG package of computer programs. 100% of the PCM-1 sequence was confirmed by analyses of both DNA strands (see Fig. 9).

Expression of Recombinant Protein

A lysogen of cDNA clone 17A1 was made by infecting E. coli strain Y1089 and plating at 32°C on LB-amp plates. Colonies were spotted onto two separate plates and one plate was grown at 32°C and the other was grown at 42°C. Colonies which showed growth at 32°C while being unable to grow at 42°C were analyzed further. Preparation of a fusion protein-containing crude lysate using the λgt11 lysogen was performed using the procedures outlined by Huynh et al. (1985).

Production of Antibodies

Samples of fusion protein in SDS sample buffer were separated by preparative gel electrophoresis in 7.5% polyacrylamide gels. The separated proteins were transferred to nitrocellulose using standard procedures (Balczon and Brinkley, 1987) and the nitrocellulose blots were stained with Ponceau S (0.1% wt/vol in 5% trichloroacetic acid). The region containing the high molecular weight fusion protein was cut out, the nitrocellulose was dried completely by incubating in an 80°C drying oven for 1 h, and then the nitrocellulose was solubilized using 100 μl DMSO. An equal volume of Freund's complete adjuvant was added and the emulsified fusion protein was injected subcutaneously into either rabbits or mice. Subsequent booster injections were performed at two week intervals using the above procedure, except that Freund's incomplete adjuvant was used. Blood was collected from either the marginal ear veins of rabbits or from the tails of mice and antibody production was assayed by immunofluorescence microscopy and immunoblot analysis.

Immunoblot Analysis

Proteins were resolved on either 5 or 7.5% polyacrylamide gels using standard procedures (Laemmli, 1974). Proteins were transferred to nitrocellulose using the methods detailed by Towbin et al. (1979) and the blots were probed using either SPJ antiserum or antibodies generated against the induced 17A1 lysogen fusion protein using methods detailed previously (Balczon and Brinkley, 1987; Balczon and West, 1991). Peroxidase-labeled secondary antibodies (anti-human IgG, anti-rabbit IgG and anti-mouse IgG; Boehringer Mannheim Biochemicals) were used at 1:1,000 dilutions. Before being developed, the blots were rinsed three times with PBS (5-min each) followed by three rinses with PBS + 0.5 M NaCl (5-min each). Blots were developed using 3, 3'-diaminobenzidine and H_2O_2.

Affinity Purification of Antibodies

For some studies, a modification of the immunoblotting procedure was used to purify anti-PCM-1 antibodies from the SPJ serum. These studies were performed essentially as described in Balczon and West (1991). Briefly, proteins present in bacterial lysates were resolved by preparative SDS-PAGE. The resolved proteins then were transferred to nitrocellulose and the nitrocellulose was stained with Ponceau S. The region of the blot containing the 17A1 high molecular weight induced fusion protein was cut out and the thin nitrocellulose strip then was used to immunopurify anti-17A1 antibodies from the SPJ serum (Balczon and West, 1991). Monospecific anti-17A1 antibodies then were used for immunofluorescent staining of cultured cells.

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed using previously published methods (Balczon and West, 1991). SPJ antiserum was used at a 1:1,000 dilution and rabbit and mouse antibodies against the 17A1 fusion protein were used at a 1:100 dilution. Monospecific anti-PCM-1 antibodies (see above) were used undiluted. FITC-labeled secondary antibodies (anti-human IgG, anti-rabbit IgG, and anti-mouse IgG; Boehringer-Mannheim Biochemicals) were used at a 1:20 dilution. Cells were mounted in PBS/glycerol (1:1) containing 25 μg/ml Hoechst 33258 dye and then observed using a Zeiss 35 M Axiovert microscope equipped for epifluorescence microscopy. Images were photographed using Kodak T-Max 400 film and negatives were developed using T-Max developer.

For some experiments, trachea were isolated from recently sacrificed rats and used for the isolation of ciliated cells. Trachea were cut in half longitudinally and then the surfaces were scraped with a razor blade to dislodge epithelial cells. The cells were collected, rinsed twice with PBS, and then centrifuged onto polylysine-coated coverslips. The cells then were fixed and processed for immunofluorescence microscopy using SPJ antiserum.

For cell cycle studies, mitotic cells were collected by mitotic shake off and then the cells were plated either on 11 × 22 glass coverslips or in T-25 flasks. For immunofluorescence studies, some of the coverslips were processed for immunofluorescence microscopy by fixing in −20°C MeOH at various times after plating and then staining with anti-17A1 antibodies followed by FITC anti–rabbit IgG. At each time point, a parallel coverslip was pulsed for 15 min with 10 μM bromodeoxyuridine (BrDU) in culture medium to determine cell cycle stages. The appropriate BrDU-treated coverslip then was fixed at room temperature in EtOH/acetic acid (3:1) for 10 min. The coverslip then was rinsed with 30% EtOH, rinsed in PBS, and then immersed in 4 N HCl for 30 min to remove histones and other chro-
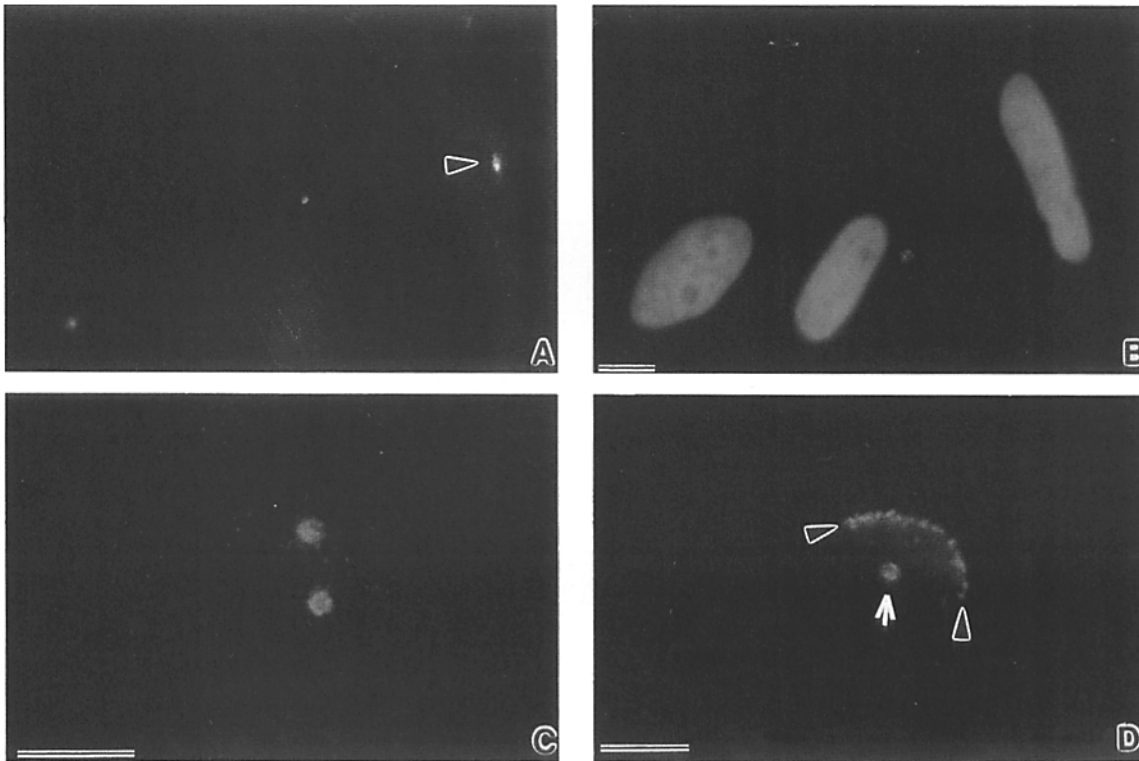
Figure 1. Immunofluorescent staining of cells using SPJ autoimmune anticentrosome antiserum at a 1:1,000 dilution. *A–C* are HeLa cells. (*A*) HeLa cells stained with SPJ serum. A single intensely stained centrosome can be observed in each interphase cell. The interphase centrosomes appear to be comprised of numerous punctate fluorescent foci (see the cell at the arrowhead). (*B*) The corresponding field of cells stained with Hoechst 33258 dye. (*C*) A mitotic cell. The spindle poles are stained intensely. Also note that numerous distinct fluorescent foci can be detected in the cytoplasm. (*D*) A ciliated tracheal epithelial cell. A fluorescent band (*arrowheads*), corresponding to basal bodies, can be observed just below the region of the cilia. Presumably, the intense fluorescent focus in the perinuclear area represents the cytoplasmic microtubule organizing center (*arrow*). Bars, 10 μms.

mosomal proteins. The coverslips then were rinsed twice with PBS followed by a 10-min incubation in PBS containing 1% BSA. The cells then were incubated in monoclonal anti-BrDU (diluted 1:50 in PBS) antibody for 60 min at room temperature followed by a PBS rinse and then a 45-min incubation in FITC-labeled goat anti-mouse IgG (1:20 dilution in PBS). After a brief rinse, the coverslips were mounted and observed. Anti-BrDU was purchased commercially from Sigma Chemical Co.

Cells that were plated into the T-25 flasks were used for immunoblot studies. For these experiments, cells were collected from the flasks at various times after plating by trypsinization. The cells were rinsed twice with PBS and then resuspended directly in sample buffer. The samples then were treated as described previously.

Results

A human autoimmune antiserum (SPJ serum) was obtained that contained antibodies which reacted specifically with antigens in the centrosome complex when cells were processed for immunofluorescence microscopy. The SPJ serum recognized antigens that were present in both interphase and mitotic centrosomes (Fig. 1), as well as antigens that were localized to basal bodies of ciliated epithelial cells. Close examination of the labeled cells (Fig. 1) revealed that the antibodies in the SPJ serum stained numerous punctate fluorescent foci in the centrosome region during interphase. In mitotic cells, spindle poles as well as numerous cytoplasmic foci were stained by the SPJ antibodies (Fig. 1 *C*). In ciliated cells, basal bodies, and a single perinuclear structure that

probably corresponds to the cytoplasmic microtubule organizing center were recognized by the human autoantibodies (Fig. 1 *D*). In a previous study it was demonstrated that antibodies present in the SPJ anticentrosome antiserum recognized centrosomal antigens of 39, 185, and ∼220 kD (Balczon and West, 1991). A function has not been attributed to any of these centrosome proteins to date.

Studies were undertaken to characterize the centrosomal autoantigens more completely. For these experiments, a human fetal liver λgt11 cDNA library was screened using the SPJ antiserum. From the initial screening, two phage clones, clones 5A1 and 17A1, were identified and purified to homogeneity. Restriction map analysis demonstrated that the 1.6-kb cDNA insert in clone 17A1 and the 1.45-kb insert in clone 5A1 encoded overlapping portions of the same cDNA (see Fig. 8). Northern blot analysis of total HeLa cell RNA was performed using radioactively labeled cDNA inserts obtained from the two clones and a single mRNA of 7.5–8.0 kb was identified on autoradiographs (Balczon, R., and W. E. Zimmer. 1990. *J. Cell Biol.* 111:180a).

A series of experiments was performed to demonstrate that clones 5A1 and 17A1 encoded portions of a centrosome protein. The probe used for screening the library was an unfractionated human serum and not an affinity-purified antibody. Therefore, it was possible that the isolated clones may have encoded an antigen that was reacting with additional, noncentrosome-specific antibodies that may be present in the
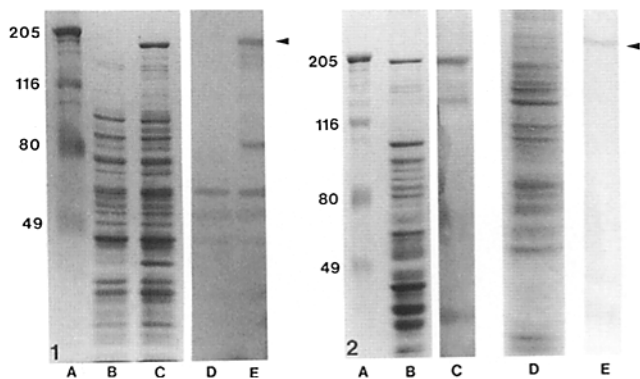
Figure 2. (Part 1) SDS-PAGE and immunoblot analyses of the 17A1 lysogen. A lysogen was generated using phage clone 17A1 and bacterial fusion protein was induced by the addition of 10 mM IPTG. Lanes A–C are a 7.5% Coomassie-stained gel and lanes D and E are the corresponding immunoblots of lanes B and C using SPJ antiserum at a 1:2,500 dilution. (Lane A) molecular weight standards (in kD). (Lane B) A gel of uninduced 17A1 lysogen. (Lane C) A gel of induced 17A1 lysogen. Note the prominent band that appeared in the induced lane near 180 kD. The size of the induced protein corresponds exactly with the size expected of a fusion protein containing the entire β-galactosidase polypeptide plus the region of PCM-1 encoded by the 17A1 cDNA. (Lane D) The corresponding immunoblot of lane B using SPJ serum. (Lane E) The corresponding immunoblot of lane C using SPJ serum. The induced fusion protein was bound strongly by the SPJ antibodies (arrowhead). The lower relative molecular mass bands directly below the 180-kD fusion protein that were recognized by the SPJ serum probably are proteolytic breakdown products of the fusion protein. (Part 2) Characterization of rabbit antiserum generated against the 17A1 fusion protein. Lanes A, B, and D are Coomassie-stained gels. Lanes C and E are immunoblots. (Lane A) A Coomassie-stained gel of the molecular weight standards (in kD). (Lane B) A Coomassie-stained gel of the 17A1 lysogen. (Lane C) The corresponding immunoblot of lane B using the rabbit anti-17A1 serum at a 1:1,000 dilution. The high molecular weight induced fusion protein was recognized by the antibodies. The lower relative molecular mass bands presumably are proteolytic breakdown products of the 17A1 fusion protein. (Lane D) A Coomassie-stained gel of total HeLa cellular proteins. (Lane E) The corresponding immunoblot of lane D using the rabbit anti-17A1 serum at a 1:400 dilution. A single reactive band near 220 kD was identified (arrowhead).

SPJ antiserum. To determine whether the clones encoded portion of a centrosome protein a lysogen was generated using clone 17A1. Bacterial fusion protein synthesis was induced and whole cell lysates were produced. Proteins from both the uninduced and IPTG-induced 17A1 lysogen were separated by SDS-PAGE, and then the resolved proteins were transferred to nitrocellulose. When the nitrocellulose blots were probed with SPJ antiserum the high molecular weight IPTG-induced fusion protein was recognized (Fig. 2, part 1) demonstrating that antibodies present in SPJ serum recognized epitopes contained within the fusion protein. As a further characterization of clone 17A1, monospecific antibodies were produced by generating antibodies against the 17A1 fusion protein in rabbits and mice. Blood was collected from immunized animals and whole sera was used to stain both CHO and HeLa cells. When cells that were stained with anti-17A1 antisera were observed it was determined that centrosomes were recognized by the antibodies (Fig. 3) demon-
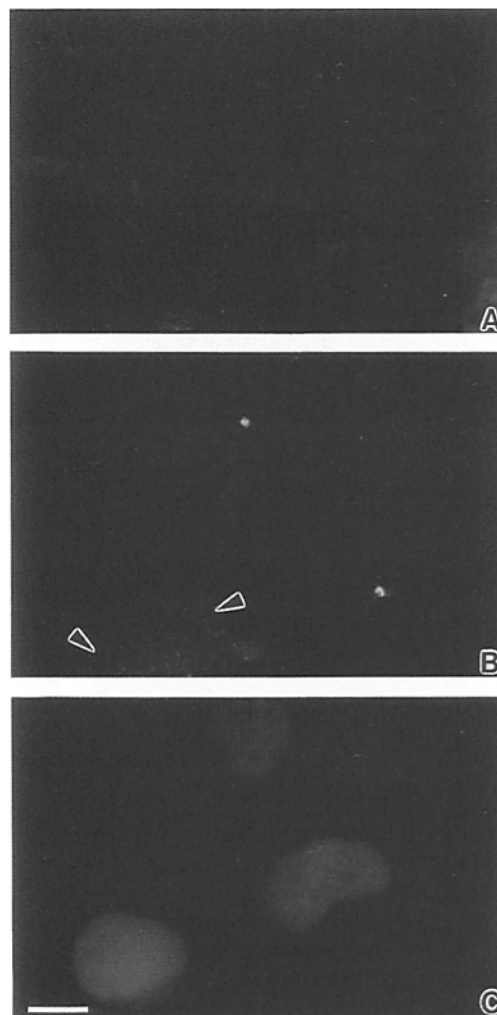

Figure 3. Immunofluorescent staining of cultured HeLa cells using anti-17A1 antibodies. Rabbits were injected with 17A1 fusion protein and blood was collected from the immunized animals. Cells then were processed for immunofluorescence using the anti-17A1 sera. (A) Staining of HeLa cells with pre-immune rabbit serum. (B) Staining of cells with rabbit anti-17A1 serum. Note that a distinct centrosome could not be identified in one of the cells in this field following staining by the anti-17A1 serum (arrowheads). However, numerous punctate fluorescent foci could be observed in the cytoplasm of that cell. A distinct centrosome could be observed in both of the other cells in this field. (C) Hoechst 33258 staining of the cells in B. Bar, 10 μms.

strating that clone 17A1 encoded portion of a centrosome protein. Interestingly, it was noted that occasionally a cell was observed that did not exhibit distinct perinuclear centrosome staining following incubation with either polyclonal mouse or rabbit anti-17A1 antisera. Instead, numerous punctate fluorescent foci were detected throughout the cytoplasm of these cells. In particular, it was noted that little centrosome staining was observed at mitotic spindle poles (see Fig. 4). Finally, anti-centrosome antibodies were not detected in any of the pre-immune sera (Fig. 3). This control was essential as it has been shown that anti-centrosome antibodies occasionally can be detected in the pre-immune sera of animals (Connolly and Kalnins, 1978).

To determine which of the centrosome autoantigens was recognized by the anti-17A1 antiserum, immunoblot analysis was performed using whole cell lysates from CHO and HeLa cells. As Fig. 2 demonstrates, anti-17A1 antiserum reacted with a protein band with ~220 kD in HeLa cells. Similar results were obtained when CHO cellular proteins were assayed (not shown). Moreover, the anti-17A1 antibodies that were generated in rabbits reacted strongly with the 17A1 IPTG-induced bacterial fusion protein on immunoblots (Fig. 2, part 2) while pre-immune serum exhibited no reactivity (now shown). Together, these results demonstrated that overlapping clones 5A1 and 17A1 encoded portion of a high molecular weight centrosome autoantigen. This protein has been named PCM-1.

As mentioned previously, occasionally cells were observed that exhibited little, if any, centrosome reactivity after being processed for immunofluorescence microscopy using anti-17A1 antiserum. In particular, mitotic spindle poles generally showed no reactivity following anti-17A1 staining, although on occasion slight spindle pole staining could be detected. This suggested that the association of the PCM-1 protein with the centrosome may be cell cycle regulated. To investigate this possibility, cells were collected by mitotic shake off and plated onto coverslips. Coverslips then were fixed at intervals after plating and processed for immunofluorescence microscopy. In addition, parallel coverslips were pulsed with BrDU and then stained with anti-BrDU monoclonal antibodies to get an approximation of cell cycle stages. As shown in Fig. 4, in G_1 phase cells the centrosome autoantigen was observed to be tightly associated with the centrosome complex. PCM-1 remained intimately associated with the centrosome complex through S phase and into G_2 phase. Midway through G_2 phase of the cell cycle, PCM-1 was observed to dissociate into numerous fluorescent foci and began to be dispersed through the cytoplasm (Fig. 4, G and H). By mitosis, the centrosome autoantigen was observed to be completely dispersed throughout the cell. Although much of the centrosome autoantigen appeared to be spindle associated in the metaphase stage cell shown in Fig. 4 I, there was little detectable staining at the spindle pole regions. These results demonstrated that the association of PCM-1 with the centrosome fluctuated during the cell cycle, with the PCM-1 protein dissociating from the centrosome as cells prepared for mitotic division.

The differences between the staining patterns of cells processed for immunofluorescence using either SPJ serum or anti-17A1 serum can be observed by directly comparing Figs. 4 to 5. Fig. 5 shows HeLa cells that were processed for immunofluorescence using SPJ serum. Like cells stained with anti-17A1 antiserum, the SPJ-reactive material was intimately associated with the centrosome region throughout most of interphase (Fig. 5 A). However, the differences between the staining patterns obtained using the two antisera became apparent in late G_2. Figs. 4 G and 5 C show late G_2 cells that were stained with anti-17A1 and SPJ sera, respectively. As shown previously, PCM-1 dissociated completely from the centrosome complex during late G_2 (Fig. 4 G). However, when late G_2 cells were stained with SPJ serum, the majority of the SPJ-reactive material remained associated with the centrosome complex with only a small percentage of the SPJ-reactive antigenic material being detected as cytoplasmic foci (Fig. 5 C). In mitotic cells that were

stained with the SPJ serum (Fig. 5 E), distinct SPJ-reactive zones could be detected at both spindle poles. Unlike anti-17A1-stained cells, only a small percentage of the SPJ-reactive material could be detected as cytosolic foci (see also Fig. 1 C). These results suggested that two different populations of anticentrosome antibodies were present in the SPJ serum — one population of antibodies that reacted with antigens present in the PCM during both interphase and mitosis and a second population of antibodies that recognized antigens that were associated with the centrosomes only during mitosis.

The previous results suggested that two populations of SPJ-reactive material existed within the centrosome. Moreover, the results shown in Figs. 4 and 5 suggested that we had cloned and sequenced the cDNA encoding a protein that comprises at least portion of one of these centrosome autoantigen subtypes — antigens that have the capacity to associate and dissociate from the PCM. An experiment was designed to test this hypothesis. For this experiment, monospecific anti-17A1 antibodies were affinity purified from the SPJ serum using 17A1 fusion protein. The purified antibodies then were used to stain HeLa cells that were in different stages of the cell cycle. As Fig. 6 demonstrates, antibodies that were purified from the SPJ serum using the IPTG-induced 17A1 fusion protein exhibited an identical staining pattern to the pattern that was observed following staining with the rabbit polyclonal anti-17A1 antiserum. Specifically, the monospecific anti-17A1 antibodies that were purified from SPJ serum stained interphase centrosomes but did not stain centrosomes in late G_2 phase and M phase cells (Fig. 6). As the telophase stage cell in Fig. 6 E shows, most of the PCM-1 antigen was dispersed throughout the cytoplasm. Note that a cell that has just completed cytokinesis also can be observed in Fig. 6 E. In this cell (Fig. 6 E, arrowhead) which has re-entered G_1 phase of the cell cycle, the PCM-1 protein has re-aggregated into a single structure in the perinuclear region. These results support the hypothesis that two populations of PCM antigens are recognized by antibodies present in SPJ serum.

Although the immunofluorescent staining patterns suggested that PCM-1 was undergoing changes in cellular distribution during the cell cycle, it is conceivable that the lack of centrosome-associated staining in late G_2 and M phase cells actually represented a change in protein abundance during the cell cycle that gave the appearance of decreased centrosome stained during the cell cycle. To test for the relative abundance of PCM-1 in cells at different stages of the cell cycle, immunoblot analysis was performed using anti-17A1 serum. For these experiments, cells were collected by mitotic shake off and then plated and allowed to progress for a period that allowed the cells to enter to either G_1 phase or late G_2/M phase. The cells then were collected and levels of PCM-1 in each population of cells was determined by immunoblot analysis. As Fig. 7 demonstrates, there was no distinct change in the relative levels of PCM-1 when G_1 phase cells were compared to late G_2/M stage HeLa cells. These results support the conclusion that the lack of anti-17A1 staining that is detected in both the centrosomal regions of late G_2 phase cells and in spindle poles of mitotic cells is due to a change in the distribution of PCM-1 within cells during the cell cycle and not to a change in PCM-1 protein levels during the cell cycle.
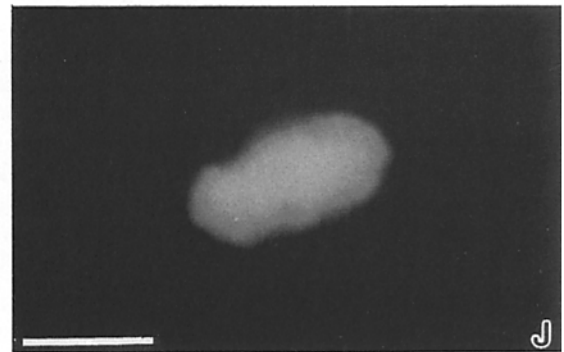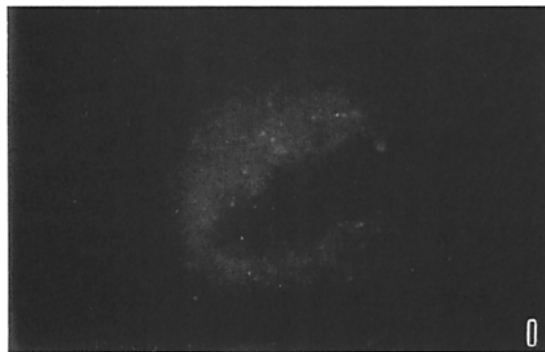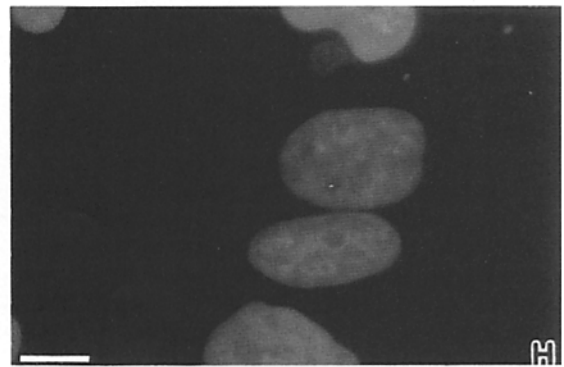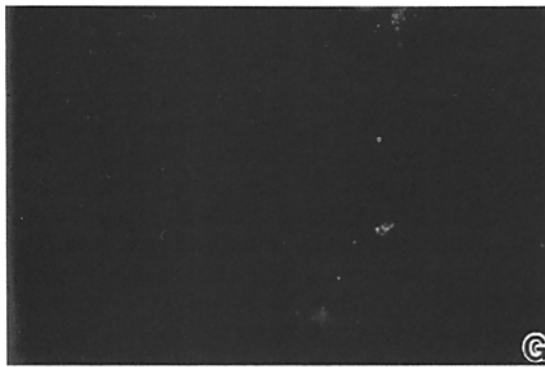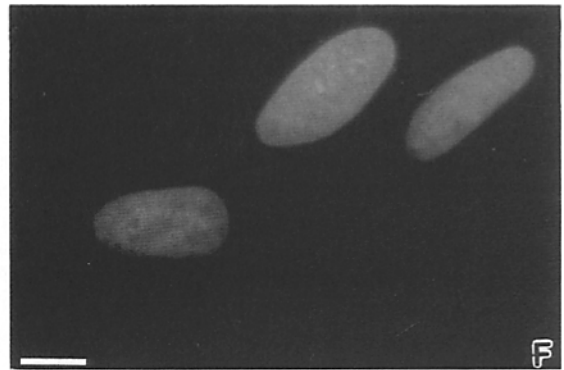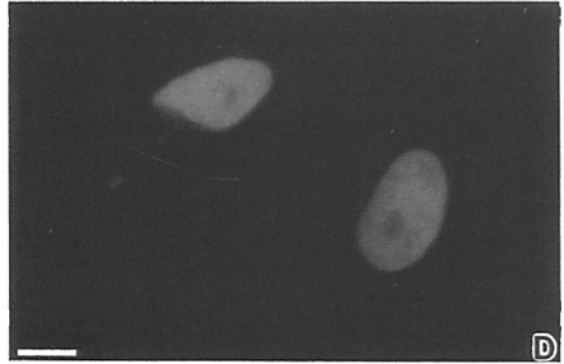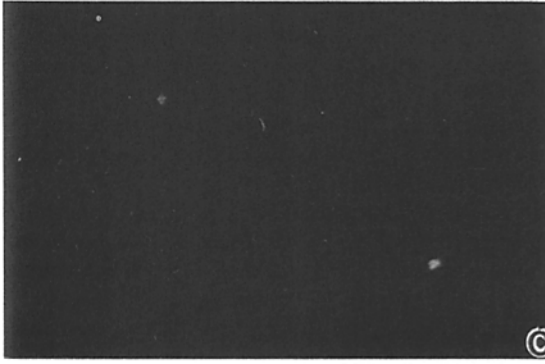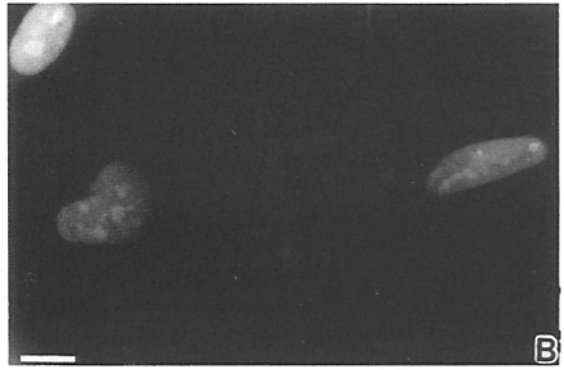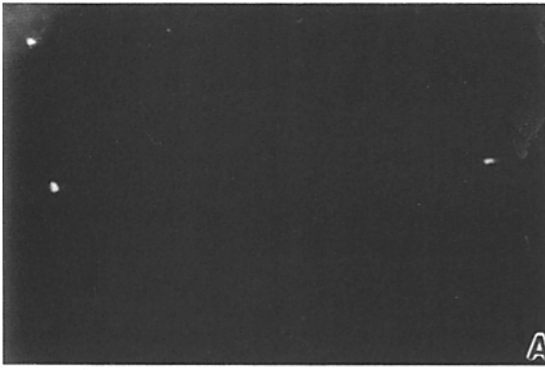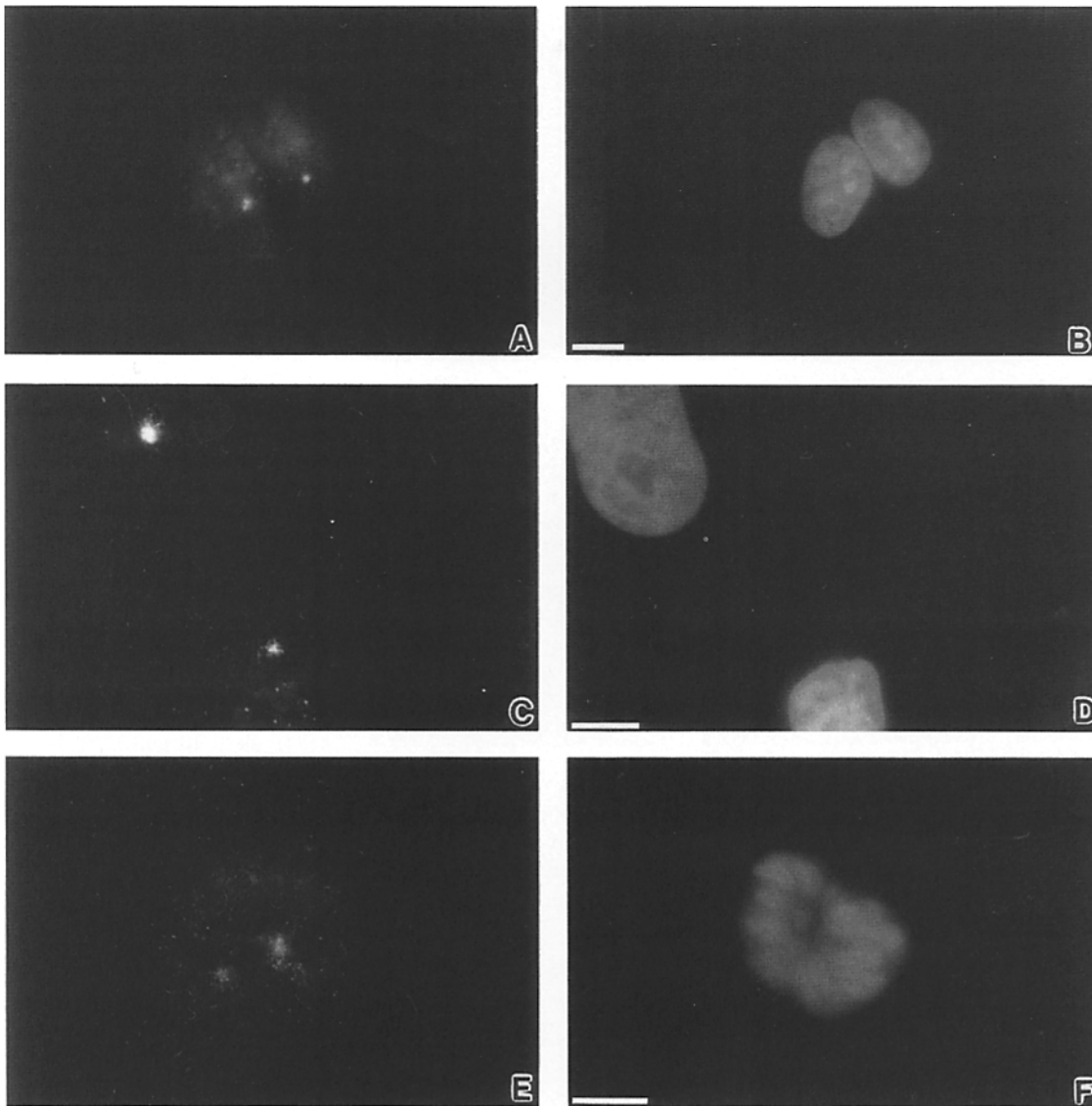
Figure 5. Cell cycle distribution of SPJ-reactive centrosome autoantigens. *A, C,* and *E* are SPJ-stained HeLa cells and *B, D,* and *F* are the corresponding Hoechst 33258–stained cells showing nuclear morphology. (*A* and *B*). G_1/S phase cells. (*C* and *D*). Late G_2 stage cells. (*E* and *F*). A mitotic (prometaphase) stage cell. Bars, 10 μms.

To isolate clones containing cDNA's encoding additional regions of the centrosome protein, the λgt11 fetal liver library was re-screened by hybridization using the 17A1 cDNA. Two additional clones, clones 5–8 and 1A2, were obtained that extended in the 3′ and 5′ directions, respectively (Fig. 8). Sequence analysis determined that clone 5–8 contained a stop codon, polyadenylation signal, and poly A-sequence (Fig. 8). Subsequently, the library was re-screened and a series of overlapping clones was obtained that encoded the entire centrosome protein. Analysis of the cloned sequence identified

an open reading frame of 6,072 nucleotides encoding 2,024 amino acids. The assigned initiator codon, corresponding to nucleotides 410-412, was preceded by a Kozak consensus (CCAXXATGG) initiation sequence (Kozak, 1986). This translation initiation sequence was preceded by a 5′ untranslated region containing multiple in-frame stop codons, with the final stop codon being at nucleotides 375-377. From the deduced amino acid sequence, the exact molecular mass of PCM-1 was calculated to be 228,705 daltons (Fig. 9).

Comparison of the nucleotide and amino acid sequences

Figure 4. Cell cycle distribution of PCM-1. HeLa cells were collected by mitotic shake off and plated onto coverslips. Cells then were collected at various stages of the cell cycle and either processed for immunofluorescence microscopy or for BrDU incorporation. This figure shows the fluorescence pattern obtained following anti-17A1 staining. *A, C, E, G,* and *I* are anti-17A1 antibody stained cells and *B, D, F, H,* and *J* are the corresponding Hoechst 33258–stained cells showing nuclear morphology. (*A* and *B*). G_1 phase cells. (*C* and *D*). S phase cells. (*E* and *F*). Early G_2 phase cells. (*G* and *H*). Late G_2 phase cells. (*I* and *J*). A mitotic (metaphase) cell. Bars, 10 μms.
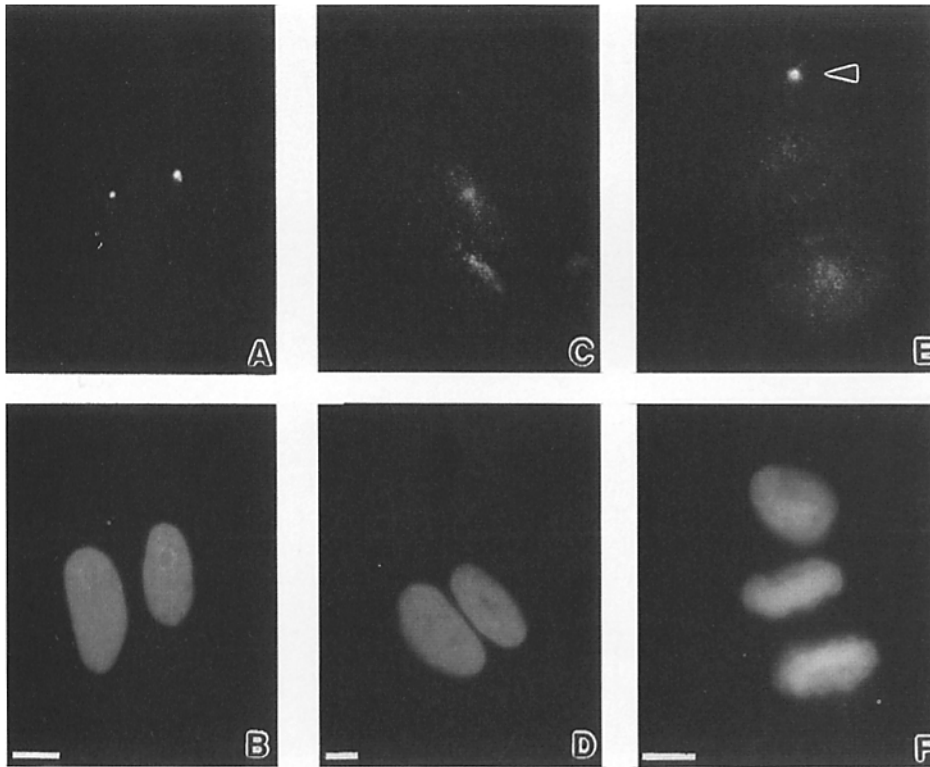
Figure 6. Monospecific anti-17A1 antibodies were purified from SPJ serum using procedures that were detailed in the Materials and Methods section. The affinity-purified human anti-17A1 antibodies then were used to stain HeLa cells. *A*, *C*, and *E* are cells stained with monospecific human anti-17A1 antibodies and *B*, *D*, and *F* are the corresponding Hoechst 33258-stained images showing nuclear morphology. (*A* and *B*). G_1/S phase cells. (*C* and *D*). Late G_2 phase cells. (*E* and *F*). A mitotic (telophase) cell and an early G_1 phase cell (*arrowhead*). Bars, 10 μms.

of the 228-kD centrosome autoantigen to known DNA sequences in databases demonstrated that the centrosome autoantigen was a unique protein (analysis performed April, 1993). From this analysis, a consensus nucleotide-binding site extending from amino acids 1,167–1,174 was found. This amino acid stretch of ARILSGKT corresponds to the consensus ATP/GTP-binding motif (A, G)-(4X)-G-K-(S, T) that has been identified in several ATPases, kinases, and GTP-binding proteins (Walker et al., 1982; Saraste et al., 1990). In addition, the sequence of PCM-1 showed several other in-

teresting features. First, the overall isoelectric point of the protein is 4.82. Moreover, the protein itself can be subdivided into four regions on the basis of charge with the NH_2-terminus 200 amino acids of the protein exhibiting a
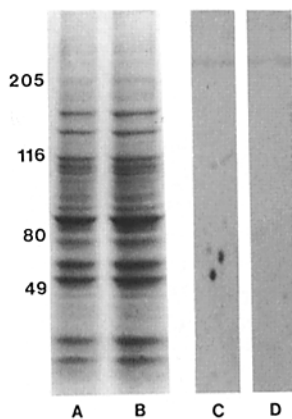
Figure 7. Immunoblot analysis of HeLa cells at different stages of the cell cycle. Cells were collected by mitotic shake-off and then plated into two separate flasks. One flask of cells was harvested after the cells completed division and entered G_1 phase of the cell cycle and the cells in the other flask were allowed to progress to late G_2/M stage before being harvested. The cellular proteins then were separated by SDS-PAGE. The resolved proteins then either were stained with Coomassie blue or transferred to nitrocellulose for Western blot analysis. Lanes *A* and *B* are a Coomassie-stained gel of cellular proteins from either G_1 phase cells (*A*) or G_2/M phase cells (*B*). Lanes *C* and *D* are the respective immunoblots of lanes *A* and *B* using rabbit anti-17A1 antiserum. The arrowhead shows the location of PCM-1. Relative molecular masses are given in kD.
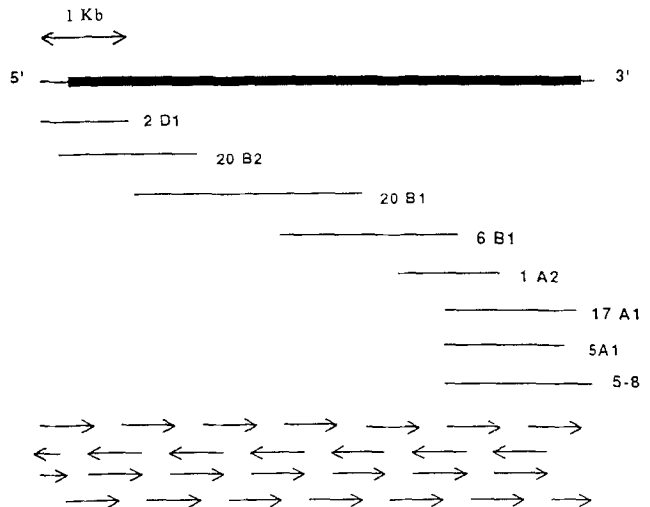
Figure 8. A λgt11 human fetal liver cDNA library was screened with SPJ serum and two clones, 5A1 and 17A1 were isolated. The library was rescreened with the cDNA insert contained in clone 17A1 and two clones, 5-8 and 1A2, were isolated. The library was then screened repetitively until the entire cDNA encoding the 228-kD centrosome autoantigen was obtained. The upper portion of this figure shows the region corresponding to the open reading frame in bold and the non-translated regions as fine lines. The middle region of this figure shows the overlapping clones that were isolated, and the lower panel shows the sequencing strategy that was used to obtain the entire sequence of both strands of the PCM-1 cDNA.

Figure 9. The nucleotide and deduced amino acid sequences of the PCM-1 cDNA and protein. Amino acids are denoted using single letter notation. The polyadenylation signal is underlined, and the nucleotide numbers are given in the left margin. The molecular mass of the protein is 228, 705 D. These sequence data are available from EMBL/GenBank/DDBJ under accession number L27841.

slightly basic P_i (P_i = 8.09). This domain is followed by two acidic domains (P_i's of 4.63 and 4.33, respectively) separated by a basic region extending from residues 1,000 to 1,400 (P_i = 9.47). Contained within the sequence of PCM-1 are several glutamic acid and aspartic acid–rich regions. For example, amino acids 619-632 are EDDEEEEEE-AEEE, amino acids 917-925 are DEEEEEEQD, and amino acids 1800-1813 are EDENEDEEMEEFEE. Other Glu and Asp-rich regions also were noted. Analysis of the amino acid composition of the centrosome protein determined that glutamic acid is the most common amino acid in PCM-1 (11.4% of residues).

Discussion

In this manuscript the cell cycle–specific distribution and nucleotide and amino acid sequences of a 228-kD centrosome protein are reported. The initial cDNA clones were obtained by screening a human fetal liver λgt11 cDNA expression library using a human autoimmune centrosome antiserum. In a previous report, it was demonstrated that the SPJ human anticentrosome serum contained antibodies that reacted specifically with centrosome proteins of 39, 185, and ~220 kD

on immunoblots (Balczon and West, 1991). It had been demonstrated previously by immunoelectron microscopy that centrosome autoantibodies reacted specifically with proteins of the pericentriolar material (Calarco-Gillam et al., 1983), and we propose the terminology PCM-1, PCM-2, and PCM-3 for the ≥220, 185, and 39-kD centrosome autoantigens, respectively. We conclude that we have cloned and sequenced the cDNA encoding PCM-1 based on the following criteria: (a) the molecular weight of the cloned centrosome protein as derived from the deduced amino acid sequence is 228 kD; (b) Antibodies generated against the 17A1 fusion protein reacted with a single polypeptide of ~220 kD on immunoblots of CHO and HeLa cellular homogenates; (c) antibodies in human anticentrosome antisera specifically bound to the 17A1 fusion protein on immunoblots; and (d) antibodies generated against the 17A1 fusion protein interacted with centrosomes when interphase cells were fixed and processed for immunofluorescence microscopy.

The PCM-1 protein exhibited a striking cell cycle distribution. As shown in Fig. 4, PCM-1 remained associated with the centrosome throughout most of interphase in HeLa cells, and then during mid to late G_2 the 228-kD centrosome autoantigen dissociated from the centrosome complex. This periodic association and dissociation of proteins with the centrosome complex has been reported previously. Rattner (1992) has observed the cell cycle stage–specific association of dense granules with the pericentriolar region of centrosomes in L929 cells. At the EM level, dense granules were only observed to be associated with interphase centrosomes and were completely absent from mitotic centrosomes (Rattner, 1992). Whether PCM-1 is a component of the dense granules remains to be determined by immunoelectron microscopy. Rao et al. (1989) reported a centrosome antigen of 43 kD that was recognized by a monoclonal antibody called MPM-13. The MPM-13 antigen could be dissociated from the pericentriolar material by treatment of cells with Colcemid. The distribution of the MPM-13 antigen following Colcemid treatment was similar to the pattern that was observed in late G_2 and M phases for PCM-1. Also, Sellito and Kuriyama (1988) reported that a centrosome-specific monoclonal antibody called CHO-1 stained interphase but not mitotic centrosomes. Likewise, Baron and Salisbury (1988) reported that a 165-kD centrin-like centrosome antigen was organized in a punctate fashion in the pericentriolar region, and that the distribution of the 165-kD protein was regulated by Ca^{2+} concentration (Baron and Salisbury, 1988, 1992). Despite the similar immunofluorescence patterns, it seems unlikely that PCM-1 is homologous to either centrin or the 165-kD protein because of a lack of sequence identity. Taken together, the results reported here for PCM-1 and the previously reported data of Rao et al. (1989), Sellito and Kuriyama (1988), Rattner (1992), and Baron and Salisbury (1988) suggest that the pericentriolar material is a dynamic substance whose composition can fluctuate during the cell cycle and following cellular perturbations. How the apparent dynamic nature of the PCM contributes to centrosome function is not clear at present, but it raises interesting questions regarding the regulation of the microtubule cytoskeleton. For example, as a cell progresses from interphase to mitosis the microtubule nucleating capacity of the centrosome increases dramatically. It has been proposed that this heightening of microtubule nucleating ability may occur as

a direct result of the phosphorylation and activation of microtubule nucleating proteins in the centrosome (Vandré et al., 1984; Verde et al., 1990). An alternative hypothesis is that the phosphorylation of centrosomal proteins results in the dissociation from the centrosome region of proteins that inhibit microtubule growth, thereby exposing additional microtubule-nucleating sites at mitosis. Alternatively, the heightened nucleating capacity of the centrosome at mitosis may be a result of both of the above events occurring. How the cell cycle–specific dissociation of PCM-1 and other proteins (Sellito and Kuriyama, 1988; Rattner, 1992) from the centrosome contributes to centrosome activity remains to be determined.

The function of the 228-kD centrosome autoantigen is unknown at this time, but the cell cycle distribution of the protein suggests several possibilities. First, the fact that PCM-1 dissociates from the centrosome at certain times argues against its being involved in microtubule formation directly. In particular, the observation that PCM-1 is not associated with centrosomes at mitosis, the period of the most rapid microtubule nucleation in cells, suggests that PCM-1 is not a microtubule-nucleating protein in the centrosome. A possible explanation for the role of PCM-1 may be as an inhibitor of microtubule growth, as was mentioned in the previous paragraph. In this putative capacity, PCM-1 would be blocking microtubule-nucleating sites in the centrosome and the release of PCM-1 from the centrosome at mitosis would expose additional microtubule-nucleating sites in the PCM. Alternatively, the 228-kD protein may have no role in the regulation of microtubule assembly, but may be involved in other centrosomal phenomena. For example, centrosomes are replicated each cell cycle in a regulated fashion. Using centriole doubling as a landmark, it has been demonstrated that centrosome replication begins in G_1, and then centrosome maturation continues through S and into G_2 (Robbins et al., 1968; Vandré et al., 1989). The mature replicated centrosomes then serve as the spindle poles during cell division. PCM-1 remains associated with the centrosome through G_1, S, and early G_2, and then dissociates from the centrosome complex. The redistribution of PCM-1 in mid to late G_2 shows a correlation to the period when centrosome replication is completed. Thus, PCM-1 may provide a structural component to the pericentriolar material which functions to maintain centrosome integrity throughout the early phases of the cell cycle and/or may play a role in the events leading to centrosome duplication. The cDNA and antibody probes that we have generated should allow for each of these possible roles of PCM-1 to be addressed.

Examination of the amino acid sequence of PCM-1 did not identify any regions of homology to tubulin-binding domains that have been identified in other microtubule-associated proteins. Moreover, both tubulin and PCM-1 are highly acidic proteins. However, analysis of the sequence of PCM-1 identified two slightly basic domains extending from residues 1–200 and 1,000–1,400 that conceivably could be involved in microtubule binding. Experimentation is underway to determine whether these basic subregions of PCM-1 have microtubule-binding activity. Although there is no direct evidence of PCM-1 binding to microtubules, studies should be performed to determine whether PCM-1 interacts either directly or indirectly with microtubules.

In summary, the cloning and sequencing of the cDNA en-

coding a 228-kD centrosome autoantigen is reported. This protein, which we have called PCM-1, shows a dynamic distribution during the cell cycle. PCM-1 antibodies stain numerous foci that are localized to the perinuclear centrosomal region during interphase of the cell cycle, and then the PCM-1 antigen dissociates from the centrosome complex as cells prepare for mitosis. These results demonstrate that the centrosome is a dynamic organelle, the molecular composition of which can be modulated during the cell cycle. The significance of this is not clear at this time, but the ability of the cell to regulate the biochemical composition of the PCM may be important for controlling the number of microtubules that are nucleated from a centrosome in interphase versus mitosis.

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