Volume 282, number 1, 65–68 FEBS 09620 April 1991

ADONIS 001457939100328 3

MAP2-mediated binding of chromaffin granules to microtubules

F.F. Severin[1], N.A. Shanina[2], S.A. Kuznetsov[2] and V.I. Gelfand[1]

[1]*Institute of Protein Research, Academy of Sciences of the USSR, Pushchino, Moscow Region, 142292 USSR* and [2]*Department of Molecular Biology, Moscow State University, Moscow 119899, USSR*

Received 8 February 1991

We have examined the interaction of chromaffin granules from bovine adrenal medulla with microtubules. Chromaffin granules were mixed with microtubules made of phosphocellulose-purified tubulin, and pelleted through a 1.6 M sucrose cushion at 12000 × *g* for 10 min. Both components (granules and microtubules) were pelleted when added together but not separately. This result indicates that granules form a heavy complex with the microtubules. Such a complex was visualized by an electron microscopy of the granule/microtubule mixture. Treatment of the granules with trypsin abolished their ability to interact with the microtubules. The binding of the granules to the microtubules: (i) was not sensitive to ATP; and (ii) was completely inhibited by the cleavage of C-terminal peptides of α- and β-subunits of tubulin with subtilisin. These relationships suggest that the granule binding is mediated by one of the structural microtubule-associated proteins rather than by microtubule-dependent translocators. For identification of protein(s) mediating the binding, the granules were solubilized with Triton X-100, soluble proteins were mixed with the microtubules, and microtubules with bound proteins were pelleted through a glycerol cushion. At least one granule protein interacting with the microtubules was found in the pellet. This protein was identified as MAP2 according to its electrophoretic mobility and reactivity with a MAP2 antibody. Affinity chromatography of solubilized proteins on a column containing taxol-stabilized microtubules also revealed MAP2 as a protein of chromaffin granules interacting with the microtubules.

Chromaffin granule; Microtubule; Membrane–microtubule interaction; MAP2; Adrenal medulla

1. INTRODUCTION

Microtubules are involved in the movement and positioning of membranous organelles within the cells (for a review see [1]). Several recent studies have demonstrated a number of microtubule-dependent translocator proteins. Kinesin, translocating the material towards the plus ends of microtubules [2], and cytoplasmic dynein, translocating it in the opposite direction [3], belong to this group of proteins. Kinesin and dynein are thought to mediate the translocation of organelles inside the cell [4–6]. Organelles, moving along the microtubules also contain some microtubule-binding proteins which exhibit no apparent translocator activity. These include 50 kDa protein from lysosomes [7], 58 kDa [8] and 110 kDa [9] proteins from the Golgi complex as well as MAP2, which was found in the mitochondrial outer membrane [10]. Chromaffin granules are the secretory vesicles of the adrenal medulla cells. These cells have a well-developed tubulin cytoskeleton [11], and contain kinesin [12] and MAPs [11]. It therefore would be tempting to suggest that chromaffin granules can bind microtubules and move along them. It was recently found that isolated chromaffin granules can move along microtubules in the presence of exogenous kinesin [13]. Below, experiments will be described showing that chromaffin granules do interact with microtubules in vitro in a MAP2-mediated fashion.

Correspondence address: F.F. Severin, Institute of Protein Research, Academy of Sciences of the USSR, Pushchino, Moscow Region, 142292, USSR

Abbreviations: S-microtubules, microtubules made of subtilisin-digested tubulin; MAPs, microtubule-associated proteins; SDS-PAGE, electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulfate; TI, soybean trypsin inhibitor; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; BSA, bovine serum albumin

2. MATERIALS AND METHODS

Chromaffin granules were obtained using the differential centrifugation method with subsequent pelleting through a sucrose cushion as described in [14]. 0.3 or 1.6 M sucrose were dissolved in buffer A (50 mM imidazole-HCl buffer, pH 6.8, containing 3 mM $MgCl_2$, 1 mM EGTA and 0.1 mM EDTA, 1 mM 2-mercaptoethanol). Microtubules and subtilisin-digested microtubules (S-microtubules) were made from phosphocellulose-purified bovine brain tubulin as described previously [15].

Trypsin treatment of the granules was performed for 20 min at room temperature. The concentration of trypsin was 1 μg/ml and that of granule protein, 3 mg/ml. The treatment was stopped by soybean trypsin inhibitor (TI) (10 μg/ml). Granules incubated with a mixture of 40 μg/ml TI and 4 μg/ml trypsin were used as a control.

For the pelleting assay, the granules (3 mg/ml protein) were suspended in 1 ml of buffer A with 1.5 M sucrose, mixed with 20–40 μl of microtubules (2 mg/ml), layered on a 1.6 M sucrose cushion in buffer A and pelleted for 10 min at 10000 rpm and 20°C in a Beckman SW 50.1 rotor.

For identification of the granule proteins interacting with the microtubules, the granules were extracted with 0.5% Triton X-100 in a 50 mM imidazole-HCl buffer, pH 6.8, containing 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA and 1 mM

2-mercaptoethanol (buffer B); the extract was clarified by centrifugation and subjected to affinity chromatography on a column with taxol-stabilized microtubules immobilized on Affi-gel 10, as described by Kellogg et al. [16]. The bound proteins were eluted with 2 mM ATP and then with 0.5 M KCl. A column with immobilized bovine serum albumin was used as a control.

For co-sedimentation of the granule proteins with microtubules, the Triton extract was mixed with taxol-stabilized microtubules and pelleted through 4 M glycerol on buffer B. SDS-PAGE was performed as in [17] and immunoblotting was carried out according to Towbin et al. [18]. An affinity-purified rabbit anti-MAP2 antibody was kindly provided by Dr V.I. Rodionov.

3. RESULTS

The pelleting assay was used to find out whether chromaffin granules interact with microtubules. The granules were mixed with MAP-free microtubules, pelleted through a 1.6 M sucrose cushion, and the pellet was analyzed by SDS-PAGE. Either granules or microtubules, taken separately, were used as a control (Fig. 1). It was found that only the granule-microtubule mixture was pelleted through the cushion (Fig. 1A, lane 6) but not the granules or the microtubules separately (Fig. 1A, lanes 1,2). Sedimentation of granules in the presence of microtubules, but not in their absence, shows that the granules form a complex with the microtubules. Electron microscopy confirms the presence of such aggregations in the granule/microtubule mixture (Fig. 2A).

The pelleting assay with trypsin-treated granules was performed in order to show which of the granule components, protein or lipid, mediates the interaction between the granules and the microtubules. Granules pretreated with trypsin did not bind to the microtubules and therefore were not pelleted (Fig. 1B, lane 1). Fig. 1B (lanes 3,4) shows that a number of high molecular weight granule proteins were cleaved by trypsin. It is also clear that the amount of the intragranule protein, chromogranin, was not altered by trypsin digestion. Inhibition of the interaction between the granules and the microtubules by trypsin treatment shows that it is mediated by granule proteins rather than lipids.

The next step of our study was to identify the protein(s) in question. Two facts were taken into consideration. (i) ATP decreases the binding of microtubule-dependent translocators to microtubules [1,2]; (ii) 4-kDa C-terminal domains of α- and β-tubulins, which can be cleaved with subtilisin, are essential for interaction of microtubule with MAPs [19], but not with kinesin and dynein [14]. First, the ATP dependence of co-pelleting was tested. It is clear from Fig. 1A that there is no difference between the pellets obtained in the absence (lane 6) or in the presence (lane 5) of ATP. Secondly, we tested the binding of the granules to S-microtubules. Fig. 1A (lanes 3,4) shows that subtilisin digestion of microtubules completely inhibited the granule–microtubule interaction. The electron microscopy assay was used for additional demonstration of the absence of interaction between S-microtubules and granules (Fig. 2B). Unlike the mixture of granules with intact microtubules (Fig. 2A), there are no microtubule/granule complexes in the case of S-microtubules. So, protein(s) mediating the granule–microtubule interaction are neither kinesin nor dynein.

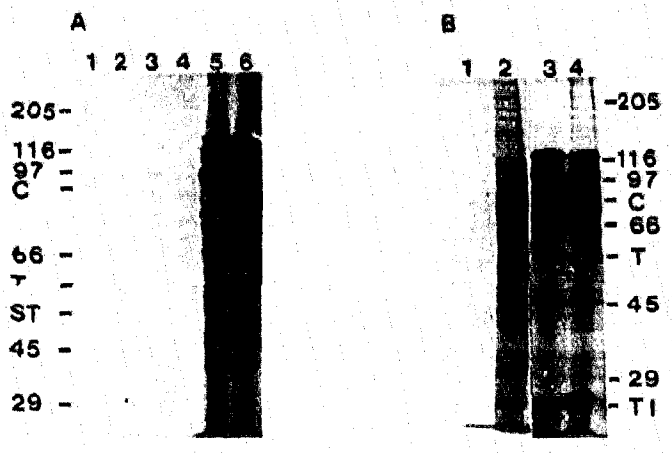

Fig. 1. Co-sedimentation of chromaffin granules with microtubules. (A) Effect of ATP and cleavage of 4 kDa C-terminal domain of tubulin on co-pelleting of granules with microtubules. (Lane 1) Granules; (lane 2) microtubules; (lane 3) S-microtubules + granules + ATP; (lane 4) S-microtubules + granules; (lane 5) microtubules + granules + ATP; (lane 6) microtubules + granules. Note that the granules cosediment with intact microtubules, and this co-sedimentation is not sensitive to ATP (lanes 5,6). Granules were not pelleted with S-microtubules (lanes 3,4). Granules and microtubules were not pelleted separately either (lanes 1,2). Arrows show the positions of tubulin (T) and chromogranin (C). (B) Effect of trypsin treatment on the binding of chromaffin granules to microtubules. Co-sedimentation of intact microtubules with trypsin-pretreated (lane 1) and intact granules (lane 2); preparations of trypsin-pretreated and intact granules (lanes 3,4, respectively). Arrows show the positions of tubulin (T), chromogranin (C) and soybean trypsin inhibitor (TI).

In order to learn which particular protein(s) promotes the granule–microtubule interaction, we used affinity chromatography on a microtubule column [16] and the pelleting assay. For affinity chromatography, the Triton extract of the granules was loaded onto the microtubule column; after washing, the two consecutive fractions were collected, 2 mM ATP eluate and 0.5 M KCl eluate. The same set of proteins was found in the ATP-eluates from BSA- and microtubule columns (Fig. 3A, lanes 2,4). Therefore, no protein from the Triton extract showed ATP-dependent specific binding to microtubules. This result is in good agreement with the data on pelleting experiments showing an ATP-independence of the granule-microtubule binding. Lanes, corresponding to KCl-eluates from microtubule and BSA columns, show that there was at least one protein which was specifically eluted from the microtubule column (Fig. 3A, lanes 3,5). All the other

Fig. 2. Electron microscopy of the mixtures of granules and microtubules (A) and with S-microtubules (B). Note, that the granules interact with intact, but not with S-microtubules. Bar = 1 μm.

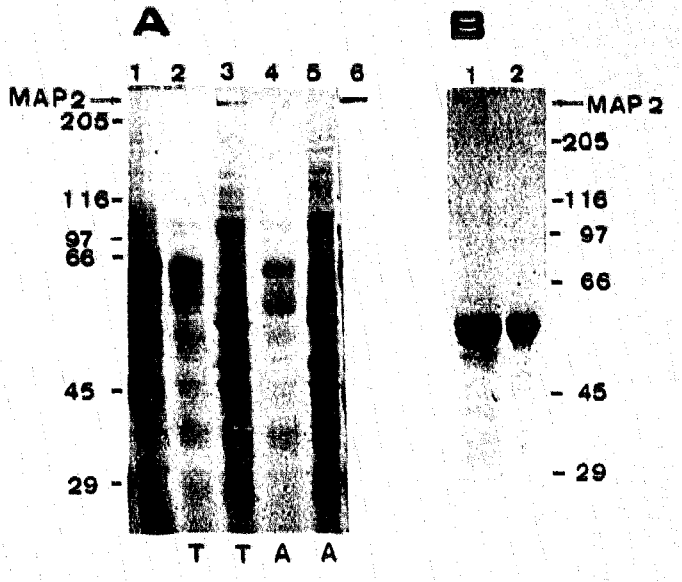

Fig. 3. Analysis of granule proteins responsible for the granule–microtubule interaction. (A) Affinity chromatography of the Triton X-100 extract of the granules on the microtubule column. Triton-solubilized proteins (lane 1); 2 mM ATP and 0.5 M KCl eluates from the microtubule column (lanes 2,3); 2 mM ATP and 0.5 M KCl eluates from BSA column (lanes 4 and 5); anti-MAP2 antibody staining of the protein blots from lane 3 (lane 6). The only protein, specifically eluted from microtubules, co-migrates with MAP2 (arrow) and is stained with an anti-MAP2 antibody. (B) Co-sedimentation of the Triton extract of the granule proteins with microtubules. Microtubules were pelleted with (lane 1) and without (lane 2) Triton-solubilized proteins. The single band is present in lane 1 in addition to tubulin. This band co-migrates with bovine brain MAP2 (arrow).

proteins were common for microtubule and BSA columns and thus seemed to be bound to the column non-specifically. The molecular weight of this protein was close to that of MAP2. The corresponding band interacted with an anti-MAP2 antibody (lane 6).

A similar result was obtained using co-pelleting of the Triton extract of the granules with microtubules. The only component found in the pellet together with the microtubules had an electrophoretic mobility similar to that of MAP2 and this band could be stained with anti-MAP2 antibodies (data not shown).

4. DISCUSSION

Our results show that purified chromaffin granules contain the microtubule-associated protein MAP2 which mediates the binding of granules to microtubules. Experiments in vitro [13] show that pure granules move along microtubules only in the presence of exogenous kinesin. This is consistent with our data showing that the granules do not bind to the S-microtubules. Thus, the granules do not contain kinesin capable of interacting with microtubules.

Most probably, binding of kinesin to chromaffin granules is reversible, and all the bound kinesin is removed during granule purification. Therefore, the putative role of MAP2 in chromaffin granules is to operate as a transitory anchor, i.e. suppress the dissociation of the granules from the microtubules. On the other hand, the transitory binding of the granules to the microtubules should not be too tight, so as to enable their movement after kinesin association. Moreover, it is even possible that the MAP2-dependent binding of the granules to the microtubules facilitates subsequent association of kinesin with the chromaffin granules: when the amount of kinesin bound to the granules reaches the threshold level, the granules start moving.

The data on secretory vesicles from Madin-Darby canine kidney (MDCK) cells also support this hypothesis. First, it has been shown that microtubule depolymerization by nocodazole slows down the in-

tracellular transport of the vesicles [1,20]. Secondly, vesicles from MDCK cells bind to microtubules; this binding is inhibited after saturation of microtubules with MAP2 [21]. But it is also known that microtubule motor proteins do not compete with MAPs for microtubule binding [15]. Thus the microtubule binding of transporting organelles is not mediated by motor proteins in MDCK cells.

This model may also be operative in other cases of the microtubule-dependent organelle movement. For example, different organelles, that are known to move along microtubules, contain different MAPs without any apparent motor activity [6–9]. The binding of organelle-associated MAPs to microtubules is relatively weak [6–9]. Probably, such weak binding is essential to let the translocator proteins move particles along microtubules, while the binding of the vesicles to the microtubules potentiates association of translocators.

Acknowledgements: We are grateful to Mrs N.V. Minina, Mrs G.A. Kuznetsova for excellent technical assistance and Dr Y.A. Kamenski who initiated our interest to chromaffin cells.

REFERENCES

[1] Kelly, R.B. (1990) Cell 61, 5–7.
[2] Vale, R.D., Reese, T.S. and Sheetz, M.P. (1985) Cell 41, 39–50.
[3] Paschal, B.M. and Vallee, R.B. (1987) Nature 330, 181–183.
[4] Hollenbeck, P.J. and Swanson, J.A. (1990) Nature 346, 864–866.
[5] Rodionov, V.I., Gyoeva, F.K. and Gelfand, V.I. (1991) Proc. Natl. Acad. Sci. USA (in press).
[6] Schroer, T.A., Steuer, E.R. and Sheetz, M.P. (1989) Cell 56, 937–946.
[7] Mitheleux, G. and Rousset, B. (1989) J. Biol. Chem. 264, 4664–4668.
[8] Bloom, G.S. and Brashear, T. (1989) J. Biol. Chem. 264, 16083–16092.
[9] Alan, V.J. and Kreis, T.E. (1986) J. Cell. Biol. 103, 2229–2239.
[10] Linden, M., Nelson, B.D., Loncar, D. and Letterier, S.F. (1989) J. Bioenerg. Biomembr. 21, 507–518.
[11] Kotani, S., Murofushi, H., Maekama, S., Sato, C. and Sakai, C. (1986) Eur. J. Biochem. 156, 23–29.
[12] Murofushi, H., Ikai, A., Okuhara, K., Kotani, S., Aizawa, H., Kumakura, K. and Sakai, H. (1988) J. Biol. Chem. 263, 12744–12750.
[13] Urrutia, R., McNiven, M.A., Albanesi, J.P., Murphy, D.B. and Kachar, B. (1991) Proc. Natl. Acad. Sci. USA (in press).
[14] Terland, O. and Flatmark, K. (1980) Biochim. Biophys. Acta 597, 318–330.
[15] Rodionov, V.I., Gyoeva, F.K., Kashina, A.S., Kuznetsov, S.A. and Gelfand, V.I. (1990) J. Biol. Chem. 265, 5702–5707.
[16] Kellog, D.R., Field, C.M. and Albrets, B.M. (1990) Methods Enzymol. (in press).
[17] Laemmli, U.K. (1970) Nature 227, 680–685.
[18] Towbin, H.T., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[19] Serrano, L., Avila, J. and Maccioni, R.B. (1984) Biochemistry 23, 4675–4681.
[20] Parczuk, K., Haase, W. and Kondor-Koch, C. (1989) J. Biol. Chem. 264, 16837–16846.
[21] Van Der Sluijs, P., Bennet, M.K., Antony, C., Simons, K. and Kreis, T.E. (1990) J. Cell. Sci. 95, 543–545.