Association of Anti-Dynein-1 Cross-Reactive Antigen with the Mitotic Spindle of Mammalian Cells

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ABSTRACT. Two anti-dynein-1 antibodies were affinity-purified from rabbit antiserum produced by immunization with dynein-1 from sea urchin sperms. One was prepared with dynein-1 as the ligand and the other with the A heavy chains of dynein-1 as the ligand. In Western blots of axonemal proteins, the former antibody reacted with the A heavy chains of dynein-1 as well as with several other smaller polypeptides, whereas the latter bound almost exclusively to the A heavy chains.

PtK₂ cells stained by indirect immunofluorescence with either of these antidyneins had identical fluorescence patterns. The interphase cell showed rather diffuse and weak fluorescence in its nucleus and perinuclear cytoplasm. Its primary cilium and its centrioles also fluoresced. During prophase and prometaphase, a more intense fluorescence was present in the asters and developing spindle. During metaphase and anaphase the half-spindles fluoresced intensely in a fibrous pattern that corresponded to that of the spindle fibers, showing less intense fluorescence in the anaphase interzone. In telophase and early interphase, the intercellular bridge on each side of the midbody also was stained. These results are evidence that dynein-1, specifically the A heavy chains and/or a related antigen, is densely packed in the mitotic spindle of PtK₂ cells.

In view of certain well-studied types of cell motility, such as cytoplasmic streaming (21) and flagellar and ciliary movements (11), search for the force-generating molecules in the mitotic apparatus might shed light on the movement of chromosomes in dividing cells. The involvement of actomyosin-based motility, however, is dubious because there is no indication that there is increased accumulation of actin in the spindle (2, 18); also, actin and myosin inhibitors have no effect on anaphase chromosome movement (3, 7, 24). It appears that cellular movements based on microtubules (MTs) merit more attention (16, 34).

Mohri et al. (27) were the first to use an antidynein antibody to cleaving sea urchin eggs. Using indirect immunofluorescence with a rabbit antiserum against fragment 1A (an ATPase-active tryptic fragment of dynein-1 (29)), they found intense fluorescence localized in the mitotic apparatus and the cortex which suggested the presence of dynein in these structures.

In collaboration with Mohri and Ogawa, we found that the same antiserum also stained the spindle of rat-kangaroo PtK₁ cells, as did another rabbit antidynein-1 prepared by Ogawa (18, 19, 31, 32). Preliminary absorption experiments indicated
that the fluorescence was due mainly to the presence of dynein-1 or a related antigen(s). In addition, immunofluorescent studies made of various types of cells using Ogawa’s antidynein revealed a similar localization pattern of fluorescence in the spindle of all the somatic cells investigated, both vertebrate and invertebrate. A third antidynein antiserum obtained in our laboratory was prepared in the same way as the second antiserum from a rabbit sensitized with purified dynein-1 isolated from sea urchins (17). We report here that two antibodies that we affinity-purified from this antiserum also stained the spindle of PtK\textsubscript{2} cells. One of these antidyneins bound almost exclusively to the A bands of dynein-1 in immunoblots of axonemal proteins, evidence that the spindle contains either the A heavy chains of dynein-1 and/or some other cross-reactive antigen(s).

**MATERIALS AND METHODS**

*Preparation of antigens for purification of anti-dynein-1s by affinity chromatography.* Two different antigens were used. One was dynein-1, the other A heavy chains eluted from a gel containing A heavy chains of dynein-1 that had been isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Spermatozoa were obtained from the sea urchin *Anthocidaris crassispina* for the preparation of the former antigen and from *Hemicentrotus pulcherrimus* for the latter by injecting 0.5 M KC\textsubscript{1} into the body cavity. The semen collected was mixed with a 2-fold volume of medium that consisted of 20 mM Tris-thioglycolate buffer (pH 8.3), 10 mM MgCl\textsubscript{2}, 0.3 M KC\textsubscript{1} and 50% glycerol, after which it was stored at $-20^\circ$C until use.

Axonemes were prepared from the glycerinated spermatozoa, and crude dynein was extracted with a high salt-extracting solution by the method of Ogawa et al. (30) with slight modifications. Axonemes were suspended in a high salt-extracting solution [10 mM Tris-HCl buffer, pH 8.0, containing 0.5 M KC\textsubscript{1}, 2 mM MgCl\textsubscript{2}, 0.1 mM EDTA (ethylenediamine tetraacetic acid) and 0.1 mM EGTA (ethylene glycol bis(β-aminoethylether)-N,N',N'-tetraacetic acid), 0.1 mM DTT (dithiothreitol), 0.1 mM PMSF (phenylmethylsulfonyl fluoride) and 10 μg/ml leupeptin]. After being shaken for 30 min at room temperature, the suspension was centrifuged at 12,000 g for 15 min to obtain the crude 0.5 M KC\textsubscript{1}-extractable dynein-1.

To purify the dynein-1 that was used for the affinity-purification of one anti-dynein-1, we dialyzed crude dynein from axonemes of *A. crassispina* against 10 mM Tris-HCl buffer, pH 8.0, containing 0.2 mM EDTA, 0.1 mM EGTA, 0.1 mM DTT, 0.1 mM PMSF and 1 μg/ml leupeptin (TED buffer). The crude dynein was concentrated with an ultrafiltration membrane (PM-10, Amicon, Danvers, MA.), then dialyzed against TED buffer, after which it was applied to a Sepharose 4B column (2.6 × 85 cm) and eluted with TED buffer. The fractions that constituted the major ATPase activity were combined and concentrated. After dialysis against TED buffer, the solution was made 2 mM (13), with respect to the calcium concentration, by the addition of 20 mM CaCl\textsubscript{2} solution, then it was applied to a calmodulin-Sepharose 4B column. The calmodulin had been purified from bovine brain by the procedure of Yazawa et al. (48) with slight modifications, and was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) by the usual method. About 4.4 mg of calmodulin was bound to 1 ml of the gel.

The column loaded with the fractions described above was washed with TED buffer containing 2 mM CaCl\textsubscript{2} and 0.5 M NaCl until the absorbance at 280 nm was nearly zero. The proteins adsorbed then were eluted with calcium-free TED buffer containing 0.5 M
NaCl. The eluate was dialyzed against TED buffer and concentrated, after which samples were layered on 5 to 20% sucrose density gradients then centrifuged at 26,500 rpm for 20 h using a Hitachi RPS 27-2 rotor. Fractions that constituted the peak of ATPase activity were combined.

To prepare the A heavy chains of dynein-1, we extracted the crude dynein from the axonemes of *Hemicentrotus pulcherrimus* with a high salt-extracting solution. The crude dynein was concentrated then partially purified by sucrose density gradient centrifugation. The ATPase-active fractions obtained were concentrated and electrophoresed on 5% polyacrylamide gel by the method of Laemmli (22). Two pieces were cut, one from each side of the gel and stained with Coomassie brilliant blue. Another piece containing the A heavy chains was excised from the part of the gel between the two stained gel slips. The A heavy chains then were eluted electrophoretically from this peice.

Antiserum and preparation of affinity-purified antidynein-1 antibodies. Antiserum to dynein-1 was raised in a rabbit by the method described elsewhere (17).

Using dynein-1, or the A heavy chains, as the ligand, two kinds of affinity-purified antibodies were obtained by the following procedures: The ligands were coupled by the usual method to Sepharose 4B activated with cyanogen bromide. The method used to prepare the affinity-purified antibodies was essentially the same as that used by Fuller *et al.* (10) to prepare antitubulin antibody. The antiserum was precipitated by adding an equal volume of saturated ammonium sulfate. After centrifugation, the precipitate was resuspended in borate-saline buffer (pH 8.3) then dialyzed against the same buffer. The solution obtained was applied to a Sepharose 4B column (1.6 × 1 cm) that contained dynein-1 or A heavy chains. After the column had been washed completely with the same buffer, the antibodies to dynein-1 were eluted from it with 0.2 M glycine-HCl (pH 2.7). The eluate was neutralized immediately with Na₂CO₃.

Preparation of affinity-purified antitubulin antibody. A rabbit was immunized with bovine brain tubulin, and the antibody was affinity-purified by the method described elsewhere (49).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot techniques. Slab gel electrophoresis was performed by the method of Laemmli (22) in a 5% polyacrylamide gel or in a 5–12%, or 5–15%, polyacrylamide gradient gel in the presence of 0.1% SDS.

The protein bands that developed were transferred to a nitrocellulose membrane as described by Towbin *et al.* (44). The membrane then was stained by the peroxidase anti-peroxidase method with the affinity-purified anti-dynein-1 antibody (10 μg/ml) or the anti-A heavy chain antibody (10 μg/ml).

Protein determination and ATPase assay. Protein was determined by the method of Lowry *et al.* (23) with some modifications. ATPase was assayed by the method described elsewhere (17).

Cells. Rat-kangaroo PtK₂ cells were grown on coverslips in Petri dishes containing Eagle's minimal essential medium (Handaibiken, Osaka) supplemented with a solution of 1 ml nonessential amino acids (Gibco, Chagrin Falls, Ohio) per 100 ml and 10% fetal calf serum. The cells then were incubated for 2 to 3 days at 37°C in a 5% CO₂ atmosphere.

Immunofluorescent staining of PtK₂ cells. PtK₂ cells grown on coverslips were fixed for 20 min with 4% paraformaldehyde in buffered saline solution (0.9% NaCl in 0.01 M phosphate buffer, PBS, pH 6.5). After being washed in PBS (pH 6.5), the PtK₂ cells were treated with methanol for 10 min at −10°C and equilibrated in PBS (pH 7.2) to make them permeable. The coverslips then were incubated for 2 h at room temperature in PBS (pH 7.2) containing 1% normal goat serum with the affinity-purified rabbit antibodies (50 μg/ml) to dynein-1, A heavy chains or tubulin. After being washed extensively in PBS (pH 7.2),
the cells were treated for 2 h at room temperature with 40-fold diluted second antibody (FITC-labeled antirabbit IgG goat serum, MBL, Nagoya, Japan). The coverslips then were washed thoroughly in PBS (pH 7.2) and mounted in glycerol containing 5% n-propyl gallate, pH 8.0. The cells were viewed through a Leitz incident-light fluorescence microscope equipped with the appropriate filters for fluorescein isothiocyanate. Photographic records were made on Kodak Tri-X film through a 100x oil immersion or 40x objective lens.

RESULTS

Characterization of two antidynein antibodies. The dynein-1 used to purify an anti-dynein-1 was composed virtually of A heavy chains only, the admixture of polypeptides related to dynein-1 (e.g., intermediate chains) being almost negligible (Fig. 1, lane 2). Results of immunoblots of 0.5 M KCl-extractable axonemal proteins

![Fig. 1. Electrophoretic patterns of high salt-extractable, axonemal proteins and purified dynein-1 (lanes 1 and 2), and immunoblot characterization of affinity-purified anti-dynein antibodies using axonemal proteins (lanes 3–6). Polyacrylamide gel electrophoresis was run in 0.1% sodium dodecyl sulfate by Laemmli's method on 5–12% (lanes 1 and 2), or 5–15% (lanes 3 and 5), polyacrylamide gradient gel. Coomassie brilliant blue-stained gels of electrophoresed, high salt-extractable, axonemal proteins are shown in lanes 1, 3 and 5. The purified dynein-1 used for affinity-purification of antibody D is shown in lane 2. Arrowheads indicate the A heavy chains of dynein-1 and the arrows tubulin. Protein bands in the replicate gels of lanes 3 and 5 were transferred to nitrocellulose membranes then stained by the peroxidase anti-peroxidase method with either of the anti-dynein antibodies. Antibody D was affinity-purified on a Sepharose column conjugated with dynein-1 and antibody A on a column conjugated with the A heavy chains of dynein-1. Lane 4 was stained with the D antibody and lane 6 with A antibody. For details see text.]
show that the affinity-purified antidynein-1 (antibody D), prepared with dynein-1 as the ligand, reacted with the A heavy chains of dynein-1 and several other poly-peptides (Fig. 1, lane 4): the first had a molecular weight of about 110 k, somewhat smaller than the intermediate chain (IC) 1; the second had a molecular weight intermediate between IC1 and IC2; the third was similar in molecular weight to IC2; but the fourth and fifth had apparent molecular weights about equal to the respective weights of IC3 and tubulin. A few additional bands with molecular weights much smaller than the weight of tubulin also were stained.

The reactivity of antibody D with proteins present in mitotic spindles (5, 12, 46) also was investigated by immunoblot analysis. Results showed that this antibody does not react with tubulin, τ factor, high molecular weight microtubule-associated proteins (HMW-MAPs), calmodulin, cyclic GMP-dependent protein kinase (G-kinase), the type II regulatory subunit of cyclic AMP-dependent protein kinase (A-kinase) or myosin light chain kinase (MLCK, data not shown).

The other antibody to dynein-1 (antibody A) was affinity-purified using A heavy chains of dynein-1 as the ligand. In immunoblots, the A antibody bound almost exclusively to the A heavy chains of dynein-1 in the crude dynein preparation (Fig. 1, lane 6).

**Immunofluorescent staining of PtK₂ cells with the D and A anti-dynein antibodies.** Indirect immunofluorescence with the D antibody produced mitotic PtK₂ cells that showed specific fluorescence on the mitotic spindle and at the intercellular bridge (Fig. 2), but no fluorescence was detected in control cells that had been treated with the IgG fraction of non-immune serum (Fig. 3). Interphase PtK₂ cells showed diffuse fluorescence in their nuclei. The centrioles and a cilium-like structure (probably the primary cilium, (39, 45)) originating from the centrioles, as well as the perinuclear cytoplasm, also were stained more intensely than the general cytoplasm (Fig. 4). In prophase and prometaphase, the fluorescence of the asters was conspicuous, and tiny fluorescent particles were present in the cytoplasm. Compared with tubulin immunofluorescence (49), the pattern of fluorescence of the aster was not clear, nor were the astral rays stained uniformly. Chromosomes showed no affinity for the antibody (Fig. 5). In metaphase, the spindle was stained intensely in a fibrous pattern that possibly corresponds to the spindle fibers. The centrosomes were also stained (Fig. 6). In anaphase, strong fluorescence was present in both half spindles, the interzonal region also being stained but with less fluorescence than the half spindles (Fig. 7). Although the astral rays were not stained with the anti-dynein during metaphase and anaphase, cells stained with antitubulin showed distinct fluorescence on the astral rays (Figs. 10-12). In telophase, some fluorescence was seen in the peripolar regions and part of the interzonal region was stained in a fibrous pattern, particularly near the chromosomes (Fig. 8). In early interphase, the two daughter cells remained
connected by a fluorescent intercellular bridge, whereas the midbody in the bridge appeared dark. Nuclear fluorescence reappeared (Fig. 9).

The patterns of fluorescence obtained by the A anti-dynein antibody that had been affinity-purified with A heavy chains of dynein-1 were very similar to those obtained with the D antidynein (Fig. 13). Briefly, the specific fluorescence was present

Figs. 10-12. Immunofluorescent patterns of PtK2 cells stained with antitubulin antibody. Fig. 10, metaphase. Fig. 11, early anaphase. Fig. 12, mid to late anaphase. Spindle fibers stained with antitubulin antibody are sharper than those stained with the anti-dyneins D and A. Astral rays are distinct although not stained with the antidyneins (Figs. 6 and 7).
in the nucleus, centrioles and cilium-like structure in interphase, in the asters in prophase and prometaphase, in the centrosomes and spindle in prometaphase through anaphase, and in the intercellular bridge in telophase and early interphase. Fluorescence of the mitotic spindle stained with the A antidynein had the same intensity, or was brighter than that, of the spindle treated with the D antidynein at the same protein concentration (50 µg/ml).

Fig. 13. Immunofluorescent patterns of PtK₂ cells stained with antidynein antibody A affinity-purified with A heavy chains as the ligand. a, interphase. The cilium-like structure is indicated by the arrowhead. b, prometaphase. c, metaphase. d, early anaphase. e, early telophase. f, late telophase. The bar in Fig. 10f represents 10 µm in Figs. 10a–f.
DISCUSSION

Two antidynein antibodies were prepared by affinity column chromatography. One, D antibody, was prepared with dynein-1 as the ligand; it reacted with A heavy chains and several other bands of crude dynein in immunoblots, but not with other antigens of spindle origin: tubulin, HMW-MAPs, τ factor, calmodulin, G-kinase, the regulatory subunit of A-kinase type II or MLCK. The second antibody, A, was separated on a column conjugated with the A heavy chains of dynein-1. Immunoblot staining of this antibody showed that it bound almost exclusively to the A heavy chains. By indirect immunofluorescence both antibodies strongly and similarly stained the mitotic spindle of PtK\(_2\) cells.

These results indicate that dynein-1, specifically its A heavy chains or an A heavy chain-like antigen is present in the mitotic spindle. Both the A\(\alpha\) and A\(\beta\) heavy chains, which make up the A heavy chains of dynein-1, have ATPase activity (4, 43, 47). We need to identify the cell components that react with these anti-dyneins, but so far none has been found in immunoblots of PtK\(_2\) cell extracts.

It must be noted here that the pattern of fluorescence of the mitotic apparatus in PtK cells stained by these affinity-purified antidyneins was indistinguishable from the patterns of fluorescence present when two other antidyneins, anti-fragment 1A (29) and Ogawa’s anti-dynein-1 (31, 32), were used (18, 19). In contrast Zieve et al. (50) reported that a variety of cultured cells including bovine fibroblasts treated with an affinity-purified antibody to dynein from bovine sperm showed no specific affinity, although the antibody reacted with HMW-polypeptides of dynein obtained from bovine sperm flagella.

The difference in the immunofluorescence results obtained in their study and our study reported here may be due to a difference in the antigenic sites of the antibodies used. The dynein or dynein-like molecules present in the mitotic apparatus may not be identical to flagellar dynein molecules. For example, the ATPase of flagellar dynein appears to be more sensitive to vanadate ions than is that of cytoplasmic dynein (1, 38). Thus, an antibody made against bovine sperm dynein may not cross-react with the dynein or dynein-like molecules in the mitotic apparatus.

Cytoplasmic dynein, or dynein-like Mg\(\cdot\)ATPase, has been isolated from unfertilized sea urchin eggs (1, 13, 15, 33, 36, 42). This Mg\(\cdot\)ATPase also has been found in the mitotic apparatus isolated from sea urchins (14, 37). Hisanaga and Sakai (13) have shown that this cytoplasmic dynein is composed of a high molecular weight polypeptide with the same electrophoretic mobility as the A\(\beta\) chain of flagellar dynein. According to Asai (1), his dynein-like Mg\(\cdot\)ATPase is composed, at least in part, of three high molecular weight polypeptides (M\(_r\) ca. 300–350K daltons), the largest two of which appear very similar to the A chains of flagellar dynein. It must be noted that the anti-fragment 1A, which stains the mitotic spindle of PtK\(_1\) cells by indirect immunofluorescence (described above), cross-reacts with the high molecular weight polypeptides of the cytoplasmic Mg\(\cdot\)ATPase reported by Asai (1). The cytoplasmic dynein or dynein-like Mg\(\cdot\)ATPase can bind to reassembled microtubules, and that binding is completely reversed on the addition of Mg\(\cdot\)ATP (1, 13, 15).

Using monoclonal antibodies against subunits of sea urchin flagellar dynein, Piperno (35) showed that three cross-reacting antigens (M\(_r\) ca. 330K, 124K and 72K) are present in sea urchin eggs during first metaphase, in fractions that contain mitotic apparatuses and in cilia. These antigens, however, are not found in unfertilized eggs. Using monoclonal antibodies against 21S dynein from sea urchin sperms, Hisanaga
*et al.* (14) showed evidence of the presence of dynein in the spindle and in the spindle poles of sea urchin eggs. All these results, together with our immunofluorescent data on PtK cells, indicate that it is highly probable that dynein or dynein-like Mg•ATPase is present in the mitotic apparatus of animal cells. Note also that our preliminary study in collaboration with Hisanaga *et al.* showed by indirect immunofluorescence (20) that one of their monoclonal antibodies also reacted with the mitotic spindle of PtK₂ cells.

Anaphase chromosome movement in isolated mitotic apparatuses of sea urchin eggs, which requires ATP and Mg²⁺, was prevented by anti-dynein (fragment 1A) antiserum (40, 41) or vanadate ions in the +5 oxidation state, which state is a potent inhibitor of ciliary and flagellar ATPase. In the permeable cell models of Cande and Wolniak (6) also, anaphase chromosome movement was stopped by +5 vanadate ions. After movement stopped, it could be restarted by converting the vanadate to the inactive +4 state. Although the chromosome movements in these models were considerably slower than in living cells, even in the absence of vanadate and anti-dynein, the experiments do indicate strongly the involvement of dynein in anaphase chromosome movement.

Based on their studies of nucleotide requirements for, and the effects of dynein inhibitors on, anaphase movement in permeable mitotic cells, more recent reports by Cande’s group (8, 9) rule out the possibility of tubulin-dynein mechanochemistry in chromosome-to-pole movement (anaphase A). Dynein or dynein-like Mg•ATPase appears, at least in part, to be responsible for the separation of the spindle poles in anaphase (anaphase B). It is not clear, however, whether dynein functions in the chromosome-to-pole movement.

Currently we are conducting microinjection experiments on mitotic PtK₂ cells using antidynein and inhibitors of dynein ATPase (20). Preliminary results show that when sodium metavanadate or erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) is injected in early anaphase, chromosome movement is inhibited, and both chromosome-to-pole movement and the separation of poles is suppressed. However, anaphase chromosome movement is normal in cells injected with the antidynein D affinity-purified in the present study with dynein-1 as the ligand. The observed effects of vanadate and EHNA are consistent with an anaphase model in which a dynein-mediated sliding mechanism is employed. (This is not necessarily limited to the sliding models (25, 26, 28) already proposed.) Any interpretation of the results of antidynein injection at this time would be premature because no data are available on such properties of the antibody used as its effect on the motility of demembranated sperm models and on the ATPase activity of dynein-1, both of which are now under investigation in our laboratory.

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