Identification of Molecular Components of the Centrosphere in the Mitotic Spindle of Sea Urchin Eggs

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ABSTRACT Monoclonal antibodies were prepared to identify molecular components specific to the mitotic apparatus of sea urchin eggs. The mitotic apparatus or asters induced within unfertilized eggs by taxol treatment were isolated from *Strongylocentrotus purpuratus* and used for immunization of mice. After fusion with spleen cells, the supernatant of hybridomas were screened in two stages by indirect immunofluorescence staining, first on isolated sea urchin mitotic spindles in 96-well microtiter plates to identify rapidly potential positive hybridomas, and second, on whole mitotic eggs on coverslips to distinguish between spindle-specific staining and adventitious contamination. Two hybridomas, SU4 and SU5, secreted antibodies reactive to microtubule-containing structures in eggs during the course of development. They preferentially stained the centrosphere both in isolated mitotic apparatus and in whole metaphase eggs, which was further confirmed by staining the isolated centrospheres with these antibodies. SU4 recognized a major 190-kD polypeptide on immunoblots as well as a species at 180 and 20 kD, whereas hybridoma SU5 stained a species at 50 kD. Thus, these polypeptides may be components of the centrosphere.

The molecular basis of mitotic poles is a major unsolved problem of mitosis. Clearly, the equal segregation of chromosomes in mitosis requires the establishment of a bipolar spindle. The poles of the spindle may be defined functionally as the loci toward which the chromosomes move in anaphase and as specifying the axis that determines the orientation and position of the plane of cleavage. Yet, the material basis of the poles remains virtually unknown.

In animal cells, the morphological expression of the poles is the centrosome, (37) which electron microscopy has shown to consist of a pair of centrioles surrounded by a cloud of amorphous substance designated the pericentriolar cloud (29). An accumulation of studies has shown that the centrioles are not required for mitosis (1, 2, 5, 6, 33), although a role for the centriole in the production or organization of the pericentriolar cloud has not been excluded.

On the other hand, the pericentriolar cloud has been shown to be the focus of microtubule organization (26) and can serve to nucleate the formation of microtubules in vitro (8). Therefore, attention has shifted to investigating the molecular composition of the cloud.

Efforts to isolate and purify the cloud have so far not been successful, principally because of the small amount of material available and the lack of a simple and reproducible assay system. Consequently, we have chosen to use hybridoma technology, combined with immunocytochemistry, to identify, from heterogeneous isolated spindle fraction, components specific to the centrosome. A number of previous studies using preimmune or immune sera (3, 4, 13, 22, 23, 25, 32) have reported labeling of centrosomes and preliminary identification of centriolar antigens (21, 35). However, these antigens were mainly common to centrioles and basal bodies (4, 13, 23, 25). One report, in which the monoclonal antibody technique was used, describes antibodies that reacted with the microtubule organizing centers of several lines of cultured animal cells (28). These antibodies additionally reacted with either *Tetrahymena* basal bodies, Chinese hamster ovary cell centrioles, or vimentin-type intermediate filaments, suggesting that they were directed toward determinants shared among several cytoskeletal elements. However, no characterization of the antigenic determinants has yet appeared.

Sea urchin eggs have traditionally been an important source

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of material for studies on mitosis since they provide a naturally synchronizable, homogeneous population of dividing cells available in substantial quantities. The mitotic centers of dividing sea urchin eggs are larger than those of cultured cells and have been referred to by a different term—the centrosphere (9; see reference 37 for review); however, the functional role is thought to be equivalent.

A preliminary report has already been made by Izant et al. (11) to identify spindle components by using the monoclonal antibody technique. We have also employed the same technique of monoclonal antibody in a "shotgun" approach to identify hitherto unknown molecular components in a heterogeneous preparation of isolated mitotic spindles. In this paper, we focus in particular on two antibodies that are directed specifically toward determinants of the centrosphere.

A brief account of this work has already appeared elsewhere (18).

MATERIALS AND METHODS

Isolation of Mitotic Apparatus, Centrosphere, and Taxol Asters from Sea Urchin Eggs

Sea urchins, *Strongylocentrotus purpuratus* and *Lytechinus pictus*, were obtained from Pacific Bio-Marine Laboratories Inc., Venice, CA; *Arbacia punctulata* were collected at the Marine Biological Laboratory, Woods Hole, MA. Gametes were obtained and fertilized as described previously (17). Taxol asters were induced by treatment of unfertilized eggs with 1–10 μg/ml taxol for 30–90 min at room temperature (31). Taxol-induced asters and mitotic apparatus at metaphase were isolated and purified as described elsewhere by using 1 M glycerol, 5 mM MES (2, [*N*-morpholino]ethane sulfonic acid), 1 mM EGTA, 2 mM $MgSO_4$, 0.05% Triton X-100 at pH 6.15 as a standard isolation medium (17). To check extraction of antigens from mitotic spindles during the course of spindle isolation, we also isolated spindle fractions in media prepared with different microtubule-stabilizing reagents and at different pH, ionic strength, and detergent concentration from the standard isolation medium. The differences included 1 M hexylene glycol-5 mM MES at pH 6.15 (12), 25% glycerol-10 mM PIPES-5 mM EGTA-0.5 mM $MgCl_2$-0.5–1% Triton X-100 at pH 6.8 (30), or 100 mM PIPES-1 mM EGTA-1 mM $MgCl_2$-0.2% Triton X-100 at pH 6.8 (27).

Centrospheres were prepared by extracting the metaphase spindles with 0.5 M KCl in the standard isolating medium for 10–30 min at room temperature (17).

Preparation of Monoclonal Antibodies

IMMUNIZATION AND CELL FUSION: Female BALB/c mice were immunized with mitotic spindles or taxol asters isolated from eggs of *Strongylocentrotus purpuratus*. About 100 μg/ml of native protein in Freund's complete adjuvant was injected intraperitoneally. During a 4–6 wk period, each mouse received three additional 100-μg injections of denatured proteins, and on the third day after the last boost, they were killed and the spleens were removed for fusion. Hybridomas were prepared by a method first described by Kohler and Milstein (16). After fusion of spleen cells and NS-1 mouse myeloma cells in 50% polyethylene glycol 1500, they were then plated into 96-well plates containing a feeder layer of mouse red blood cells, and grown for 1–2 wk in medium containing hypoxanthine, amethopterin, and thymidine.

SCREENING: Two stages of screening were done to identify monoclonal antibodies specific to the sea urchin mitotic spindle. For both stages, indirect immunofluorescence was applied to test material adsorbed to a solid substratum, but in the first stage, the test materials were isolated mitotic spindles, and in the second stage, the test materials were whole sea urchin eggs at first mitosis. Isolated mitotic apparati were fixed and stored in methanol, and whole eggs (10) were stored in 50 mM EGTA in methanol at −20°C until use. After rehydration with phosphate-buffered saline (PBS), plates or coverslips were incubated with test supernatants for 1–1.5 h at 37°C, rinsed thoroughly with PBS, and then stained with second antibodies as described in a previous paper (19).

The purpose of the first stage of screening was to rapidly identify potential hybridomas positive for spindle antibody production. Isolated spindles were adsorbed to the bottom of wells of a 96-well microtiter dish (Costar, Cambridge, MA), and hybridoma supernatants were added and assayed by indirect immunofluorescence using a 16× phase neofluar objective on a Zeiss inverted microscope (Carl Zeiss, Inc., Oberkochen, FRG) equipped for epifluorescence. In this way, the screening could be done directly on the microtiter dish. Whole eggs were not suitable for the initial screening because they did not adhere tightly to the wells and would be removed during the washing steps and changes of solution. Of 1,820 wells in the initial plating, 1,172 contained growing hybridomas after 1–2 wk and, of these, 94 were positive in the first screening. These were immediately screened by indirect immunofluorescence on whole eggs adsorbed to coverslips.

The purpose of the second stage of screening was to confirm spindle-specific antibodies. Isolated spindles might have stained positively if they had trapped some cytoplasmic component toward which the antibody was directed. If this were the case, their staining in the whole eggs would be distributed throughout the cytoplasm and not confined to the spindle. By examining the staining in whole eggs, we could distinguish between antibodies directed toward bona fide components of the spindle and adventitious contaminants. The staining patterns in whole eggs were examined by epifluorescence using 25× or 40× phase neofluar objectives. Objectives of higher magnification were not useful on the whole eggs because of their limited depth of focus. Positive hybridomas were defined as showing clearly brighter staining in the spindle region than in the cytoplasm.

Of the 94 positive wells in the first screening, five hybridomas were deemed to exhibit specific staining of the mitotic apparatus in whole eggs. In addition, one hybridoma, selected from the group of 89, was positive in the first screening but negative in the second screening. These six hybridomas were subcloned twice by the limited dilution method and assayed in each instance by indirect immunofluorescence using isolated spindles adsorbed to microtiter plates. Two hybridomas (SU1 and SU2) stained the chromosomes, two hybridomas (SU4 and SU5) stained the centrosphere, one hybridoma (SU7) stained the fibers of the spindle, and the hybridoma obtained from the group of 89 (SU6) stained the cytoplasm and especially the cortex. This paper will deal principally with the centrospheric antibodies SU4 and SU5.

ANTIBODY CLASS: After subcloning, the antibody class of the monoclonals was determined by immunoprecipitation in an Ochterlony test. Hybridoma SU4 produced IgM, whereas SU5 produced IgG immunoglobulins (data not shown). The IgM class of SU4 was exploited in double label immunofluorescence with monoclonal antitubulin (see below) since the antitubulin was of the IgG class and the two antibodies could be distinguished by class-specific, fluorophore-conjugated secondary antibodies.

CLONING AND LARGE SCALE PREPARATION OF MONOCLONAL ANTIBODIES: Positive hybridomas were subcloned by limited dilution with feeder cells at 3–4 wk after fusion. After a second subcloning, hybridomas were retained if they showed a proportion of positive response ≥60%. To obtain large quantities of antibodies, ascites fluid was prepared by injection of twice-subcloned hybridomas into mice.

FLUORESCENCE MICROSCOPY: Isolated mitotic apparatus, centrospheres, or taxol asters and whole eggs at various stages of development were mounted on polylysine-coated coverslips and fixed with cold methanol with or without 50 mM EGTA as above. Before fixation, mitotic cells on coverslips were sometimes extracted with 0.4 M PIPES, 1 mM EGTA, 2 mM $MgSO_4$, 0.05% Triton X-100 at pH 6.7 for several minutes. Extraction of metaphase eggs with this detergent buffer did not alter the staining pattern with antitubulin, SU4, and SU5 antibodies at all. After the coverslips were washed three times with PBS, they were then incubated 1–2 h with 3% gelatin dissolved in PBS. They were next treated with diluted ascites fluid containing monoclonal sea urchin spindle antibodies or monoclonal antibody raised against yeast tubulin (15) (generous gift from Dr. J. V. Kilmartin, Medical Research Council, Laboratory of Molecular Biology, Cambridge, England). For negative controls, we treated whole eggs or isolated structures with either PBS or supernatants secreted by negative hybridoma clones.

For double immunofluorescence staining of the mitotic apparatus, coverslips with adsorbed mitotic apparatus were first treated with antispindle antibody, then with fluorescein-conjugated goat anti-mouse IgM (mu chain specific) (Miles Laboratories Inc., Naperville, IL). After the coverslips were thoroughly washed with PBS, antitubulin antibody was added, then rhodamine-conjugated rabbit anti-rat IgG (gamma chain specific).

Microscope observations were made under a Zeiss Inverted or Zeiss Universal microscope (Carl Zeiss, Inc.) equipped with epifluorescence optics, and photographed with Kodak Tri-X film.

IMMUNOBLOTTING: Pellets of whole mitotic eggs or isolated spindle and centrosphere fractions were resuspended and boiled in SDS sample buffer as described in a previous paper (19). Gradient polyacrylamide gels (5–15%) were prepared according to the method of Laemmli (20). Proteins on the gels were electrophoretically transferred (34) to 0.15 or 0.45-μm nitrocellulose membranes (Schleicher and Schuell, Inc., Keene, NH) in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol at pH 8.5) with 0.2% SDS overnight, followed by transfer in the same buffer without detergent for 2 h at

4°C. To identify total protein, some lanes of the nitrocellulose membranes were stained with 0.2% Brilliant Black (Pelikan AG D-3000, Hanover 1, Germany) in 45% methanol, 7% acetic acid, and the staining was stopped with distilled water. Other parallel lanes were first blocked with 3% gelatin dissolved in 20 mM Tris-HCl, 500 mM NaCl at pH 7.5 (TBS) for 2 h and incubated with monoclonal antibodies in TBS containing 1% gelatin. The nitrocellulose strips were rinsed in TBS for 20–30 min, then further incubated for 2 h with second antibody of either peroxidase-conjugated IgG fraction, goat anti-mouse IgM (mu chain specific), or peroxidase-conjugated affinity purified goat anti-mouse IgG (heavy and light chain specific) (Cappel Laboratories, Cochranville, PA) in a medium of 1% gelatin containing TBS. Immunoreactive polypeptides were visualized by incubation of the nitrocellulose strips with 4-chloro-1-naphthol to develop the color of substrate against peroxidase according to the procedure of Bio-Rad Immun-Blot Assay Kit (Bio-Rad Laboratories, Richmond, CA).

RESULTS

Morphology of Antibody Staining

Immunofluorescence microscopy of monoclonal antibody staining of whole mitotic eggs is presented in Fig. 1, both at low magnification (*A*, *C*, and *E*) to show the uniformity of the staining reaction, and at high magnification (*B*, *D*, and *F*) to show structural details. Monoclonal antitubulin (*A* and *B*) bound to fibers of the mitotic spindle, whereas antibodies of US4 (*C* and *D*) and SU5 (*E* and *F*) bound primarily to the central region of the aster resulting in the staining pattern of a dumb-bell shape. However, the patterns of SU4 and SU5 were slightly different from each other in that SU5 also showed staining in the spindle region (the region between the asters), whereas the staining by SU4 was confined to the astral region.

The centrospheres develop as the egg progresses toward division. Antibodies to the centrosphere would be expected to show an equivalent developmental pattern. Therefore, the temporal specificity of the antibody reaction was assayed during the process from fertilization to first cleavage. As has already been reported (10), antitubulin labeled the microtubule fibers of the sperm aster (Fig. 2*A*), cortical microtubules in interphase (not shown), and first cleavage spindles at various stages (Fig. 2, *B*, *C*, and *D*). With the monoclonal antibodies SU4 and SU5, staining first appeared as two concentrations in the perinuclear region. Unlike the antitubulin staining, no fibrous patterns were obvious. The staining regions enlarged and then moved apart as the egg passed from metaphase to telophase, which is in good agreement with light (10, 37) and electron (7) microscopic observations of changes in the centrospheric region during mitosis. Thus, the temporal sequence of changes in staining with SU4 and SU5 were consistent with their reaction with centrospheric components

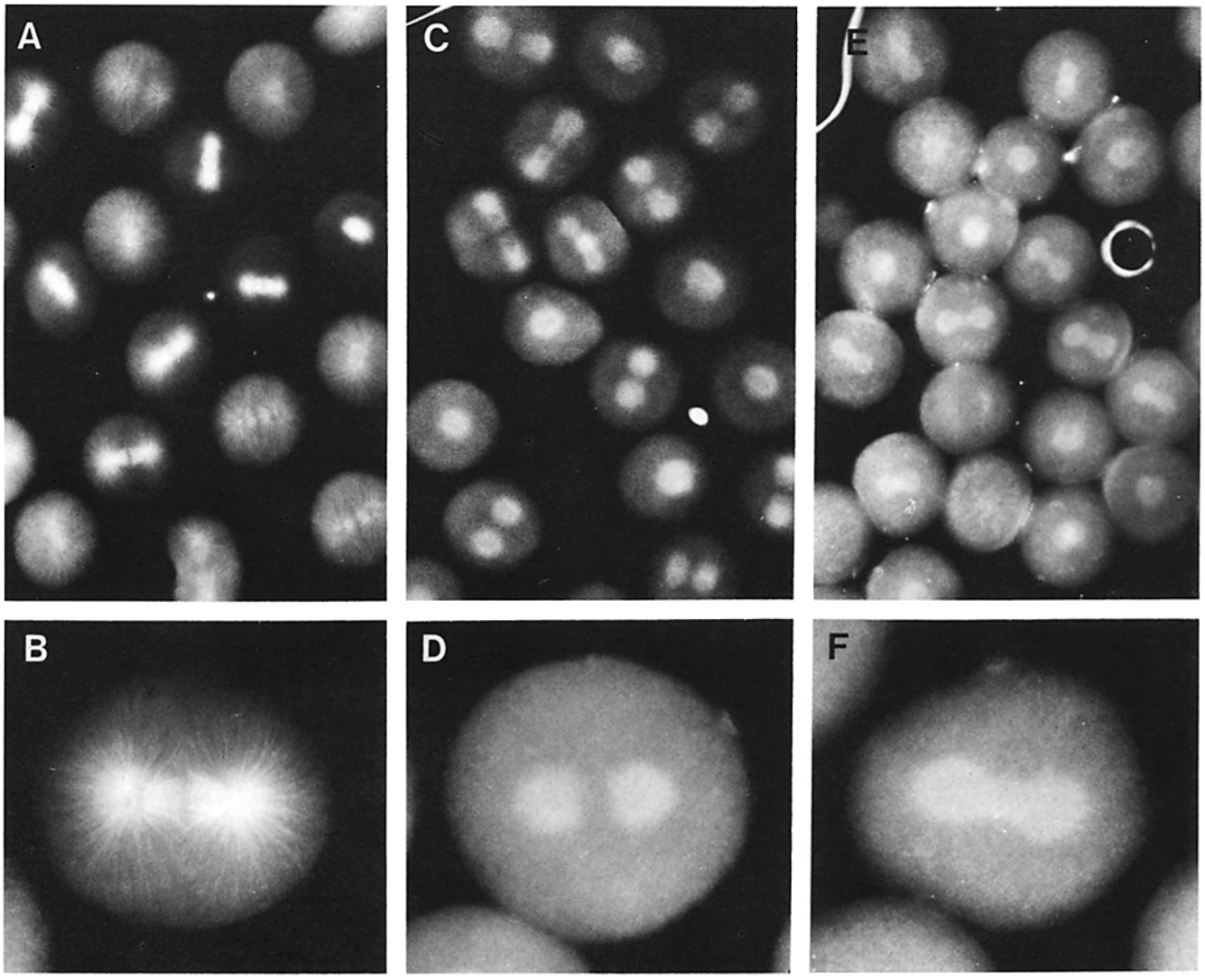

FIGURE 1 Immunofluorescence micrographs of mitotic sea urchin eggs stained with monoclonal antitubulin (*A* and *B*), SU4 (*C* and *D*), or SU5 (*E* and *F*) antibodies. (*A*, *C*, and *E*) × 160; (*B*) × 450; (*D* and *F*) × 460.

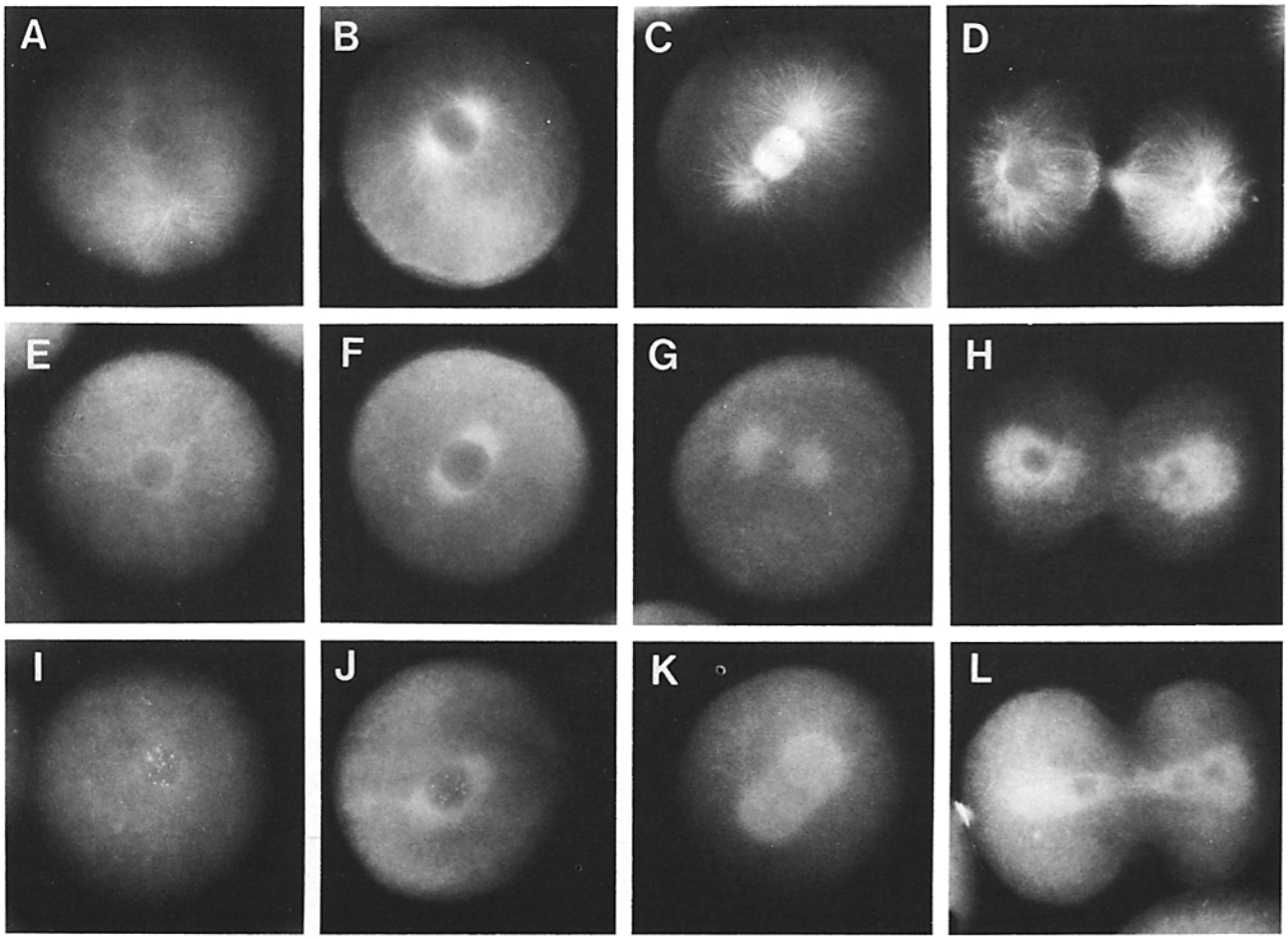

FIGURE 2 Immunofluorescence micrographs of sea urchin eggs stained with monoclonal antitubulin (*A–D*), SU4 (*E–H*), or SU5 (*I–L*) antibodies. Photographs were taken according to the process of egg development from fertilization to first cleavage. (*A, B, E, G, H,* and *K*) × 350; (*C*) × 300; (*D*) × 310; (*F* and *L*) × 340; (*I*) × 380; (*J*) × 320.

(7). In addition, dot staining with SU5 was observed over the nucleus (Fig. 2, *I* and *J*). Although the same type of intranuclear staining has been observed in sea urchin eggs after incubating with antitubulin antibody (10), we detected the dot staining only in eggs treated with SU5 antibody. The significance of this reaction is not known.

In order to study the specificity of antibody localization in more detail, isolated mitotic spindles were immunofluorescently stained and examined with a high numerical aperture objective (63×, 1.4 numerical aperture phase planapochromat). The results are shown in Figs. 3 and 4. In Fig. 3, the staining patterns of spindles incubated with antitubulin (*A, B*), SU4 (*C* and *D*), and SU5 (*E* and *F*) antibodies are illustrated. Panels are paired to show the same isolated spindles as seen by phase-contrast (*A, C,* and *E*) and fluorescence (*B, D,* and *F*) microscopy. As expected, the fibrous microtubular structures in the spindle and asters are evident after antitubulin staining. Because of a higher density of microtubules at the central region of asters, especially bright fluorescence is seen at the rim of the centrosphere. Monoclonal antibodies SU4 and SU5 seemed to react primarily with the centrospheric region of the isolated spindle, although weaker staining was also evident throughout the spindle structure. In contrast to the antitubulin staining, fibrous patterns were weak and barely discernible in the spindles stained with these antibodies. The isolated spindle in Fig. 3, *C* and *D* was somewhat compressed in the pole-to-pole direction, and formed "D"-shaped rings of fluorescence at each pole. While SU4 showed dot staining along the astral and spindle fibers (Fig. 3, *D*), some continuous fibrous staining was detectable in the isolated spindle treated with antibody SU5 after adjusting the planes of focus (Fig. 3, *F*).

To check whether there were immunoreactive components readily extractable from the spindle during the isolation step, we prepared mitotic spindles from metaphase eggs with media different in pH, ionic strength, and detergent concentration from the standard solution. Variations in the isolation medium included: (*a*) 5 mM MES, 1 M hexylene glycol at pH 6.15 (12); (*b*) 25% glycerol, 10 mM PIPES, 5 mM EGTA, 0.5–1 mM $MgCl_2$, 0.5–1% Triton X-100 at pH 6.8 (30); (*c*) 100 mM, 1 mM EGTA, 1 mM $MgCl_2$, 0.2% Triton X-100 at pH 6.8 (27). Identical patterns of antibody staining were obtained for mitotic apparatus isolated with control as well as three different media (data not shown). Mitotic apparatus isolated with the standard isolation medium were also further treated either with 1% Triton X-100, 0.5 M KCl, 10 mM EDTA, or 10 mM EGTA in the standard isolation medium for 15–60 min at room temperature. Neither treatment resulted in loss or change in the staining of mitotic apparatus with SU4 and SU5 antibodies. Salmon and Segall have already reported that the isolated spindles with medium *b* were highly extracted and contained no membranes (30). Also, the treatment of isolated spindles with 1% Triton X-100 resulted in the prominent feature of fibrous elements in spindle and

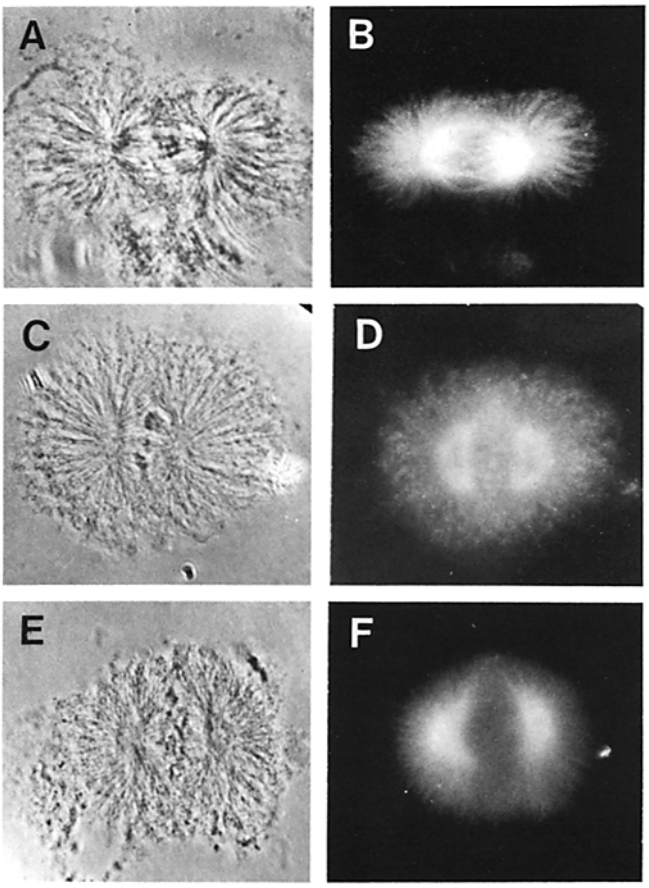

FIGURE 3 Light micrographs of isolated mitotic apparatus. The micrographs are paired to show the same mitotic apparatus in phase-contrast (*A*, *C*, and *E*) and fluorescence (*B*, *D*, and *F*) microscopy. Mitotic apparatus was stained with anti-tubulin (*A* and *B*), SU4 (*C* and *D*), or SU5 (*E* and *F*) antibodies. (*A* and *B*) × 540; (*C*, *D*, *E*, and *F*) × 620.

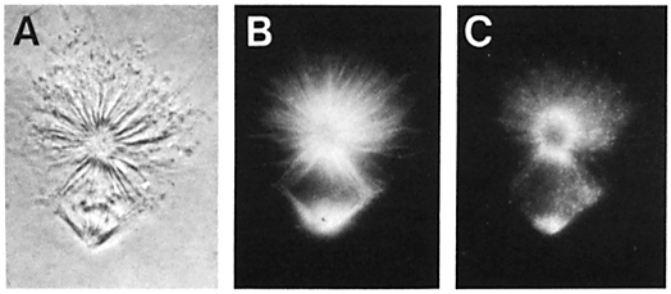

FIGURE 4 Light micrographs of isolated mitotic apparatus as seen by phase-contrast (*A*) and fluorescence (*B* and *C*) microscopy. The same mitotic apparatus was stained with antitubulin (*B*) and SU4 (*C*) antibodies. × 600.

asters. Therefore, it would be unlikely that the antibodies recognized components of membranes associated with the poles; rather, they appeared to react with integral components of the centrosphere specifically.

To delineate more clearly the difference in localization patterns, double indirect immunofluorescence staining was done with antitubulin and SU4 antibodies as outlined in Materials and Methods. The isolated structure presented in Fig. 4 lacked an aster at one side and was selected to demonstrate clearly the staining patterns in the polar region with the different antibodies. Again, while spindle and astral microtubules were manifest with tubulin staining (Fig. 4 *B*), only the centrospheric area was well visualized after staining with SU4 monoclonal antibody (Fig. 4 *C*).

Centrospheres were isolated from metaphase spindles to further test the specific binding capacity of these structures. Both SU4 (Fig. 5) and SU5 (data not shown) stained the isolated centrospheres brightly, showing clearly that the staining reaction was not dependent on the presence of microtubules and was retained in the isolated centrospheres. These isolated centrospheres have already been demonstrated to be able to nucleate microtubule assembly in vitro (17).

Asters induced in unfertilized eggs by treatment with taxol were also examined. In contrast to the asters at the poles of spindles, no particular staining was observed with SU4 or SU5 at the center of the taxol aster. However, as with natural asters, weak punctate staining with SU4 and continuous staining with SU5 was observed along the astral fibers (data not shown).

The species specificity of the staining reaction was examined with isolated spindles of *Lytechinus pictus* and *Arbacia punctulata*. Although spindles of both sea urchins stained strongly with the monoclonal atitubulin, no cross-reaction was detected with the SU4 and SU5 antibodies raised against *Strongylocentrotus purpuratus*.

Molecular Identity of Antigens

The molecular specificity of antibodies SU4 and SU5 was examined by the immunoblotting technique. Proteins separated on one-dimensional polyacrylamide gels were transferred to nitrocellulose membranes. Lane *BB* in Fig. 6 shows the Brilliant Black–stained microtubule proteins (*MT*), and samples containing whole lysate of metaphase eggs (*L*), isolated mitotic spindles (*S*), and isolated centrospheres (*C*), respectively. Panel *SU4* demonstrates identification of immunoreactive polypeptides to antibody SU4. The major reactive species was 190 kD. In addition, reactive species at 180, 80, and 20 kD were detected. These polypeptides were not detectable in the fraction of whole cell lysate. When spindles were isolated in a medium containing 0.3 mg/ml of the protease inhibitor, phenylmethylsulfonyl fluoride, the same blotting pattern as lane *S* was obtained. Therefore, it seems unlikely that proteolytic degradation of antigens during sample preparation was the cause of the multiple reactive species. The 80-kD polypeptide was not retained in the isolated fraction of centrospheres, suggesting that it is not a centrospheric component but may be a microtubule-associated protein of the spindle.

A 50-kD polypeptide was a band that reacted with antibody

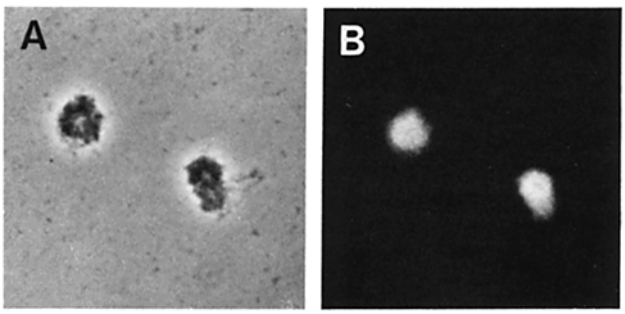

FIGURE 5 Light micrographs of isolated centrospheres as seen by phase-contrast (*A*) and fluorescence (*B*) microscopy. Centrospheres were stained with SU4 antibody. × 600.

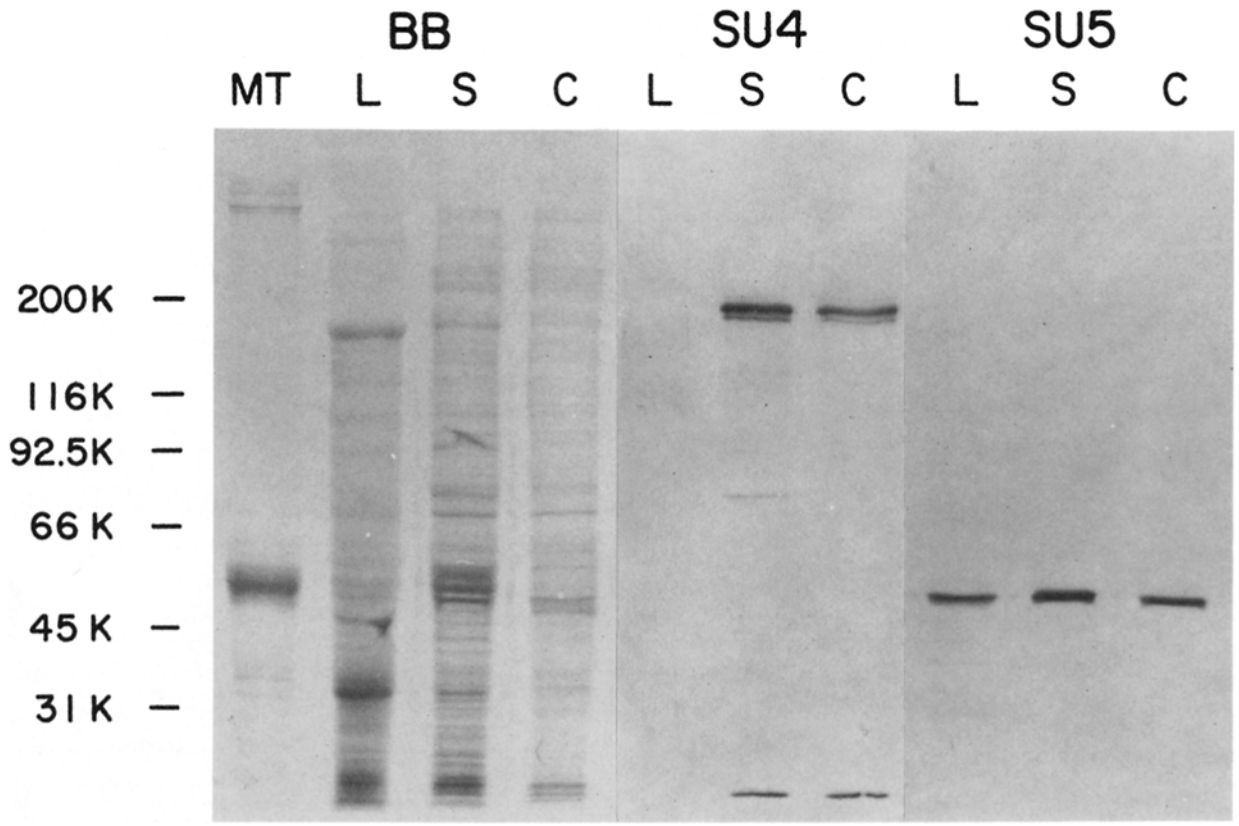

FIGURE 6 Immunoelectrophoretic detection of antigens reactive with the monoclonal antibodies SU4 and SU5. *BB*, nitrocellulose membrane stained with Brilliant Black after electrophoretic transfer of proteins. *SU4*, immunoreaction of nitrocellulose blot with antibody SU4. *SU5*, immunoreaction of nitrocellulose blot with antibody SU5. Lane *MT*, high-speed supernatant fraction of porcine brain microtubule protein purified by two cycles of assembly and disassembly. Lane *L*, whole lysate fraction of metaphase eggs. Lane *S*, fraction of isolated mitotic spindles. Lane *C*, fraction of isolated centrospheres. Numbers at left indicate positions of molecular weight markers myosin (200 kD), β-galactosidase (116 kD), phosphorylase B (92.5 kD), BSA (66 kD), ovalbumin (45 kD), and carbonic anhydrase (31 kD).

SU5 in whole cell lysate, isolated spindles, and centrospheres. It should be noted here that, unlike SU4 antigens in whole cell lysate (Fig. 6, lane *L* in panel *SU4*), the 50-kD polypeptide antigen of SU5 was clearly identified in the whole cell lysate (lane *L* in panel *SU5*). Since the background fluorescence of the cytoplasm had a tendency to be higher in SU5-treated cells than in eggs treated with antitubulin or SU4 antibody (Fig. 1, *A*, *C*, and *E*), it is not clear whether the 50-kD antigen is localized in the cytoplasm as well as in the mitotic spindle in whole metaphase eggs.

The bands appearing on the immunoblots were not prominent on the gels and nitrocellulose membranes stained with Coomassie Blue and Brilliant Black, respectively, suggesting that the components recognized by antibodies SU4 and SU5 were not present at high concentrations in the mitotic spindle.

DISCUSSION

We here report the first preparation of monoclonal antibodies against previously unidentified components of the centrosphere in the mitotic apparatus of sea urchin eggs. It has been observed that the spindle and astral microtubules of sea urchin eggs appear to end at the periphery of the centrosphere where an array of dense granules is seen (9). The centrosphere, containing randomly oriented microtubule fragments, enlarges during the progress of mitosis. Since the microtubule density increases as the focal point is approached, the enlarging centrosphere can be visualized by antitubulin staining as an expanding bright rim around the pole (10). In contrast, monoclonal antibodies SU4 and SU5 stained the isolated mitotic apparatus quite differently. Fibrous patterns were weak or absent after SU4 and SU5 staining, whereas the centrospheric region was stained brightly. A strong positive reaction of the antibodies to the isolated centrosphere that no longer reacted with antitubulin antibody (data not shown) further supports the presence of antigens specific to this area.

It is important to discuss the structural components of the spindle/centrosphere that are reactive with SU4 and SU5 antibodies. The preparation of mitotic spindles with a high concentration of detergent or extensive extraction of isolated spindles with detergent resulted in the disappearance of almost all membranous structures (30). Extracted spindles with detergent, however, could still react with the antibodies, suggesting that these putative structural components of the spindle are not membranes associated with the poles, but rather integral component(s) of the centrosphere. Furthermore, the antigenic determinants of the centrosphere were resistant to extraction by a high ionic strength or by a high concentration of chelating reagents such as EGTA or EDTA. It would be interesting to know whether those antigens are involved in the ability of centrospheres to initiate microtubule nucleation both in vivo and in vitro (17).

The staining pattern of isolated spindles was different in detail from that of whole mitotic eggs. The astral area was

stained evenly in whole eggs, resulting in a dumb-bell-shaped staining pattern. Interestingly, the same kind of dumb-bell image, although much weaker in staining intensity than with SU4 and SU5 staining, was obtained by incubation of whole metaphase eggs with human autoimmune sera (3) or with antimicrotubulin-associated protein 1 antibody (32) (Kuriyama, R., unpublished result). Since both of those antibodies are already known to react specifically with centrosomal components in cultured mammalian cells, the dumb-bell image in mitotic sea urchin eggs might be common to several kinds of anticentrosomal antibodies. In contrast, the centrosphere was frequently seen to contain a nonstaining lumen in isolated spindles. Further, while SU5 stained the entire spindle region in whole eggs, only the centrospheres were strongly stained in the isolated apparatus, with the area between the centrospheres weakly fluorescent. Since sea urchin eggs are quite large, it might be hard to detect the precise pattern of antigen distribution within spindles unless the immunoreactive structures are distinct enough to define in vivo, such as the microtubular fibers. Therefore, it seems difficult to conclude here whether these differences in staining of whole eggs and isolated structures simply result from limited resolution in images of whole eggs, or whether the distribution of antigens in the spindles was modified during the isolation process.

The monoclonal antibodies SU4 and SU5 reacted with distinct and different molecular species, the major ones being 190 and 50 kD, respectively. It is of interest to know whether they are related to the polypeptides of high molecular weight microtubule-associated protein 1 (32), 200 kD (28), 50 kD (35), or 14 and 17 kD (21) reported as centriole-associated proteins in a diverse variety of cell lines. Antibody SU4 also reacted with the 80-kD band, which was extracted when the spindle microtubules were depolymerized by high salt treatment. This polypeptide may be related to the 80-kD (14) or 77-kD (36) proteins identified as a microtubule-associated protein of the spindles in the same sea urchin species. Further analysis of these antigens would be desirable from a biochemical and functional point of view.

The production of hybridomas is now a widespread laboratory procedure, and antibodies obtained by this method are especially powerful probes for unknown molecules in heterogeneous cell fractions as shown in this study. However, it is important to point out that monoclonal antibodies generally recognize only a single determinant of a given protein molecule and it frequently happens that they cross-react with essentially nonrelated proteins that nevertheless happen to share the determinant of the immunogenic peptide (24). Because of the possibility of adventitious cross-reaction via common determinants, it is not possible to conclude from the immunoblot data derived from a single monoclonal antibody that all of the reactive polypeptides are indeed constituents of the structures. Further immunocytochemical studies will be required before a definitive identification of centrospheric components can be made.

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