Vol. 115, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1983 Pages 303-311

TUBULIN IS A MAJOR PROTEIN CONSTITUENT OF BOVINE BRAIN COATED VESICLES

BERTRAM WIEDENMANN and LARRY T. MIMMS

Harvard University, The Biological Laboratories, 16 Divinity Ave., Cambridge, MA 02138

Received July 20, 1983

SUMMARY: A major protein constituent of coated vesicles derived from bovine brain is present as a M_r = 55,000 protein doublet on SDS-polyacrylamide gels. This protein comigrates with purified calf brain tubulin after two-dimensional electrophoresis, binds tubulin antibody and has two-dimensional tryptic peptide maps identical to those of purified tubulin. The results indicate that tubulin is a major protein constituent of coated vesicles. Coated vesicles had no significant effect on the polymerization rate of purified tubulin. Therefore, the physiological relevance of tubulin associated with coated vesicles remains to be determined.

Coated vesicles are involved in the transport of macromolecules among various intracellular compartments and between intracellular organelles and the plasma membrane (for reviews see 1. and 2.). These vesicles consist of a plasma membrane surrounded by a polyhedral protein lattice (3) whose assembly unit is a triskelion of three clathrin heavy chains and three light chains (4). The other major protein constituents of coated vesicles that can be separated by SDS-PAGE include at least 4 polypeptides in the M_r = 100,000 region, a protein doublet at M_r = 55,000 and a polypeptide at M_r = 50,000. Proteins in the 100,000 dalton region are believed to provide the binding site for clathrin on the coated vesicle membrane (5), but the identity and function of the 55,000 protein doublet has not been determined. Available data suggests that the 55,000 doublet and the

ABBREVATIONS: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(B-aminoethly ether)-N,N,N',N'-tetra acetic acid; IEF, isoelectric focusing; MES, 2-(N-morpholine) ethane sulfonic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethyl sulfonyl fluoride; SDS, sodium dodecyl sulfate

0006-291X/83 $1.50

50,000 polypeptide can be phosphorylated by an endogenous protein kinase associated with coated vesicles (6).

In this paper we identify the 55,000 dalton protein doublet as tubulin. Furthermore, we present evidence that coated vesicles have no significant effect on the rate of polymerization of purified tubulin.

MATERIALS AND METHODS

All standard chemical reagents used were of the highest purity available commercially. Phosphocellulose P-11 was from Whatman, England. GTP was purchased from Sigma. Ampholines were LKB-Products. Na ^{125}I was obtained from New England Nuclear. Calf brains were obtained from a local slaughterhouse, transported to the laboratory on ice and used immediately.

Coated vesicles were isolated from calf brains according to Altstiel and Branton (7) or by a modification of Pearse's technique (8) in which one continuous 90% ^2H_2O - 20% Ficoll to 9% ^2H_2O - 2% Ficoll gradient (28 ml) in buffer A (100 mM MES pH 6.5, 1.0 mM EGTA, 0.5 mM MgCl , 0.1 mM DTT, 0.005% PMSF and 0.2% NaN) was used in place of four sucrose density gradients. The turbid coated vesicle band above the pellet was collected, diluted at least four-fold with buffer A and concentrated by 1 hour centrifugation at 40,000 RPM in a Sorvall A641 rotor. Coated vesicles were resuspended and homogenized in buffer A. Prior to chromatography coated vesicles were centrifuged for 10 minutes at 10,000 RPM, to remove large aggregates. Coated vesicles were further purified by a single passage through a Sephacryl S-1000 column and the purity was determined as described previously (7). This procedure resulted in a two-fold higher yield of coated vesicles compared to the method using sucrose gradients. Electron microscopy showed that 95% or more of the vesicles in a preparation were coated vesicles. Stripped vesicles were prepared according to Unanue et al. (6).

Tubulin was isolated by the method of Shelanski (9) or, in some instances, by the method of Lee (10). The protein was used after three assembly-dissassembly cycles in buffer A containing 0.5 mM GTP throughout. To separate tubulin from microtubule associated proteins, tubulin was chromatographed on a phosphocellulose P-11 column according to Weingarten et al. (11). Column purified tubulin was free of any other protein contamination as determined by SDS-PAGE. Polymerization of tubulin was measured by light scattering at 350 nm, as described by Gaskin et al. (12).

One-dimensional polyacrylamide gel electrophoresis was performed by the procedure of Laemmli (13) using 6 - 15% linear gradient gel or a 10% gel system. For two-dimensional gels, the first dimension was run with a wide-range pH gradient. Isoelectric focusing gels contained 1.6% pH 3-10, 0.4% pH 6-8 ampholines (LKB), 9.5 M urea, 2.5% Nonidet-P 40, 3% acrylamide-bisacrylamide (28.4 : 1.6). Gels were equilibrated with an initial voltage of 125 volts which was increased incrementally to 500 volts over a period of 2 hours. Slab gels for the second dimension consisted of a 5% acrylamide stacking gel over a 10% running gel. Sample buffer was prepared as described by O'Farrell (14), with the exception that 8% Nonidet P-40 rather than 2.5% was used. This is a detergent concentration high enough to displace SDS from solubilized samples (15). Prior to the addition of sample buffer, we found it necessary to dissolve coated vesicles by

incubation at 70°C for 30 minutes in the presence of 1% SDS and 5% mercaptoethanol. Afterwards, samples were mixed with sample buffer at a ratio of 1 : 2, although ratios of even 4 : 1 did not effect the resolution. Non-equilibrium gel electrophoresis was performed according to O'Farrell (16) with the above described gel system.

Silver staining followed the procedure of Eschenbruch et al. (17). The light chains of coated vesicles derived from brain or liver were not stained by this procedure and in fact excluded the silver stain yielding colorless spots against the brownish background of gels with or without prior Coomassie blue staining.

Peptide mapping followed the procedure of Luna et al. (18).

Immunoblotting was performed as described by Burnette (19). Affinity purified tubulin antibody was kindly provided by Dr. Frank Solomon, MIT.

Protein concentration was measured either by Lowry et al. (20) or Bradford (21).

RESULTS

When purified coated vesicles are subjected to SDS-PAGE, several major polypeptide domains of M_r = 180,000, 100,000, 50,000, 36,000, 33,000, and a closely spaced doublet of M_r = 55,000 are found after Coomassie blue staining. This 55K band of brain coated vesicles is present after all known purification procedures(agarose gel electrophoresis, sucrose gradients, ^2H_2O-Ficoll gradients, and Sephacryl S-1000 column purifications) and represents about 10% of the total protein. Neither low ionic strength, pH 8.0 treatments, which remove clathrin leaving the stripped vesicle behind, nor high ionic strength (0.5 M TRIS or 0.5 KCl, pH 8.0), which partially removes the 100,000 and 50,000 dalton bands dissociated this 55,000 doublet from the vesicles. Since brain homogenates contain substantial amounts of tubulin, which appears as a 55,000 dalton doublet in our SDS-PAGE system, we investigated whether this coated vesicle associated doublet was in fact tubulin.

The protein doublet at M_r = 56,000 and 54,000 on coated vesicles is identical to tubulin by several criteria. First, purified tubulin comigrates with a protein doublet on both one- and two-dimensional gel systems (Fig. 1,2).

Secondly, monospecific tubulin antibody cross-reacts with the protein doublet as determined by immuno blot analysis (Fig. 3).

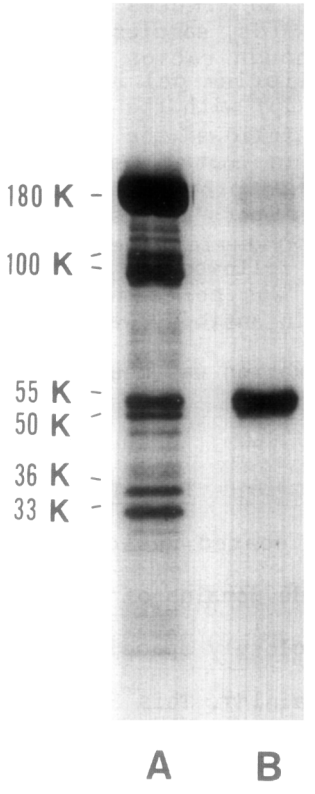

Figure 1: SDS-polyacrylamide gel electrophoresis of coated vesicles (A) and tubulin (B) stained with Coomassie blue.

Finally, tryptic peptide maps of tubulin and the 55,000 doublet excised from gels are almost identical (Fig. 4). These data, taken together, show that the 56,000 and 54,000 dalton doublet of coated vesicles is tubulin.

Interestingly, two dimensional gels show that much of the 180,000, 100,000 and 50,000 dalton polypeptides comigrate as a complex in the isoclectrofocusing dimension (Fig. 2, inset). The same comigration was also observed in liver derived coated vesicles (data not shown). In addition, a certain proportion of coated vesicle protein never entered the IEF-gel, either because the sample was not completely solubilized or the protein was too basic to be focused under these conditions. To test the latter possibility, we performed two-dimensional electrophoresis under non-equilibrium conditions as

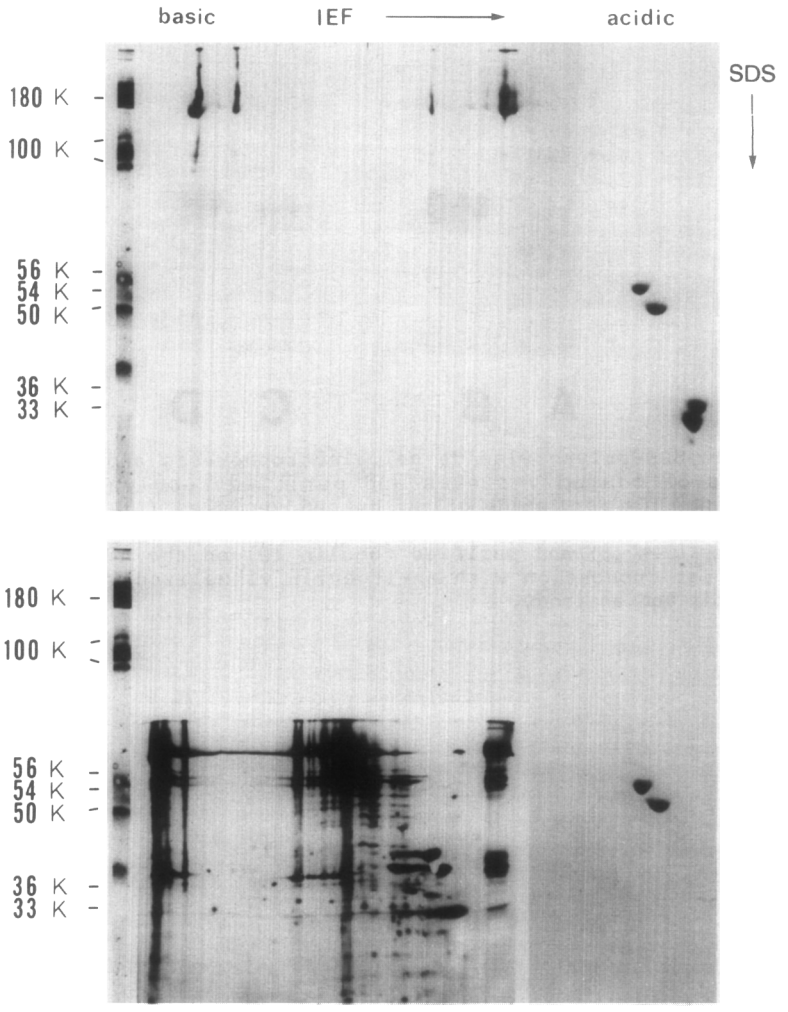

Figure 2: Two-dimensional polyacrylamide gel electrophoresis of coated vesicles (upper panel) and purified tubulin (lower panel) stained with Coomassie blue. Coated vesicles were also stained with silver (inset). Wide-range isoelectric focusing was performed as described in Materials and Methods.

described by O'Farrell (16). No additional proteins in the 55,000 dalton region were detected (data not shown).

To determine if coated vesicles serve as nucleation sites for tubulin, purified tubulin was incubated with coated vesicles under polymerization conditions. The presence of coated vesicles or stripped vesicles (up to 1.8 mg/ml or 0.75 mg/ml respectively) did not enhance the polymerization of either assembly purified tubulin (Fig. 5) or tubulin purified on phosphocellulose (not shown). In contrast,

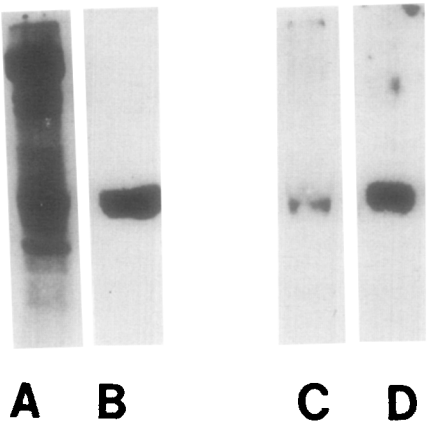

Figure 3: SDS-polyacrylamide gel electrophoresis and immuno blot analysis of coated vesicles and purified tubulin. Coomassie blue-stained 10% acrylamide gel of coated vesicles (A) and purified tubulin (C) prior to transfer to nitrocelluose. Autoradiographs of coated vesicles (B) and purified tubulin (D) on the nitrocellulose sheet after incubation with antitubulin visualized with ^{125}I-goat anti-rabbit IgG antibody.

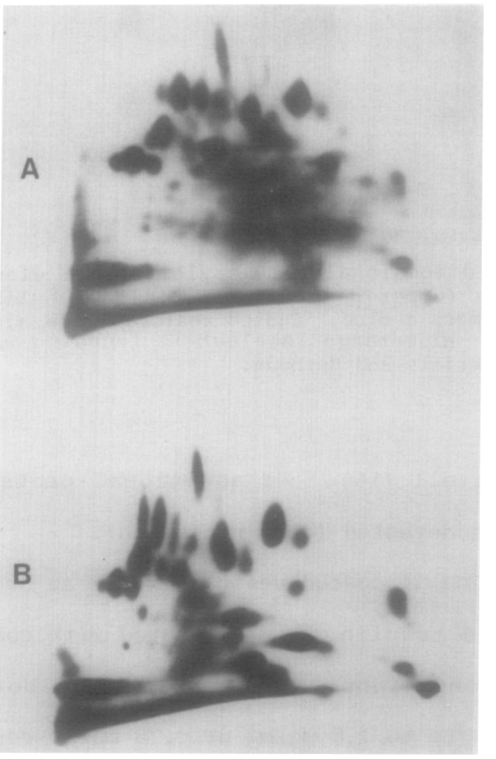

Figure 4: Comparison of the ^{125}I-labeled tryptic peptide maps of 55K peptides of coated vesicles (A) and tubulin (B). Electrophoresis was performed from right to left, chromatography was from bottom to top.

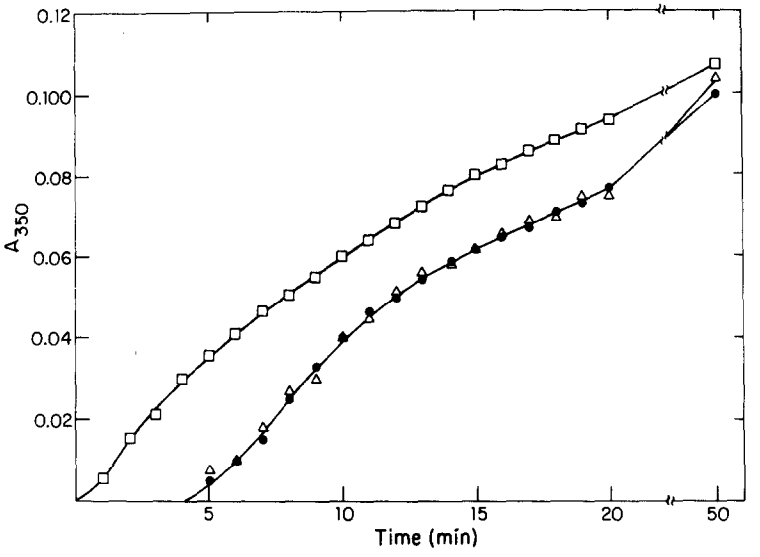

Figure 5: Effect of coated vesicles and sonicated microtubule fragments (seeds) on the polymerization of assembly-purified tubulin as determined by light scattering at 350 nm. 250 microliters of buffer A (control (circle)), coated vesicles (triangle), 2.8 mg/ml, or seeds (square) 0.2 mg/ml, were added to 750 microliters of 3 times assembly-purified tubulin (1.2 mg/ml) containing assembly buffer (buffer A and 0.5 mM GTP). Temperature, 20.5° C. The turbidity of coated vesicles or seeds in assembly buffer alone was subtracted from polymerizing tubulin and was unchanged over a period of more than one hour.

an amount of microtubule fragments (seeds) equal in concentration to coated vesicle associated tubulin completely abolished the polymerization lag phase of assembly purified tubulin (Fig. 5).

DISCUSSION

Tubulin is known to be tightly associated with various membranes (22, 23), many proteins (24) and also lipids (25, 26). Tubulin associated with purified coated vesicles is found by several methods, but the nature of the association of tubulin with coated vesicles is not yet understood. What we do know is that treatments that remove clathrin and its light chains from the vesicle do not dissociate tubulin from the vesicle. Also the addition of 0.5 M TRIS, which removes much of the 100,000 proteins and a 50,000 protein, leaves most of the tubulin associated with the vesicle (M. Hanspal, personal communication). Therefore, the association of tubulin with

coated vesicles does not appear to require these proteins. Finally, treatment of coated vesicles or stripped vesicles with Triton X 100 did not result in any removal of tubulin (unpublished observation). Thus tubulin is very tightly associated with the vesicle.

Under our conditions, the coated vesicle associated tubulin does not serve as a nucleation site for tubulin polymerization. Although we do not know the state of tubulin on the coated vesicles, this tubulin is clearly not able to eliminate the polymerization lag phase as do tubulin seeds. Because the enhanced polymerization rates depend on several parameters (the presence of either preformed microtubules, microtubule associated proteins, or a combination of both as well as the concentration of these constituents), it is premature to draw firm conclusions about the inability of coated vesicle associated tubulin to stimulate the polymerization of exogenous tubulin.

ACKNOWLEDGEMENTS: We are especially grateful to Dr. F. Solomon for helpful discussions. We would also like to thank Cheryl O'Connor for typing our manuscript.

REFERENCES

1. Goldstein, J.L., Anderson, R.G.W. and Brown, M. (1979) Nature **279**, 679-685.
2. Steinman, R.M., Mellman, I.S., Muller, W.A. and Cohn, Z.A. (1983) J. Cell Biol. **96**, 1-27.
3. Kanaseki, T. and Kadota, J. (1969) J. Cell Biol. **42**, 202-220.
4. Ungewickell, E. and Branton, D. (1981) Nature **289**, 420-422.
5. Unanue, E.R., Ungewickell, E. and Branton, D. (1981) Cell **26**, 439-446.
6. Pauloin, A., Bernier, I. and Jolles, P. (1982) Nature **298**, 574-576.
7. Altstiel, L. and Branton, D. (1983) Cell **32**, 921-929.
8. Pearse, B.M.F. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 451-455.
9. Shelanski, M.L., Gaskin, F. and Cantor, C.R. (1973) Proc. Natl. Acad. Sci. U.S.A. **70**, 765-768.
10. Lee, J.C. (1982) Meth. Cell Biol. **24**, Pt. A., 9-29.
11. Weingarten, M.D., Lockwood, A.H., Hwo, S.-Y. and Kirschner, M.W. (1975) Proc. Natl. Acad. Sci. U.S.A. **72**, 1858-1862.
12. Gaskin, F., Cantor, C.R. and Shelanski, M.L. (1974) J. Mol. Biol. **89**, 737-758.
13. Laemmli, U.K. (1970) Nature **227**, 680-xxx.
14. O'Farrell, P.H. (1975) J. Biol. Chem. **250**, 4007-4021.
15. Harell, D. and Morrison, M. (1979) Arch. Biochem. Biophys. **193**, 158-168.
16. O'Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H. (1977) Cell **12**, 1133-1142.
17. Eschenbruch, M. and Burk, R.R. (1982) Anal. Biochem. **125**, 96-99.

18. Luna, E.J., Kidd, G.H. and Branton, D. (1979) J. Biol. Chem. 254, 2526-2532.
19. Burnette, W.N. (1981) Anal. Biochem. 112, 195-203.
20. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
21. Bradford, M. (1976) Anal. Biochem. 72, 248-254.
22. Sherline, P., Lee, Y.-L. and Jacobs, L.S. (1977) J. Cell Biol. 72, 380-389.
23. Bhattacharyya, B. and Wolff, J. (1975) J. Biol. Chem. 250, 7639-7646.
24. Solomon, F. (1981) Cold Spring Harbor Symp. 46, 17-22.
25. Caron, J.M. and Berlin, R.D. (1979) J. Cell Biol. , 665-671.
26. Kumar, N., Blumenthal, R. Henkart, M., Weinstein, J.N. and Klausner, R.D. (1982) J. Biol. Chem. 257, 15137-15144.