IDENTIFICATION AND LOCALIZATION OF MICROTUBULE-ASSOCIATED PROTEINS IN SEA URCHIN (STRONGYLOCENTROTUS DROEBACHIENSIS) COELOMOCYTES.

John H. Henson and Michael J. Smith
Department of Biology, Dickinson College, Carlisle, PA, 17013

Microtubules are a major cytoskeletal element within cells and help mediate a number of fundamental cell processes including cell motility, the intracellular trafficking and distribution of organelles and the migration of chromosomes during mitosis. The functions and assembly properties of microtubules are regulated by a number of microtubule-associated proteins (MAPs, reviewed by Vale, Ann. Rev. Cell Biol. 3:347, 1987; Wiche, J. Cell Sci. 259:1, 1989). MAPs have been extensively studied in sea urchin eggs and embryos, particularly with reference to possible roles in mitosis (Vallee & Bloom, Proc. Natl. Acad. Sci. 80:6259, 1983; Hirokawa et al., J. Cell Biol. 101:1858, 1985; Scholey et al., Nature 318:483, 1985; Hirokawa & Hisanaga, J. Cell Biol. 104:1553, 1987). The MAPs previously identified in embryos include structural MAPs, such as the high molecular weight proteins (> 200 kDa) and a 77 kDa protein, and the microtubule motor proteins kinesin and cytoplasmic dynein. Several of these MAPs have been immunolocalized in the mitotic apparatus during sea urchin embryo division (Bloom et al., Cell Motil. 5:431, 1985; Leslie et al., Proc. Natl. Acad. Sci. 84:2771, 1987). However, the actual functional roles of these proteins during mitosis or interphase remains largely unclear.

We decided to study MAPs in sea urchin phagocytic amoebocytes which are a subpopulation of the coelomocytes inhabiting the perivisceral fluid of echinoderms. These terminally differentiated, nonmitotic cells have an elaborate cytoskeleton composed of actin filaments and microtubules, undergo dramatic actin-mediated shape changes and are involved in phagocytosis of foreign matter and the clotting of the coelomic fluid following wounding (Edds, J. Cell Biol. 73:479, 1977). The amoebocyte represents an excellent experimental system since they can be isolated as a homogeneous population, obtained in quantities sufficient for biochemical studies and are extremely thin cells ideal for the immunofluorescent localization of proteins. We reasoned that the study of MAPs in amoebocytes would shed light on the non-mitotic functions of these proteins and would allow for important comparisons between the totipotent embryo and a differentiated cell type. Our previous studies have indicated the existence of both microtubule-dependent organelle movements and microtubule bundles in amoebocytes (Henson et al., J. Cell Biol. 111:414a, 1990). Therefore, we hypothesized that these cells should contain intracellular transport and structural MAPs, such as kinesin and the 77 kDa protein.

MAPs were initially identified through co-purification with microtubules in taxol (Wani et al., J. Am. Chem. Soc. 93:2325, 1971) stabilized microtubule preparations (following the methods of Vallee & Bloom, loc. cit.) from amoebocytes isolated from the coelomic fluid of approximately 100 S. droebachiensis. In brief, amoebocytes were isolated by centrifugation through a sucrose cushion and homogenized in a Dounce homogenizer. Microtubules in the high speed supernate (100,000 x g) of the amoebocyte extract were polymerized and stabilized by the addition of GTP and taxol. Centrifugation of this solution resulted in a pellet containing polymerized microtubules and MAPs. In order to optimize for the presence of kinesin, adenylyl imidodiphosphate (AMP-PNP) was added to half of the polymerization solution. In the presence of this non-hydrolyzable analog of ATP, kinesin becomes bound to microtubules. Samples for SDS polyacrylamide gel electrophoresis were taken during the preparation process and run on either 7.5% acrylamide gels or 5-15% acrylamide gradient gels.

Figure 1 shows a silver stained gel of a taxol-stabilized microtubule preparation from amoebocytes. A specific enrichment for tubulin is clearly evident in the microtubule pellet sample (Fig. 1, panel A, lanes 4 & 5) along with other major proteins at the following molecular weights:

33 kDa, 60 kDa, 78 kDa, 240 kDa, 260 kDa and several bands > 260 kDa. Strict identification of these proteins as MAPs must be approached cautiously due to the large degree of contaminating

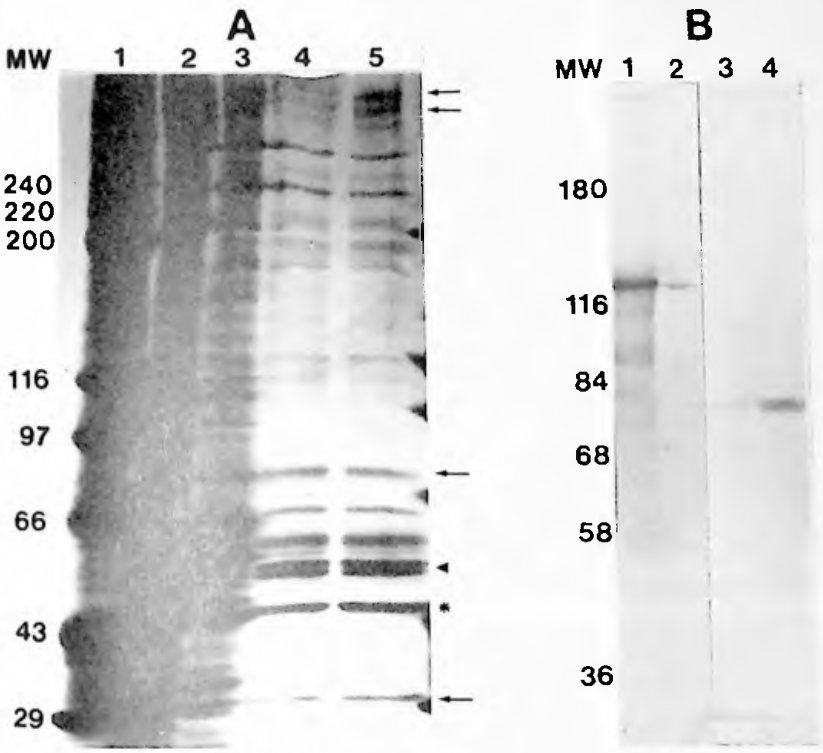

Figure 1. Panel A = A 5-15% acrylamide gel of a taxol stabilized microtubule preparation from amoebocytes. Samples consist of molecular weight markers (lane 1), whole cell homogenate (lane 2), high speed supernatant (lane 3) and the microtubule pellet plus (lane 4) and minus (lane 5) AMP-PNP. Tubulin is indicated by an arrowhead, potential MAPs by arrows and actin by an *. Panel B = Immunoblots of amoebocyte extracts with monoclonal antibodies against egg kinesin and egg 77 kDa protein. The anti-kinesin reacts with a band in amoebocytes (lane 2) which comigrates with a proteolyzed sample of purified egg kinesin (lane 1). The anti-77 kDa recognizes a band of appropriate molecular weight in amoebocytes from the species S. purpuratus (lane 3) and S. droebachiensis (lane 4).

actin and actin binding proteins (ABPs) in the microtubule pellets. It is likely that the 60 kDa protein corresponds to the ABP fascin, and the 240 and 260 kDa proteins represent the two subunits of the ABP spectrin. This leaves potential MAPs at 33 kDa, 78 kDa and several > 260 kDa. MAPs at similar molecular weights have been previously identified in microtubule preparations from sea urchin eggs and embryos (Vallee & Bloom, loc. cit.). A potential kinesin-like molecule (expected molecular weight 130 kDa) was not evident in the microtubule pellet in the presence (lane 5) or absence (lane 4) of AMP-PNP. The presence of the 77 kDa MAP and kinesin in amoebocytes was further investigated by performing immunoblots (western blots) on amoebocyte extracts using antibodies against the two proteins. Site specific monoclonal antibodies against kinesin were obtained from my collaborators Brent Wright and Jonathan Scholey (University of California, Davis), while a monoclonal antibody against the 77 kDa egg MAP was generously provided by Richard Vallee (Worcester Foundation for Experimental Biology). On immunoblots, the kinesin monoclonals recognized an immunoreactive band of approximately 130 kDa which comigrated with a proteolyzed sample of purified sea urchin egg kinesin (Fig. 1, panel B, lanes 1 & 2). The anti-77 kDa antibodies reacted with a band at approximately 77 kDa in amoebocyte samples from the two sea urchin species S. droebachiensis and S. purpuratus (Fig. 1, panel B, lanes 3 & 4).

Given the results of the microtubule preparations and the immunoblots, we next attempted the

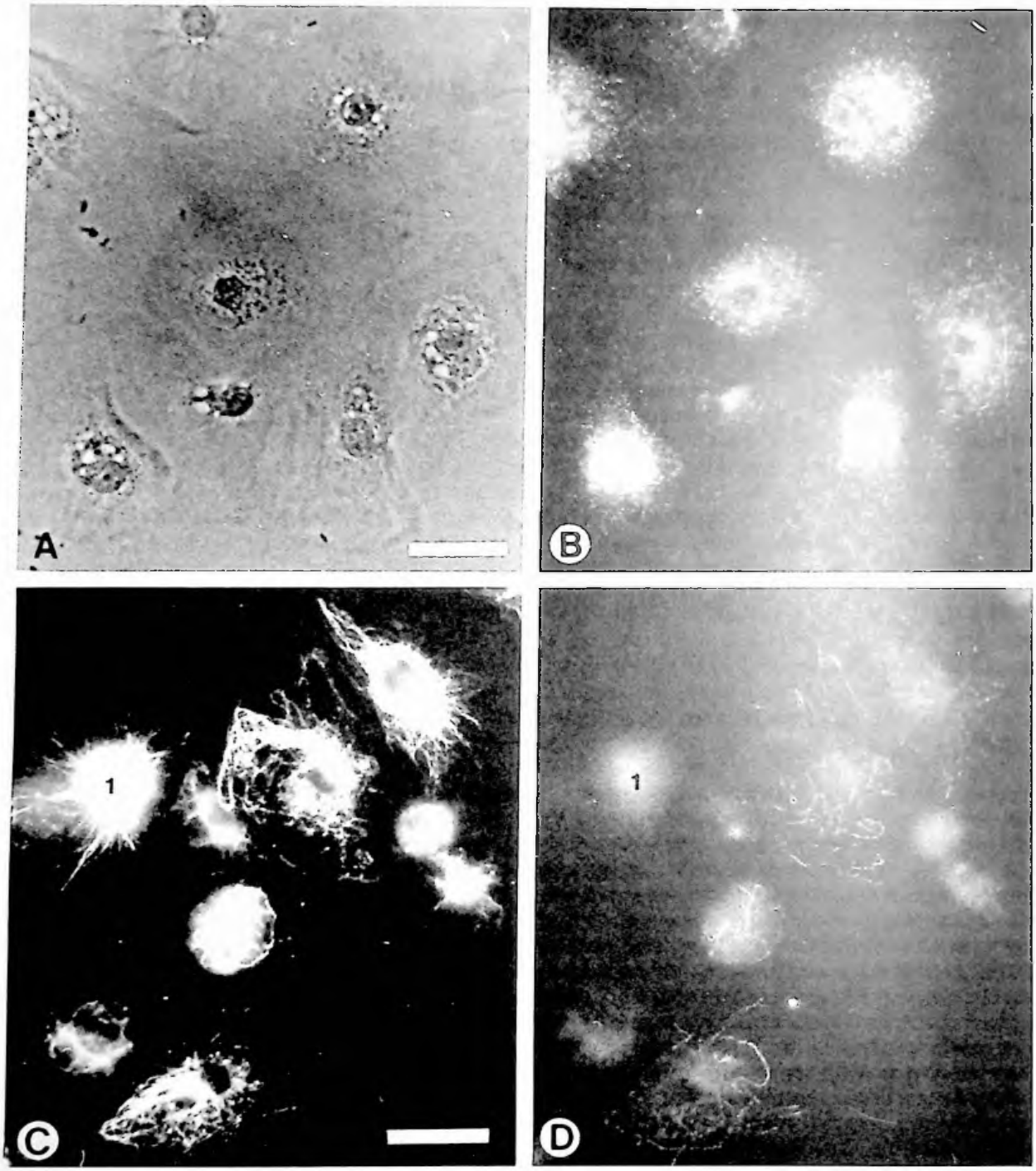

Figure 2. Panels A & B: Phase contrast (A) and fluorescence (B) views of amoebocytes stained with antibodies to kinesin. Panels C & D: Double label of amoebocytes with anti-tubulin (C) and anti-77 kDa (D) antibodies. Note the absence of 77 kDa staining along microtubles in some cells (cell labeled with the #1 in C & D). Bar = 10 μm.

subcellular localization of kinesin and the 77 kDa MAP in amoebocytes using immunofluorescent staining. Isolated amoebocytes were allowed to settle onto poly-lysine coated coverslips and fixed in ice cold methanol. The coverslips were incubated in primary antibody followed by a fluorophore conjugated secondary antibody. For double labeling of MAPs and microtubles, the cells were also stained with a polyclonal antiserum raised against sea urchin egg tubulin. Figure 2 shows the results of the immunofluorescence experiments. Anti-kinesin stains a perinuclear array of cytoplasmic, vesicle-like structures (Fig. 2 A & B). The staining did not appear to extend into the actin-rich cortex of the cells and was consistent with the known distribution of microtubules (Edds, Cell Motil. 4:269, 1984). On occasion, the particles appear in almost linear arrays. The staining pattern was eliminated in cells exposed to prefixation extraction with Triton X-100 under microtubule stabilizing conditions. Taken together, these data suggest that kinesin is present on membrane-bound and microtubule associated structures. The localization of kinesin in other cell types has varied greatly, however the patterns in amoebocytes most closely resemble the Triton-extractable vesicle patterns apparent in some cultured mammalian cells (Pfister et al., J. Cell Biol. 108:1453, 1989). In an attempt to determine the identity of these structures we double labeled cells with kinesin and an antiserum against the protein sea urchin calsequestrin which serves as a marker for the endoplasmic reticulum (ER) (Henson et al., J. Cell Biol. 109:149, 1989). A previous study on mammalian tissue culture cells had indicated a colocalization of kinesin and the ER (Hollenbeck, J. Cell Biol. 108:2335, 1989), ER-like structures have been created in vitro from the combination of kinesin, microtubules and membranes (Vale & Hotoni, J. Cell Biol. 107:2223, 1988), and our studies on the sea urchin embryo demonstrate a codistribution of kinesin and calsequestrin (Wright et al., J. Cell Biol. 111:418a, 1990). The staining results obtained with amoebocytes showed no clear similarity between the ER and kinesin patterns suggesting the absence of a strict codistribution in these cells.

Double label immunofluorescent staining of microtubles and the 77 kDa MAP (Fig. 2 C & D) revealed that the 77 kDa protein localizes in a discontinuous fashion on microtubles in only a subpopulation of cells. Cells are clearly evident which contain microtubules but do not show any staining with the anti-77 kDa (Fig. 2, #1 C & D). In addition, it appears that the 77 kDa MAP does not associate with all of the microtubules in a given cell.

In previous studies by other groups, both the 77 kDa MAP and kinesin had been localized to the mitotic apparatus in early sea urchin embryos (Scholey et al., loc. cit.; Hirokawa & Hisanaga, loc. cit.; Bloom et al., loc. cit.). The 77 kDa MAP was speculated to have a role in stabilizing mitotic microtubles, while kinesin was inferred to have a possible role in karyokinesis. The results of our experiments on the presence and localization of these two MAPs in amoebocytes clearly indicates nonmitotic functions for both of these proteins. The 77 kDa protein may be stabilizing select microtubules in a subpopulation of amoebocytes. Kinesin in these cells appears to be in the position to mediate the intracellular movement of membrane bound organelles on microtubules. Interestingly, recent sequence analysis of the kinesin heavy chain gene from sea urchin eggs indicates that this protein is more homologous with the kinesin isoforms responsible for vesicle transport in axons than the kinesin-like proteins which appear essential for mitosis (Wright et al., loc. cit.; Scholey, Nature 343:118, 1990). Future experiments will concentrate on determining whether the 77 kDa MAP is actually stabilizing microtubules in amoebocytes and the identity of the intracellular structures labeled by the kinesin antibodies.

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