Journal of Cellular Biochemistry 35:83–92 (1987)

Microtubule-Associated Proteins Present in Different Developmental Stages of *Drosophila melanogaster*

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Microtubule-associated proteins (MAPs) have been isolated from different development stages of *Drosophila melanogaster* and characterized by their association to tubulin, but not to tubulin lacking its 4-kD carboxy terminal region (S-tubulin), and by their ability to promote tubulin polymerization. Following these criteria some peptides of Mr 255, 205, and 180 kD were identified as MAPs.

By means of immunological analogy we have identified a peptide related to mammalian brain MAP known as tau factor.

Key words: microtubule polymerization, tubulin binding proteins, tau factor

Morphogenesis in *Drosophila melanogaster* follows a sequence of developmental stages (embryo, larva, pupa, and imago) during which dramatic changes in cellular proliferation and differentiation take place. Microtubules, together with other cytoskeletal components, have been implicated in the determination of cell shape (morphology) and also in cell division. These two functions are based on the capacity of microtubules to polymerize from their dimeric subunits and interact with other structures. In higher eukaryotic cells, microtubules are composed of tubulin and microtubule-associated proteins (MAPs); these may facilitate the polymerization of microtubules and their interaction with other subcellular structures. Since microtubule functions may change, it has been suggested that different microtubule populations may exist depending on cell type and the presence of different tubulin isoforms [1] or MAPs [2,3].

In *D melanogaster* four different genes for both α and β tubulin [4,5] as well as four isoforms of each subunit [6,7] have been identified. Also, different MAPs

Abbreviations used: PMSF, phenylmethylsulfonyl fluoride; MAPs, microtubule-associated proteins; Pc-tubulin, tubulin purified by phosphocellulose chromatography; S-tubulin, tubulin obtained by partial substilisin digestion; EGTA, ethylenebis (oxyethylenenitrilo) tetraacetic acid; MES, morpholine-ethane sulphonic acid.

Received October 8, 1986; revised and accepted February 4, 1987.

have been described in embryos [8]. Recently Goldstein et al [9] have characterized as a MAP a high molecular weight protein in *Drosophila* cultured cells.

In this paper we have identified MAPs from *D melanogaster* by the criteria of (1) association with tubulin and microtubules, (2) promotion of tubulin polymerization, and (3) immunological relationship with porcine tau factor.

MATERIALS AND METHODS

Embryos (0–12 hr), larvae, and flies of *D melanogaster* (Vallecas, wild-type strain) were obtained from Dr. Ripoll. Embryos and larvae were collected and washed with 150 mM sodium phosphate pH 7.5 and 0.01% Triton X-100 (ST buffer). The embryos were dechorionated with 5% sodium hypoclorite for 5 min and then washed with ST buffer.

Purification of Microtubule Protein

Embryos, larvae, and flies were homogenized at 4°C in isoosmotic buffer (0.32 M sucrose, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM sodium phosphate pH 7) in a ratio of 3/1 (w/v), adding 1 mM PMSF, 1 mM chloroquine, 1 μg/ml leupeptine, 2 μg/ml trypsin inhibitor, and 2 μg/ml pepstatin as protease inhibitors.

The homogenates were centrifuged at 100,000g for 35 min at 2°C and the supernatant was taken. The polymerized protein was obtained upon addition of dimethylsulfoxide (10%) [10] or in the presence of taxol 10 μM [11], always in a buffer containing 100 mM MES, 1 mM $MgCl_2$, 1 mM EGTA pH 6.8 (buffer A), and 1 mM GTP. The mixture was incubated for 30 min at 30°C and the assembled protein was obtained by centrifugation at 100,000g for 30 min at 24°C, and the pellets stored at −70°C.

Purified pig brain tubulin was obtained from the microtubule protein fractions by phosphocellulose chromatography [12]. Tau protein were obtained from heat-stable MAPs by filtration on a Sepharose 4B-CL column [13]. S-tubulin was obtained by proteolytic digestion of tubulin with 1% w/w subtilisin protease as described [14].

Radioimmunoassay (RIA)

Radioimmunoassay using antibodies against tau proteins from pig brain was carried out essentially as described previously [15]. Tau protein was iodinated with ^{125}I with the chloramine T procedure as described by Montejo de Garcini et al [15].

Electrophoresis and Immunoblotting

The peptide composition of microtubule protein was analyzed in 7.5% or 5–15% gradient polyacrylamide gels [16]. The gels were stained with Coomassie blue. Porcine brain MAP 2 (270 kD), neurofilament peptides (210 kD, 180 kD, 68 kD), and trypsin inhibitor (20 kD) have been used as molecular weight markers. For immunoblotting, the peptides were fractionated on polyacrylamide gels and electrophoretically transferred to nitrocellulose sheet [17]. The nitrocellulose replicas were washed with phosphate-buffered saline containing 1% gelatine, 0.1% sodium azide before being incubated with the monospecific antibody anti-tau, purified by affinity chromatography. The nitrocellulose sheets were incubated with the antibody diluted 1:200, washed with phosphate buffer containing 1% gelatine, and then incubated with ^{125}I-protein A (100,000 cpm/ml) in saline buffer with gelatine.

The dot blots was performed as follows: the samples to test were placed on a nitrocellulose paper disc (Millipore 0.4 μm), and after drying them the same protocol as that for immunoblotting was followed.

Chromatography on Tubulin-Sepharose

Twenty-five milliliters of a soluble protein extract (15 mg/ml) obtained from adult flies were chromatographied on a tubulin-Sepharose column (5 × 10 cm). The column was washed with a solution containing 100 mM MES, (pH 6.5), 10% glycerol 1 mM PMSF, 1 mM cloroquine, 1 μg/ml pepstatin, and 1 μg/ml leupeptin and then eluted with the same buffer, containing 0.5 M NaCl. The samples were pooled, dialyzed against buffer A, and stored at −70°C.

Chromatography on DEAE-Cellulose

The proteins bound to the tubulin-Sepharose column after dialysis were applied to a DEAE-cellulose column (5 × 10 cm) in 0.1 M MES (pH 6.5), 0.5 mM EGTA, 1 mM PMSF, and washed with the same buffer before being eluted by the addition of the same buffer plus 0.2 M NaCl [18]. They were pooled, dialyzed against buffer A, quantified by Lowry et al [19] and stored at −70°C.

Electron Microscopy

Tubulin polymers were stained with a 1% (w/v) aqueous solution of uranyl acetate and visualized in a JEOL 100 B electron microscope.

RESULTS

Assembly of Microtubules From *D melanogaster* at Different Developmental Stages

Embryos (0–12), third-instar larvae, and adult flies were homogenized and cell extracts from each stage were incubated in the presence of either dimethylsulfoxide or taxol to promote microtubule polymerization. Microtubule assembly was achieved in extracts from embryos or adults in the presence of either compound, but only taxol was successful for larval extracts. Figure 1 shows an example of microtubules from different developmental stages polymerized with taxol. A normal morphology, tubules perfectly closed, was observed for the microtubules from embryo and adult extracts, while microtubules from larval extract had an altered morphology (Fig. 1B), showing tubules not completely closed (see arrowheads Fig. 1B) and forming clusters or bundles.

Characterization of Microtubule Protein

Microtubule protein from different developmental stages of *D melanogaster* were characterized by gel electrophoresis. Figure 2 shows the presence of both tubulin subunits in each case, together with other proteins of variable molecular weight.

Peptides from embryonic microtubules included two high molecular weight 255-kD and 205-kD peptides; three peptides (more abundance) of 80 kD, 100 kD, and 42 kD; and eventually some peptides of 140–150 kD (Fig. 2, lane 1).

Preparations from larval microtubules contain a large number of peptides, the most abundant corresponding in mobility to larval serum proteins (77–83 kD) [20]. Besides, there are present peptides of 205 kD, 150 kD, and 42 kD, similar to those

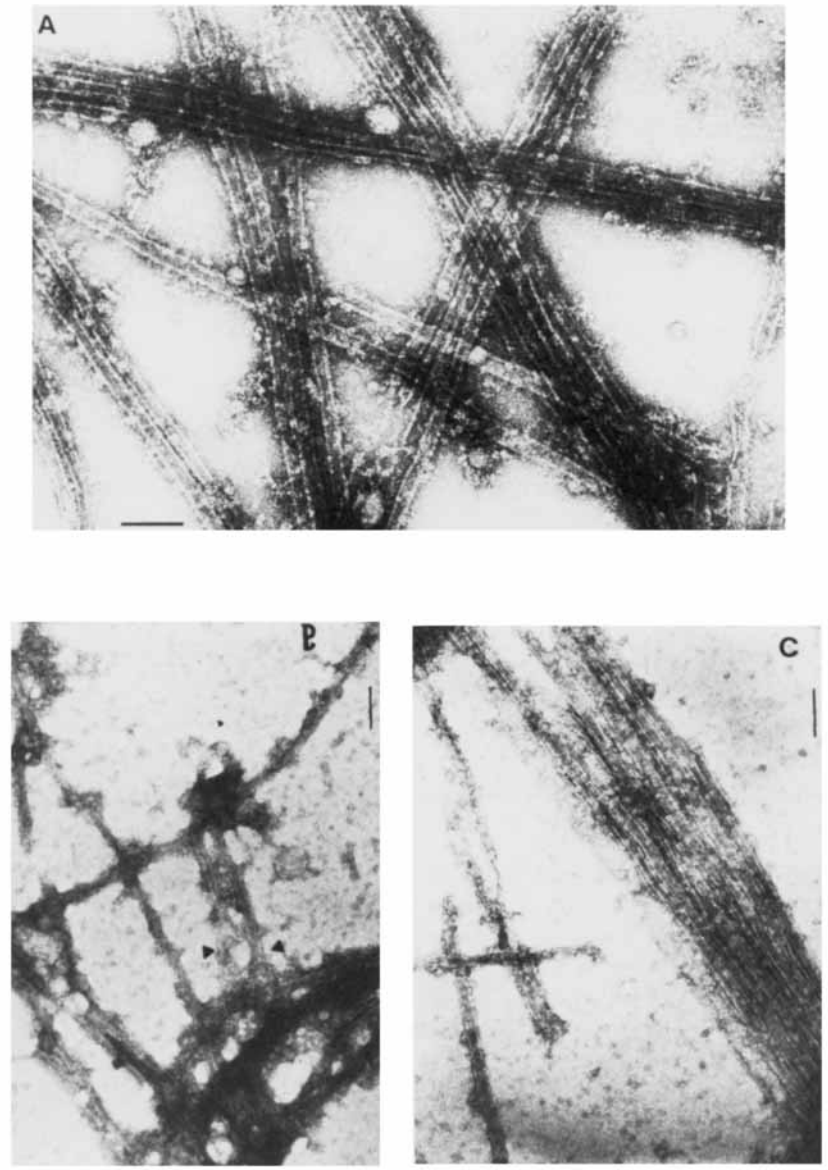

Fig. 1. Characterization of *D melanogaster* polymers. *D melanogaster* tubulin from embryo (**A**), larva (**B**) or adult (**C**) extracts was assembled in the presence of 10 μM taxol and the polymers obtained were characterized by electron microscopy. The bars indicate 0.1 μm.

found in embryonic samples, and some new ones of MW 135 kD, 90–95 kD, 38 kD, and 35 kD (Fig. 2, lane 2).

Finally, microtubular preparations from adult flies contained some peptides (255 kD, 205 kD, 80 kD, 42 kD) similar to those present in larval or embryonic extracts but some with a different molecular weight (mainly 180 kD, 150–160 kD, 130 kD, 110 kD) (Fig. 2, lane 3).

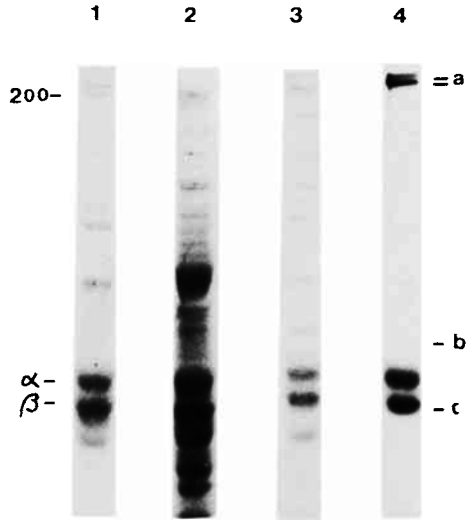

Fig. 2. Characterization of *D melanogaster* microtubule protein. Shown is the peptide composition of the tubulin polymers indicated in Figure 1, as determined by electrophoresis in sodium dodecyl sulphate/polyacrylamide gels containing 7.5% acrylamide. **Lanes 1, 2, 3** indicate the electrophoretic analysis of embryo, larva, and adult microtubule protein. **Lane 4** represents microtubules obtained from pig brain. The lines indicate the molecular weight of (a) MAP_1, 300 kD, and MAP_2, 270 kD; (b) bovine serum albumin (68 kD), and (c) β-tubulin, 52 kD, used as markers.

Isolation of Tubulin-Binding Proteins by Affinity Chromatography

Tubulin-binding proteins were purified, taking advantage of their ability to associate to purified tubulin. Soluble protein from a extracts obtained by homogenization of adult flies were run through a tubulin-Sepharose column and the bound protein was eluted with 0.5 M NaCl (Fig. 3A). These fractions were taken and analyzed by gel electrophoresis (Fig. 3B).

Chromatography on DEAE-Cellulose

The proteins obtained from the chromatography on tubulin-Sepharose were passed through a DEAE-cellulose column. The proteins bound to the resin were eluted with 0.2 M NaCl as described for brain MAPs [21]. Bound tubulin was eluted with 0.8 M NaCl.

The proteins eluted with 0.2 M salt were characterized by gel electrophoresis. As shown in Figure 4 several high molecular weight peptides are recovered in this fraction, the most abundant being peptides of 255 kD, 205 kD, and 170 kD.

Stimulable Polymerization of Tubulin by Proteins Obtained From DEAE-Cellulose

To analyze the effect of these proteins on the polymerization dynamics of purified MAP-free porcine tubulin, the latter was mixed with equal volumes of MES buffer (100 mM, pH 6.5, 1 mM EGTA, 1 mM PMSF) containing increasing amounts of the 0.2 M salt-eluted protein fraction from DEAE-cellulose chromatography. After inducing polymerization, the assembled protein was isolated by centrifugation, quantified (Fig. 5A), and analyzed by gel electrophoresis (Fig. 5B).

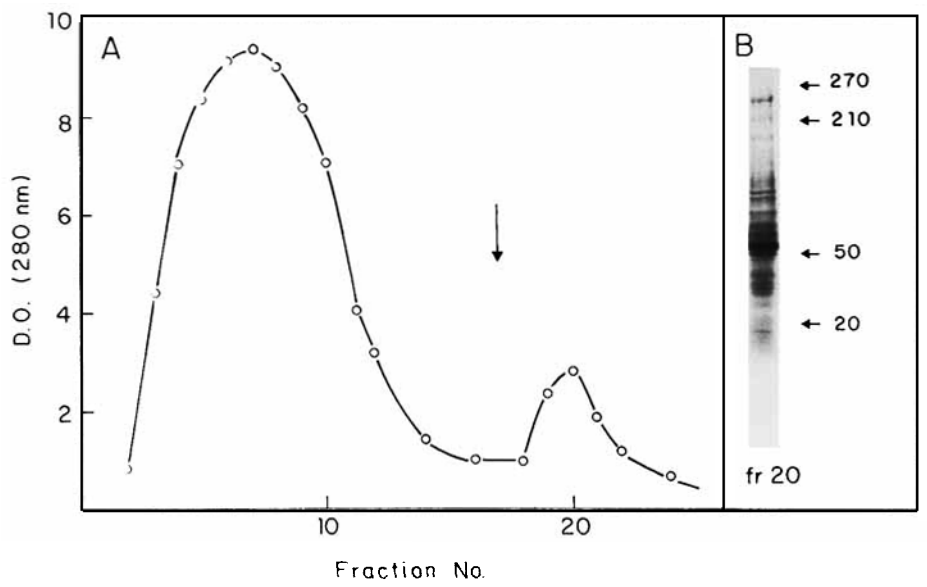

Fig. 3. Tubulin-Sepharose chromatography of *D melanogaster* proteins. **A)**: An extract obtained from adult cells was chromatographed on a tubulin-Sepharose column (5 × 2 cm) containing 2 mg of tubulin/ml volume. The bound protein was eluted upon the addition of 0.5 M NaCl (↓) and fraction number 20 was characterized by gel electrophoresis (**B**). The arrows indicate the electrophoretic mobility of markers of known molecular weight (MAP_2, neurofilament 210-kD peptide, tubulin, and soybean trypsin inhibitor).

Fig. 4. Characterization of the polypeptides purified by DEAE chromatography. The peptides bound to the tubulin-Sepharose column were chromatographed in a DEAE-cellulose column (5 × 2 cm) to remove tubulin. The MAPs were eluted with 0.2 M NaCl (it was necessary to add 0.8 M salt to elute bound tubulin). The arrowheads indicate the MW of *D melanogaster* peptides, calculated with respect to MAP_2 (270 kD) and neurofilament peptides (210 kD, 180 kD, and 68 kD).

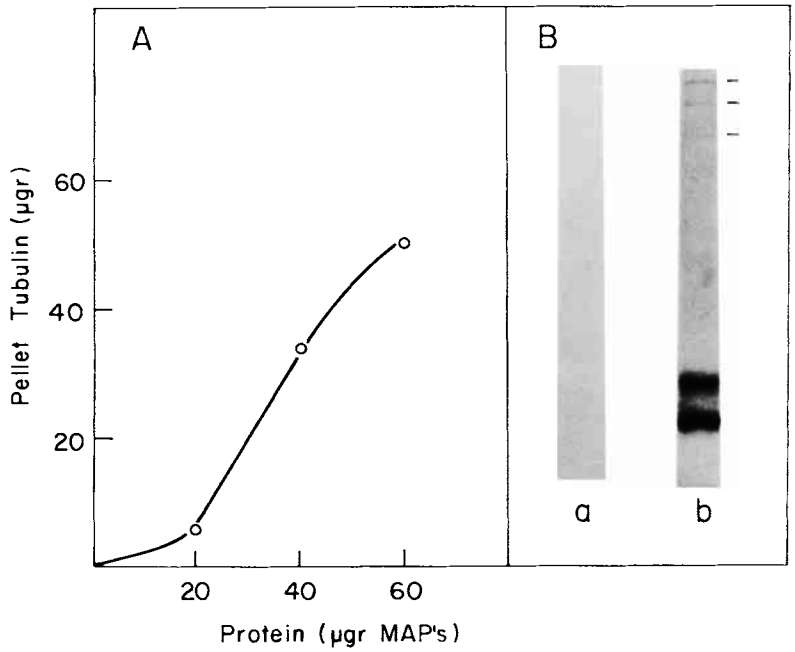

Fig. 5. Effect of the addition of DEAE-purified proteins on tubulin assembly. **A**) Increasing amounts of the fraction isolated by DEAE chromatography (0–60 µg) were mixed with porcine brain tubulin depleted of MAPs (100 µg) and tubulin polymerization was promoted by addition of 1 mM GTP. The polymerized protein was isolated by centrifugation in a Beckman airfuge and quantified. **B**) The polymerized protein obtained upon addition of *D melanogaster* (50 µg) MAPs (**b**) was characterized by gel electrophoresis, using a 7% acrylamide gel. In the absence of MAPs no polymerized protein was found (**a**).

Only peptides of 255 kD, 205 kD, and 170 kD routinely show the higher capacity to copolymerize with tubulin.

Lack of Binding of DEAE-Purified Proteins to Subtilisin-Digested Tubulin

The association of several mammalian MAPs (MAP_1, MAP_2, and tau) to tubulin has been recently shown to occur through a region located at the carboxy terminal region of both subunits [22,23]. This region can be removed after limited proteolysis of tubulin with subtilisin, yielding tubulin lacking the carboxy-terminal region (S-tubulin), having a polymerization capacity similar to that needed for undigested tubulin in the presence of MAPs [22].

We performed a similar experiment to determine whether *D melanogaster* DEAE-eluted proteins behaved in this respect as porcine MAPs. As indicated in Figure 6, *D melanogaster* proteins eluted from DEAE did not copolymerize with subtilisin-digested tubulin.

Relationship Between Mammalian and *D melanogaster* MAPs

To test if there is in *Drosophila* a protein related to the mammalian brain tau factor, a polyclonal monospecific antibody against pig brain tau factor was used as a probe. Immunological crossreactivity was found between tau proteins and some proteins present in *D melanogaster* microtubules as determined by radioimmunoassay (RIA) dot blot and immunoblotting (Fig. 7A–C, respectively).

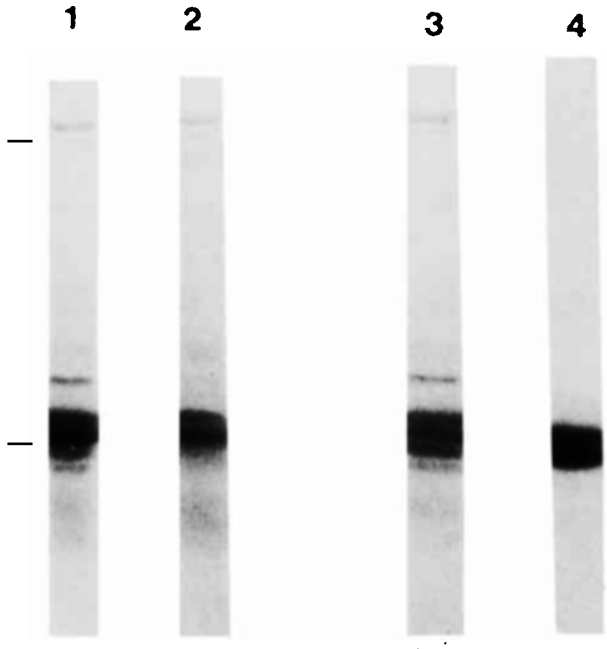

Fig. 6. Polymerization of purified tubulin or S-tubulin in the presence of *D melanogaster* MAPs. S-tubulin obtained by limited proteolysis with subtilisin or undigested tubulin (100 μg) was polymerized in the presence of purified *Drosophila* MAPs (40 μg). The polymerized protein from each fraction was isolated by centrifugation and characterized by gel electrophoresis. **1** and **2** indicate the supernatant (unpolymerized) and pellet (polymerized) of PC-tubulin, and **3** and **4** the supernatant and pellet of S-tubulin, respectively. The lines indicate the molecular weight markers (210 kD and 50 kD).

The tau-related proteins present in the extract obtained from adult flies showed a competition curve parallel to that of the standard protein, indicating a high degree of homology. This relationship was confirmed by "dot blot" (Fig. 7B) and immunoblotting (Fig. 7C), which showed that at least two peptides present in *D melanogaster* microtubules, purified in the presence of taxol, reacted with tau antibody. These peptides showed a similar molecular weight to those of porcine tau proteins.

DISCUSSION

MAPs are functionally defined by their binding to tubulin polymers [2]. In this work two criteria have been followed to purify and characterize MAPs from *D melanogaster*: (1) Polymerization in the presence of taxol and (2) affinity chromatography on tubulin-Sepharose. The former was used since it has been suggested that this procedure is suitable in culture cells to identify polypeptides that are associated with microtubules not only in vitro but also in vivo [2,24]. For example, sera against MAPs from mammalian tissues obtained from microtubules polymerized in the presence of taxol also react with antigens present in the microtubules of cultured cells [2]. The purification of taxol microtubules at different developmental stages indicated the presence of some common peptides with an MW of 205, 150, 80, and 42 kD. Other proteins were also found, depending of the development stages, such as 255 kD in embryos and adults, or 70–80-kD peptides, which probably correspond to the local serum proteins, in larvae.

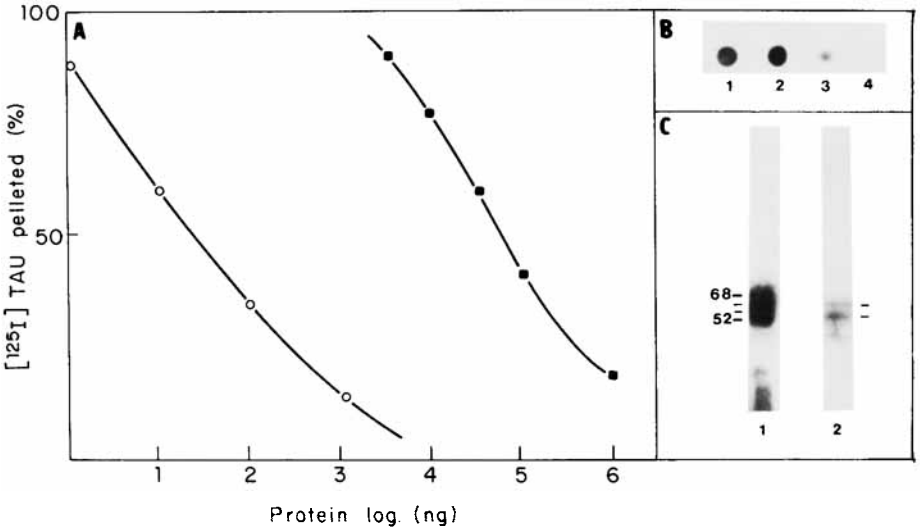

Fig. 7. Characterization of tau-like peptides on *D melanogaster* adult fly extracts. **A)** Radioimmunoassay. Purified tau labeled with ^{125}I (10,000 cpm ^{125}I-tau) was incubated with different concentrations of unlabeled brain tau (○) or with *D melanogaster* extracts (■) and incubated with antibody against tau protein diluted 1:200 as indicated in Materials and Methods. The mixtures were allowed to react 1 hr at 4°C. Afterward 15 μl of a suspension of *Staphylocorrus aureus* (Pansonbin, Calbiochem-Boehringer) diluted 1/5 was added to the mixture. The solution was centrifuged at 5,000g for 2 min and radioactivity associated to the pelleted protein was measured. **B)** Dot-blot. Different protein preparations incubated with antibody against tau factor followed by addition of ^{125}I protein A as indicated in Materials and Methods. Spot 1: pig brain microtubules 15 μg; spot 2: heat-stable pig brain MAPs (10 μg); spot 3: *D melanogaster* microtubules (15 μg); spot 4: bovine serum albumin (10 μg). **C)** Immunoblotting of pig brain microtubules (**lane 1**) and *D melanogaster* microtubules (**lane 2**) using a monospecific tau antibody (see Materials and Methods). The lines indicate the molecular weight of pig brain tau proteins (52 kD to 68 kD) (lane 1) and the tau-like proteins in D *melanogaster* (lane 2).

MAPs (tubulin binding proteins) isolated by affinity chromatography on tubulin-Sepharose included, in the case of adults, major peptides with MWs of 255, 205, and 170. Peptides of 80 kD, 68 kD, and 42 kD showed very low copolymerization capacity and for this reason we have not considered them as MAPs. In these preparations the protein of 255 kD was found in some but not in all cases. These differences could be due to the great susceptibility of this protein to proteolytic cleavage, as is the case with other high MW proteins. Some discrepancies can be found between our results and those described in previous reports. For example, Green et al [8] found embryo-stage polypeptides with MWs of 82, 61, 52, 48, and 46 kD. The latter peptide could correspond to the 42-kD protein indicated in our work, and may be actin, as suggested by Goldstein et al [9]. These authors detected MAPs with MWs of 255, 205, 150, 80, and 60 kD in *D melanogaster* cultured cells. These values are similar to those found in our work, after the affinity and ion exchange chromatography steps. Goldstein et al [9] show that antiserum against the 205-kD protein binds to an antigen located in the microtubule network in cultured cells, confirming the validity of the taxol procedure to isolate MAPs.

We cannot discard the possibility that some of those proteins present only embryo (100 kD), larvae (135 kD) or fly (130 kD) could represent MAPs developmental stage specific, but this idea needs more work trying to confirm this possibility.

On the other hand, the capacity of these peptides (255 kD, 250 kD, and 170 kD) to stimulate the polymerization of tubulin and their inability to bind to S-tubulin suggest that they behave as pig brain MAPs [22,23].

We have also detected low amounts of a couple of 60-kD peptides which react with a monospecific tau antibody, suggesting the presence of tau-related proteins in *D melanogaster*. Since *D melanogaster* is a suitable organism for genetic studies, the presence of tau proteins in this organism could facilitate its genetic dissection and the study of its function.

Finally, we should point out that the pattern of MAPs was more complex for larvae and adults than for embryos, in a similar fashion to that found during the development of mammalian organisms [25,26].

ACKNOWLEDGMENTS

We thank Drs. Hargreaves and R. Ripoll for critical reading of the manuscript and C. Hermoso for typing it. Monospecific antibodies against tau and tau protein were generous gifts from Dr. E. Montejo de Garcini. We thank Dr. L. Goldstein for sending us his manuscript containing unpublished results.

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