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Identification of ubiquitous high-molecular-mass, heat-stable microtubule-associated proteins (MAPs) that are related to the *Drosophila* 205-kDa MAP but are not related to the mammalian MAP-4

(*Dictyostelium*/monoclonal antibody)

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ABSTRACT **AX3, a monoclonal antibody raised against isolated microtubule-organizing centers of *Dictyostelium discoideum*, stains microtubule-containing structures in species ranging from *Dictyostelium* to human. On immunoblots, the AX3 antibody recognizes heat-stable proteins in the 260- to 280-kDa molecular-mass range in a number of different species. The AX3 antigens from HeLa and embryonic mouse fibroblast cells coprecipitate with microtubules *in vitro*, indicating that these antigens are, indeed, MAPs. The AX3 antigens are not immunologically related to the mammalian MAP-2 or MAP-4 but are related to the 205-kDa MAP of *Drosophila*. This report describes a structural-type MAP in *Dictyostelium* and a MAP that is detected in a wide variety of species. The *Drosophila* 205-kDa MAP had previously been proposed to represent a member of the MAP-4 class of proteins. From the results reported here, however, it is suggested that proteins recognized by AX3 monoclonal antibody, including the *Drosophila* 205-kDa MAP, represent a distinct class of MAPs that has been widely conserved through evolution.**

The role of microtubules in a variety of cellular processes appears to be regulated, in part, by the many proteins that associate with microtubules. Microtubule-associated proteins (MAPs) have been characterized in a number of different systems and represent a diverse group of proteins that are generally classified according to their apparent molecular masses or biochemical properties (for reviews, see refs. 1–4). The MAP-2, MAP-4, and τ classes include proteins that are fibrous in structure and are stable at elevated temperatures. Of these, the largest number of proteins so far described has been in the MAP-4 class, members of which range from 180 to 240 kDa and which includes mouse MAP-4 (5), HeLa 210-kDa MAP (6, 7), *Drosophila* 205-kDa MAP (8), the 190-kDa MAPs identified in bovine adrenal gland (9) and rat liver (10), and rat MAP-3 (11).

Complete or partial DNA sequence has now been reported for the genes encoding four of the MAP-4 class of proteins (12–15). Comparison of the predicted amino acid sequences of the HeLa, bovine, and mouse MAP-4 proteins has shown an overall sequence identity of ≈70%; the conserved residues of these three proteins are clustered in four regions, one of which includes the microtubule-binding domain (15). In contrast, the predicted amino acid sequence of the *Drosophila* 205-kDa MAP is not similar to either the mammalian MAP-4 proteins or to any other MAP for which sequence data is available (12).

Using a monoclonal antibody (mAb; AX3) raised against microtubule-organizing centers from the cellular slime mold *Dictyostelium discoideum*, we have identified a high-molecular mass, heat-stable MAP that occurs in species ranging from *Dictyostelium* to humans (16). We show here that the proteins recognized by mAb AX3 in mouse and human are distinct from the previously identified MAP-2 and MAP-4 proteins. However, the AX3 antigens in *Dictyostelium* and mammals are immunologically related to the 205-kDa MAP of *Drosophila*, suggesting that the 205-kDa MAP and the AX3 antigens define a distinct class of heat-stable MAPs.

MATERIALS AND METHODS

Antibodies. The AX3 mAb was raised against an isolated nucleus/nucleus-associated body (the primary microtubule-organizing center of *Dictyostelium*) fraction from the Ax-3 strain of *Dictyostelium discoideum*. The production and preliminary characterization of this mAb are described elsewhere (16).

The antibodies against mouse MAP-4, HeLa 210-kDa MAP, *Drosophila* 205-kDa MAP, and mouse MAP-2 used in this study were provided by J. B. Olmsted (17), G. G. Borisy (6), L. S. B. Goldstein (8), and L. I. Binder (University of Alabama, Birmingham), respectively. Anti-tubulin antibodies used were either an antibody raised against yeast tyrosinated α-tubulin (from J. V. Kilmartin) (18) or an anti-chicken β-tubulin mAb (Amersham).

Cell Culture and Protein Fractionation. *Dictyostelium* cells grown in suspension were collected by centrifugation and resuspended in 8–10 vol of homogenization buffer [0.1 M Pipes, pH 6.9/10% (vol/vol) glycerol/2.5 mM $MgSO_4$/5 mM EGTA/0.5 mM EDTA/leupeptin at 10 μg/ml/chymostatin at 5 μg/ml/N^{α}-(*p*-tosyl)lysine chloromethyl ketone (TLCK) at 30 μg/ml/1.5 mM phenylmethylsulfonyl fluoride]. After cell disruption by sonication, cell debris was removed by centrifugation at 30,000 × *g* for 30 min, and supernatants cleared by additional centrifugation at 100,000 × *g* for 30 min were heated in a boiling water bath for 5–10 min. Heat-stable proteins in the supernatant, obtained by centrifugation at 30,000 × *g* for 15 min, were further fractionated by precipitation with 25% saturated ammonium sulfate.

HeLa and embryonic mouse fibroblast (EMF) cells were cultured as before (19, 20). *Drosophila* embryos were obtained from T. Hays (University of Minnesota, St. Paul). Heat-stable proteins were prepared essentially as described

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Abbreviations: TLCK, N^{α}-(*p*-tosyl)lysine chloromethyl ketone; MAP, microtubule-associated protein; mAb, monoclonal antibody; EMF, embryonic mouse fibroblasts.

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above, except that three to five vol of homogenization buffer, in which pepstatin A at 2 μg/ml substituted for the TLCK, was used. The *Drosophila* embryos were disrupted in a Dounce homogenizer, whereas the HeLa and EMF cells were disrupted by using a Polytron.

***In Vitro* Microtubule Polymerization.** Microtubules were polymerized *in vitro* from extracts of either mitotic or interphase HeLa cells or interphase EMF cultures. Mitotic HeLa cells were prepared by adding nocodazole at 0.1 μg/ml as before (19). After washing twice with cold phosphate-buffered saline, cells were disrupted in three-pellet volumes of microtubule-assembly buffer (0.1 M Pipes, pH 6.8/1 mM $MgSO_4$/2 mM EGTA/2 mM dithiothreitol/0.1 mM GTP/aprotinin at 1.35 trypsin inhibitory units per ml/leupeptin at 30 μg/ml/chymostatin at 10 μg/ml/1 mM phenylmethylsulfonyl fluoride/TLCK at 67 μg/ml). The homogenates were centrifuged at 60,000 × *g* for 1 hr, and the final concentration of GTP and taxol in the supernatant was adjusted to 1.0 mM and 20 μg/ml, respectively. Microtubules polymerized at 37°C for 30 min were sedimented through a sucrose cushion (5% sucrose in assembly buffer plus taxol) at 40,000 × *g* for 30 min and then washed once in taxol-containing assembly buffer.

Immunofluorescence Staining and Immunoblot Analysis. *Dictyostelium* amoebae grown in suspension to a final density of 1–5 × 10^5 were allowed to attach to polylysine-coated coverslips and fixed with 3.7% (vol/vol) formaldehyde in a cytoskeleton buffer (16), according to the two-step fixation protocol of Roos (21). HeLa and EMF cells were cultured directly on coverslips in Dulbecco's minimal Eagle's medium or F-10 medium, washed two times with phosphate-buffered saline, and then fixed for 10 min with 4% formaldehyde in 100 mM Pipes (pH 6.9)/5 mM EGTA/2 mM $MgCl_2$/0.2% Triton X-100. Incubation of cells with antibodies and detection of the fluorescent signal were as described (16, 19).

For immunoblot analysis, protein samples separated on 7 or 10% acrylamide gels were transferred to nitrocellulose and further incubated with the primary antibodies as before (16, 19). Bound antibodies were detected by using alkaline phosphatase-conjugated secondary antibodies, with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indoyl phosphate as enzyme substrates (19).

RESULTS

AX3 mAb Stains Microtubules in a Wide Variety of Species. The AX3 mAb was raised against an isolated microtubule-organizing-center fraction of *Dictyostelium* (16). When used for immunofluorescence staining of *Dictyostelium* amoebae, the mAb reveals microtubules in both interphase (Fig. 1*A*) and mitotic cells (Fig. 1*B'*) in a pattern indistinguishable from that of tubulin staining (Fig. 1 *A'* and *B''*). Previously we reported that AX3 mAb labels mitotic centrosomes in cultured mammalian cells (16). The staining of cells with this antibody has recently been found to be very sensitive to fixation conditions. That is, in cells fixed with −20°C methanol, only centrosomes are intensely stained (16), whereas in cells fixed with formaldehyde or glutaraldehyde, interphase and spindle microtubules are also stained. Fig. 2 shows mitotic HeLa and interphase EMF cells at both low and high magnification double-stained with AX3 mAb (Fig. 1 *A–C*) and tubulin (Fig. 1 *A'–C'*) antibodies. We have also observed AX3 mAb staining of microtubules in chicken embryonic fibroblasts and ganglia, in CHO, and in PtK_2 cells (data not shown), indicating that the AX3 mAb recognizes microtubule protein(s) conserved in species ranging from fungi to humans.

AX3 Antigen Is a High-Molecular-Mass, Heat-Stable MAP. To identify the protein recognized by AX3 mAb, protein fractions were prepared from *Dictyostelium*, HeLa, and EMF cells and examined by SDS/PAGE and immunoblot analysis. For *Dictyostelium* amoebae, procedures for the *in vitro* assembly of microtubules have not yet been established; therefore, a crude method for the fractionation of whole-cell proteins was followed. Although no proteins specifically reactive to the AX3 mAb were detected in whole-cell lysates, a high-molecular-mass 280-kDa protein was seen in a high-speed supernatant from lysed cells. This same 280-kDa protein was also detected in the heat-stable fraction and could be concentrated by precipitation with 25% saturated ammonium sulfate (Fig. 3*A*, lane 1). Lanes 2 and 3 show immunoblots of ammonium sulfate-precipitated heat-stable proteins from HeLa and EMF cells, respectively. As in *Dictyostelium*, AX3 mAb recognizes high-molecular-mass proteins with apparent molecular masses of 260 and 265 kDa. A number of less intensely stained bands are also seen; almost all of them are also stained by the secondary antibody alone (lanes 2′ and 3′) and thus represent nonspecific background. A low-molecular-mass species seen in both HeLa and EMF samples (asterisks) appears to be specifically stained by AX3 mAb. However, this protein does not coprecipitate with microtubules (see below) and, therefore, is not a likely candidate for the AX3 MAP.

To verify that the proteins identified on immunoblots are indeed MAPs, we tested whether the AX3 antigens of EMF

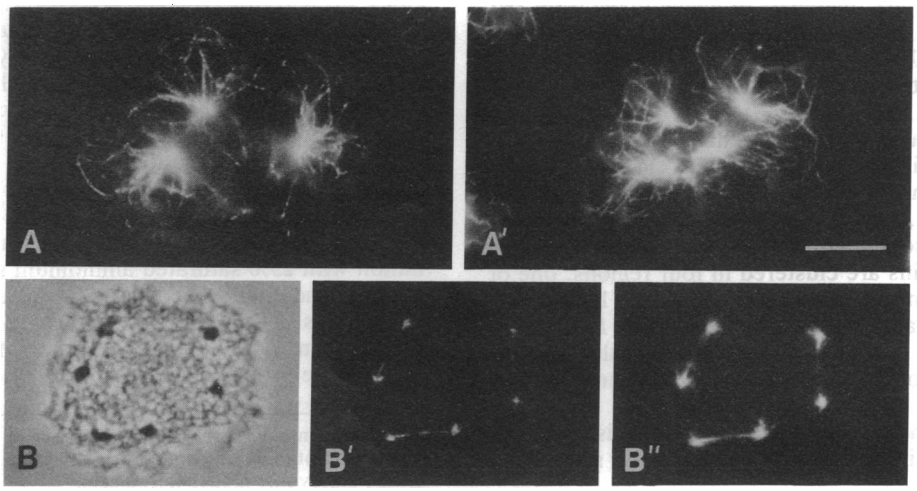

FIG. 1. AX3 mAb stains interphase and mitotic microtubules in *Dictyostelium* amoebae. Interphase cells stained with AX3 mAb (*A*) and anti-yeast tubulin antibody (*A'*). Same mitotic cell seen by phase contrast (*B*) and by fluorescence microscopy after double-staining with AX3 mAb (*B'*) and anti-tubulin antibody (*B''*). (Bar = 10 μm.)

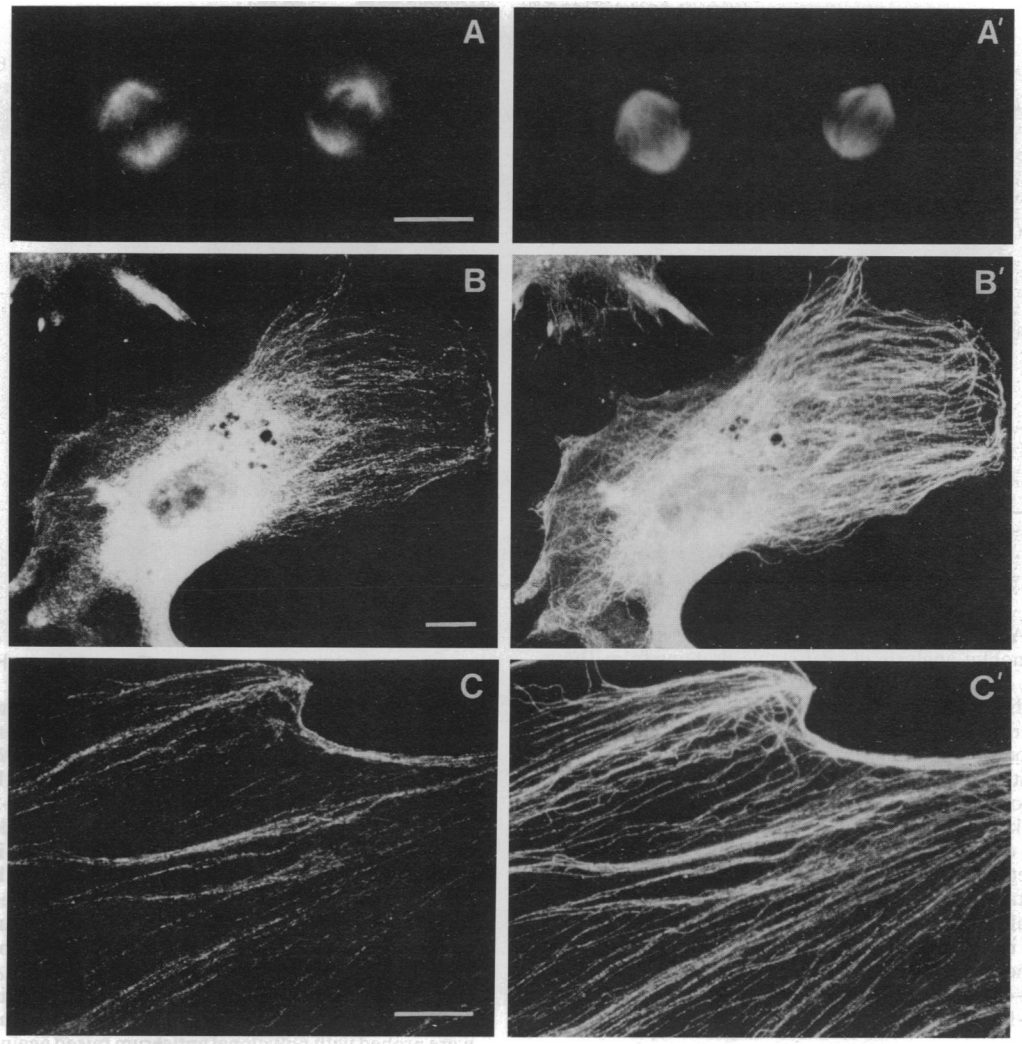

FIG. 2. Microtubules in cultured mammalian cells are also stained by AX3 mAb. Mitotic HeLa cells double-stained with AX3 mAb (*A*) and anti-yeast tubulin (*A'*) antibody. Low- and high-magnification images of EMF cells double-stained with AX3 mAb (*B* and *C*) and with anti-chicken tubulin antibody (*B'* and *C'*). (Bars = 10 μm.)

and HeLa cells could coprecipitate with microtubules. Microtubules were assembled *in vitro* from cell lysates by incubation at 37°C with GTP and taxol and were collected by centrifugation through a sucrose cushion. Samples of the washed microtubule pellets from mitotic HeLa and interphase EMF lysates, probed with AX3 mAb, are shown in Fig. 3*B*, lanes 1 and 5, respectively. Both 260- and 265-kDa AX3 antigens coprecipitate with microtubules *in vitro*, whereas the lower-molecular-mass proteins recognized by AX3 mAb (asterisks in Fig. 3*A*) do not. The AX3 antigen was also present in the microtubule-depleted supernatants. This amount is, however, quite minor because the antigen in such supernatant fractions could be detected only after concentration by heat treatment and ammonium sulfate precipitation (lane 3). These results indicate that the AX3 antigen is enriched in the microtubule pellets and confirm that the AX3 antigens of HeLa and EMF cells are, indeed, MAPs.

The AX3 Antigen Is Not Related to Mammalian MAP-4 and MAP-2 Proteins. To determine whether the proteins recognized by mAb AX3 in HeLa and EMF cells are related to the high-molecular-mass, heat-stable MAPs previously identified in these species, protein samples from the microtubule-assembly experiments were probed with antisera raised against HeLa 210-kDa MAP (6) or mouse MAP-4 (17). As described (6), the anti-HeLa 210-kDa antiserum recognized two bands that coprecipitate with microtubules (Fig. 3*B*, lane 2): the 210-kDa protein and a higher-molecular-mass band. The latter has been designated as the 255-kDa protein but migrates with an apparent molecular mass of 280–300 kDa in our gel system. Anti-mouse MAP-4 antiserum recognized a high-molecular-mass doublet in the microtubule pellet fraction (lane 6), which corresponds to the published molecular mass of mouse MAP-4 (215–240 kDa) (17). The lower-molecular-mass protein recognized by the anti-mouse MAP-4 antiserum (lane 6) is also recognized by preimmune serum from the same rabbit (arrowhead in lane 6'). Comparison of staining between lanes 1 and 2 and between lanes 5 and 6 indicates that the AX3 antigens from both cell types are electrophoretically distinct from the MAP-4 proteins. The results also show that the AX3 antigens are not recognized by either of the MAP-4 polyclonal antisera, implying that these proteins are not immunologically related to MAP-4. Thus, assembled microtubules in HeLa and EMF cells are associated with two distinct heat-stable, high-molecular-mass MAPs.

The similarity in apparent molecular mass of the AX3 antigens, 260–280 kDa, to that of MAP-2 and the report that a MAP-2-related protein is expressed at low levels in HeLa cells (22) suggested that these proteins might be related to MAP-2. To test this hypothesis we probed heat-stable proteins from *Dictyostelium*, HeLa cells, and EMF cells with four different mAbs raised against rat brain MAP-2. None of

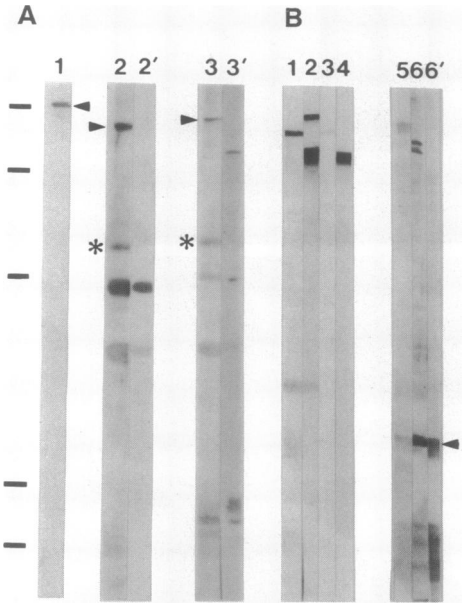

FIG. 3. The AX3 antigen is a high-molecular-mass, heat-stable MAP. (*A*) Immunoblot analysis of heat-stable proteins from *Dictyostelium* (lane 1), HeLa (lane 2), and EMF (lane 3) probed with AX3 mAb. Arrowheads indicate position of AX3 antigen in each species. HeLa (lane 2′) and EMF (lane 3′) proteins were also probed with secondary antibody alone. Asterisks indicate low-molecular-mass AX3 antigens that do not copurify with microtubules (see text). Bars at left indicate positions of brain MAP-2, myosin (200 kDa), β-galactosidase (116 kDa), and α- and β-tubulins. (*B*) AX3 antigens of HeLa and EMF coprecipitate with microtubules. Lanes: 1 and 2, microtubule pellet from HeLa cells; 3 and 4, postmicrotubule supernatant after heat treatment and ammonium sulfate precipitation; 5, 6, and 6′, microtubule pellet from EMF cells. Nitrocellulose strips were probed with AX3 mAb (lanes 1, 3, and 5), anti-210-kDa protein (lanes 2 and 4), anti-mouse MAP-4 (lane 6), or preimmune serum for anti-mouse MAP-4 (lane 6′). The lower-molecular-mass band recognized by the anti-MAP-4 serum (arrowhead) is unrelated to MAP-4.

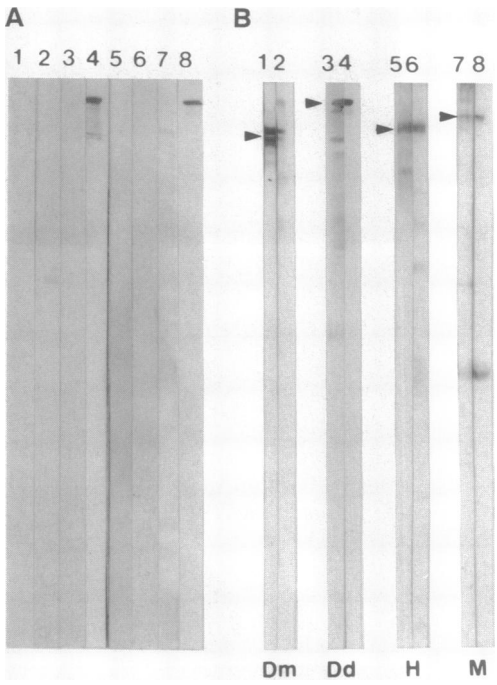

FIG. 4. Relationship of AX3 antigens to mammalian MAP-2 and *Drosophila* 205-kDa MAP. (*A*) AX3 antigens are not related to MAP-2. Lanes: 1–4, Heat-stable proteins from *Dictyostelium* (lane 1), HeLa cells (lane 2), EMF cells (lane 3), and pig brain microtubule proteins (lane 4) are stained with an anti-rat MAP-2 mAb. The mAb does not recognize AX3 antigens (lanes 1–3) but does recognize pig brain MAP-2 (lane 4). Faintly stained bands seen in lane 2 are from nonspecific staining by secondary antibodies. Pig brain microtubule proteins were probed with AX3 mAb (lane 5). Heat-stable proteins from *Dictyostelium* immunostained with anti-HeLa 210-kDa protein (lane 6), anti-mouse MAP-4 (lane 7), and AX3 mAb (lane 8). (*B*) AX3 antigens are immunologically related to 205-kDa MAP of *Drosophila*. Heat-stable, ammonium sulfate-fractionated proteins from *Drosophila* (Dm), *Dictyostelium* (Dd), HeLa cells (H), and EMF cells (M) were probed with polyclonal antiserum raised against a fusion protein containing the carboxyl-terminal region of *Drosophila* 205-kDa MAP (lanes 1, 3, 5, and 7) and AX3 mAb (lanes 2, 4, 6, and 8). Arrowheads indicate positions of a protein recognized by both antibodies.

the four mAbs recognized the AX3 antigens (Fig. 4*A*, lanes 1–3), although all four antibodies could react with MAP-2 in a preparation of twice-cycled pig brain microtubules (Fig. 4*A*, lane 4). Lane 5 shows that AX3 mAb is not reactive to pig brain MAP-2 (lane 5). In addition, neither the anti-210-kDa (lane 6) nor the anti-mouse MAP-4 (lane 7) antisera recognize the AX3 antigen of *Dictyostelium*. The many faintly staining bands seen in the *Dictyostelium* heat-stable protein fraction, when probed with mouse MAP-4 antiserum (lane 7), were also stained by preimmune serum from the same rabbit (data not shown). These results show that the AX3 antigens are distinct from the high-molecular-mass, heat-stable MAPs previously identified in mammalian systems.

AX3 Antigens Are Related to the 205-kDa MAP of *Drosophila*. The AX3 antigens are, however, immunologically related to the 205-kDa MAP of *Drosophila*. Fig. 4*B* shows nitrocellulose strips of heat-stable proteins probed with AX3 mAb (even-numbered lanes), or a polyclonal antiserum raised against the carboxyl-terminal portion of the 205-kDa MAP (odd-numbered lanes). The AX3 mAb recognizes the 205-kDa MAP (arrowhead in lane 1) in *Drosophila* embryo heat-stable proteins (Dm), and the *Drosophila* MAP antiserum recognizes the AX3 antigens (indicated by arrowheads) of *Dictyostelium* (Dd), HeLa (H), and EMF (M). In addition to the AX3 bands, a number of other bands are detected in the lanes probed with the anti-205-kDa antiserum. Some of these bands appear to be from nonspecific staining by the secondary antibodies (compare with lanes 2′ and 3′, Fig. 3*A*), whereas other bands may be from components of the crude serum unrelated to the 205-kDa antibodies. Although staining of some AX3 bands shown in Fig. 4*B* is rather weak, the fact that antibodies raised against proteins from two very different organisms (*Dictyostelium* and *Drosophila*) recognize the same proteins in all four species suggests that the staining is significant.

DISCUSSION

We have identified a high-molecular-mass, heat-stable MAP in *Dictyostelium discoideum*. This MAP was identified by using a mAb (AX3), which when used for immunofluorescence staining of *Dictyostelium* amoebae, stains microtubules in both mitotic and interphase cells. On immunoblots the AX3 mAb recognizes a heat-stable protein having an apparent molecular mass of 280 kDa. The colocalization with microtubules, the high-molecular-mass, and the heat stability of AX3 antigen suggest that this protein belongs to the class of fibrous, heat-stable MAPs, which includes MAP-2, MAP-4, and τ proteins (4). Although fibrous MAPs have been studied in several different systems, the precise *in vivo* functions of these proteins are still unknown. Identification of MAPs in *Dictyostelium* presents an opportunity to probe the *in vivo* functions of these proteins in a relatively simple system that is amenable to genetic manipulation. In particular, the use of gene disruption (23, 24) and antisense RNA (25) to block expression of MAPs should provide powerful tools for probing MAP function *in vivo*.

AX3 mAb labels interphase and mitotic microtubules in a number of different vertebrate cell lines, including HeLa, EMF, CHO, PtK_2 and chicken embryonic fibroblasts and ganglion cells. As in *Dictyostelium*, the AX3 antigens in these cells are high-molecular-mass, heat-stable proteins, and we have shown that the AX3 antigens of HeLa and EMF cells coprecipitate with microtubules. Preliminary results from protein blot analysis suggest that AX3 antigens may also be present in sea urchin embryos and *Chlamydomonas* (data not shown). The AX3 antigens are electrophoretically and immunologically distinct from mammalian MAP-2 and MAP-4 but are related to the *Drosophila* 205-kDa MAP.

Based on apparent molecular mass and a preliminary report that antibodies raised against *Drosophila* 205-kDa MAP recognized HeLa 210-kDa MAP, the 205-kDa MAP of *Drosophila* had been suggested to represent a MAP-4-type protein (26). However, sequence analysis of the gene encoding this protein has shown that the 205-kDa MAP does not share any significant sequence similarity with either the MAP-2 or the MAP-4 proteins of mammals (12). During this study we have not detected immunological cross-reactivity of either the AX3 mAb or the anti-205-kDa antibodies with MAPs previously identified in the cell types analyzed. We suggest that the AX3 antigens and the *Drosophila* 205-kDa MAP define a distinct class of MAPs that is represented throughout the eukaryotes.

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