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Interaction of microtubules and microtubule-associated proteins (MAPs) with rat brain mitochondria

Microtubules perform a variety of functions in neuron cells, and it has now been demonstrated that they are the substrate for axonal transport of membranous organelles [1]. The system of crossbridges observed between microtubules and membranous organelles appears to serve as means of transport, direction of movement and fixation of organelles [2,3]. It has been suggested that the microtubule-associated proteins (MAPs) participate in the formation of such crossbridges [4]. In a recent paper [5], we have shown that the two major brain MAPs, MAP2 and the tau proteins, are able to bind to purified rat brain mitochondria. We observed that ^{32}P-labelled MAP2 and tau bound to mitochondria could be displaced by phosphorylated non-radioactive MAP2. We also provided evidence that MAP2 bound to the mitochondria predominantly via its microtubule-binding domain.

Similar studies have been carried out by Lindén *et al.* [6]. One of the major conclusions of that paper is that mitochondria preferentially interact with the high-molecular-mass MAPs and not with tau proteins. This has been derived from experiments in which mitochondria were incubated with thermostable MAPs (a mixture of MAP2 and tau proteins), at a concentration of 0.15 mg/ml. Since the ratio of MAP2 to tau in the preparation is not indicated, we might assume that the concentration of the two proteins corresponds to their reported radioactivity, given as 90% MAP2 and 10% tau. Thus 0.15 mg/ml thermostable MAPs corresponds approximately to 4.8×10^{-7} M-MAP2 and 2.6×10^{-7} M-tau (average molecular masses of 280 and 58 kDa respectively), which means that the concentration of MAP2 is five times higher than its IC_{50} value (0.9×10^{-7} M) [5]. Consequently, it is not surprising that under these experimental conditions the amount of tau proteins bound to mitochondria is under the threshold level detectable by autoradiography. Our previous findings [5] and those reported here (Fig. 1*a*) clearly demonstrate that purified tau proteins are able to bind to rat brain mitochondria.

Lindén *et al.* [6] considered the arm-like projection of MAPs [7] as the most plausible candidate for establishing association with mitochondria. This was inferred from the lack of binding of tau proteins (discussed above) and from the finding that pure tubulin inhibits only slightly the binding of MAPs to mitochondria. This result, again, is not surprising, since as reported in [8] purified tubulin is able to bind to rat liver mitochondria with a K_d of 3.7×10^{-8} M. Thus, liver mitochondrial membranes seem to possess specific binding sites for tubulin. Though the binding of tubulin to rat brain mitochondria was not studied, conclusions for an interaction between the two organelles through a site located on the projection domain of MAPs need to be drawn carefully. We feel that there are no data in [6] that are not consistent with our proposal that MAP2 and tau proteins interact with mitochondria preferentially via their microtubule-binding domain [5].

Finally, Linden *et al.* [6] claimed that if taxol-stabilized microtubules are incubated with mitochondria and then recovered by mild centrifugation conditions, only the high-molecular-mass MAPs (MAP1 and MAP2), and not tubulin, are recovered in the mitochondrial pellets. We have been carrying out similar experiments, by utilizing radioactivity measurements or autoradiography of the ^{125}I-labelled microtubular proteins [9] for the detection of proteins bound to mitochondria. We have found that in the absence of mitochondria the non-specific pelleting (for experimental conditions see Fig. 1*b*) of total ^{125}I-taxol-treated microtubules in the presence or absence of endogenous MAPs was respectively 14 ± 3% and 8 ± 1.8%. When incubated with mitochondria the radioactivity which cosedimented increases to 37 ± 1% for total microtubules and 16 ± 1.5% for tubulin free of MAPs. Furthermore, Fig. 1(*b*) clearly shows by autoradiographic visualization that tubulin is able to bind mitochondria when mitochondria are incubated

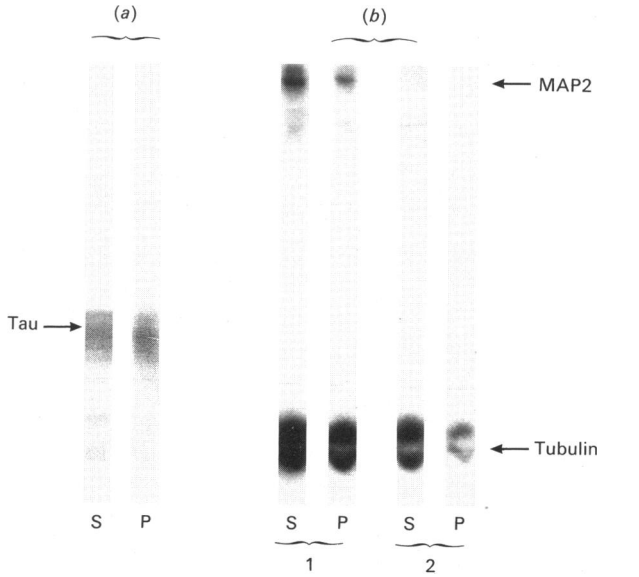

Fig. 1. Binding of tau proteins, MAP2 and tubulin to rat brain mitochondria

(*a*) ^{32}P-labelled proteins (26 μg/ml) and mitochondria (4.5 mg/ml) prepared as in [5] were incubated for 10 min at 37 °C in buffer A: 0.1 M-Mes/1 mM-$MgCl_2$/1 mM-EGTA/0.32 M-sucrose, pH 6.8, supplemented with a mixture of protease inhibitors (*p*-tosyl-L-arginine methyl ester, 0.1 mg/ml; aprotinin, 0.05 unit/ml; pepstatin, 1 mM; leupeptin, 1 mM; trypsin inhibitor, 0.1 mM; phenylmethane sulphonyl fluoride, 1 mM; NaF, 0.1 mM). After incubation, the reaction mixtures were layered on a 0.5 M-sucrose cushion and centrifuged at 12000 *g* for 10 min. Supernatants (S) and pellets (P) were resolved by LDS/PAGE (10% acrylamide). For autoradiography the dried gels were exposed to Kodak X-Omat AR5 film using two intensifying screens at −70 °C for 24 h (S) and 96 h (P). (*b*) Microtubule proteins were radioiodinated by conjugation with the ^{125}I Bolton–Hunter reagent as in [9]. ^{125}I-labelled microtubules (0.77 mg/ml) (1) or DEAE-Sephadex-purified ^{125}I-tubulin (2) were polymerized in the presence of 20 μM-taxol and incubated for 30 min at 37 °C with mitochondria (7.7 mg/ml) in buffer A supplemented with the protease inhibitor mixture. After centrifugation at 12000 *g* for 10 min, supernatants (S) and pellets (P) were resolved by LDS/PAGE (7.5% acrylamide). Protein bands were revealed by autoradiography at −70 °C for 96 h.

with taxol-stabilized microtubules either in the presence or absence of endogenous MAPs.

These data, taken together, support the idea of Martz *et al.* [10] that the mitochondrial membrane is multivalent. That is, it contains multiple sites capable of interacting with the various components of the cytoskeletal filaments. There is controversy between our results and those of Lindén *et al.* [6] concerning the nature of the cytoskeletal components capable of binding to mitochondria. Our conclusion is that, under *in vitro* conditions, brain mitochondria have the capacity to interact not only with the microtubule-associated proteins MAP1 and MAP2 but also with the tau proteins, as well as with tubulin.

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How do microtubules interact *in vitro* with purified subcellular organelles?

In two recent papers, we described the interaction of brain mitochondria with microtubule (MT) proteins [1,2]. The conclusions drawn from these studies are: (*a*) specific high-affinity binding sites for MAP2 exist on the surface of the mitochondria, probably on the outer membrane, and (*b*) that MAP2 interacts with an outer membrane domain which contains porin, or that MAP2 binding influences the physical structure of the porin-containing domain. (*c*) We also pointed out in our discussion that several experiments are consistent with the idea that the projection domain (in contrast with the MT-binding domain) of MAP2 binds to the mitochondrial surface.

In a subsequent study, Jancsik *et al.* [3] confirmed point (*a*) above. However, in the preceding letter [4] they challenge our conclusions in point (*c*) concerning the MAP2-binding domain which recognizes the mitochondrial surface. In doing so, they have focused upon a minor point of discussion in our original paper [1] and have amplified it into an issue for debate. However, since this question is, in fact, an important one, we feel it worthwhile to reiterate our views.

We reported four experimental findings which led to our tentative conclusion that the projection domain on MAP2 might bind to mitochondria [1,2].

1. MAP1 and 2 were transfered from taxol-stabilized MT to mitochondria. Since MAPs are attached to MT via the MT-binding domain, we assumed that the free projection domain interacts with mitochondria.

2. Pure tubulin did not compete for MAP2 binding to mitochondria, suggesting that the projection domain, and not the MT-binding domain of MAP 2, interacts with mitochondria.

3. Upon addition of a mixture of MAP2 and Tau to mitochondria, only MAP2 was observed to bind. This result is not expected if the MT-binding domain on Tau and MAP2 interacts with the mitochondrial surface.

4. Electron microscopy disclosed cross-bridge structures which are consistent with MAP2 and MAP1 acting as the link between MT and mitochondria. If one assumes that the MT-binding domain of MAPs, is, in fact, bound to MT, then the projection domain must be bound to mitochondria.

Rendon *et al.* argue [4] that MAP2 bind to mitochondria via the MT-binding domain on the MAP2 molecule because (*a*) the IC_{50} for the replacement of radioactive MAP2 or tau by unlabelled MAP2 were similar [3], and (*b*) that the MT-binding domain isolated from MAP2 replaced 40% of the bound MAP2 whereas the projection domain had no effect [3].

However, these arguments are not justified since, in (*a*) competition was done under conditions in which the Tau binding sites on mitochondria were saturated while the MAP2 sites were only 20% saturated, and in (*b*) the authors have used isolated projection fragments consisting of several degradative products after proteolytic treatment. In fact, their demonstration that the number of binding sites for the MT-binding fragment of MAP2 is 3-fold higher than that of the intact molecule [3] suggests that the binding characteristics of both fragments (containing MT-binding and projection domains) have been altered during preparation. The invalidity of this latter argument was, in fact, recognized in their original paper [3]. Thus, the conclusion that MAP2 binds to mitochondria via the MT-binding domain is not rigidly proven by Jancsik *et al.* [3].

In the preceding letter [4], Rendon *et al.* take issue with some of our experiments that we suggest support binding of MAP2 via its projection domain (see points 1–4 above). For the first, they point out that pure Tau protein can bind to specific sites on mitochondria. This finding raises some questions about the validity of our ideas expressed in point 3 above. Their second objection concerns experiments in which we observed no effect of tubulin on MAP2 binding (point 2 above). Rendon *et al.* [4] express the opinion that since tubulin binds *in vitro* to liver mitochondria [5] it should also bind to brain mitochondria. Furthermore, Rendon *et al.* [4] argue that bound tubulin cannot compete for MAP2. The latter thinking is faulty, however, since even if tubulin binds to brain mitochondria there are three possible results depending on the nature of the interaction, i.e. tubulin could enhance, inhibit, or, as suggested by Rendon *et al.* [4], have no effect on, MAP2 binding. The only situation in which mitochondria-bound tubulin would not affect binding of MAP2 is if MAP2 does not interact with this membranous tubulin. The association of tubulin monomers with membranous compartments including mitochondria has been known for several years [5–7]. The physiological significance of this binding is not understood, but it is clear that the membranes do not promote the polymerization of tubulin [5,7].

Finally, Rendon *et al.* [4] present an experiment which they suggest counters our ideas presented in point 1 above. However, we do not feel that this experiment invalidates our suggestion since Rendon *et al.* [4] used more drastic centrifugation conditions than ours [1], which lead to considerable aggregation of taxol-stabilized MT (14.3% and 8% for MAPs–MT and pure tubulin–MT respectively). The values increased by 2.6 and 2.0-