The specific binding of the microtubule-associated protein 2 (MAP2) to the outer membrane of rat brain mitochondria

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Purified mitochondria from rat brain contain microtubule-associated proteins (MAPs) bound to the outer membrane. Studies of binding *in vitro* performed with microtubules and with purified microtubule proteins showed that mitochondria preferentially interact with the high-molecular-mass MAPs (and not with Tau protein). Incubation of intact mitochondria with Taxol-stabilized microtubules resulted in the selective trapping of both MAPs 1 and 2 on mitochondria, indicating that an interaction between the two organelles occurred through a site on the arm-like projection of MAPs. Two MAP-binding sites were located on intact mitochondria. The lower-affinity MAP2-binding site ($K_d = 2 \times 10^{-7}$ M) was preserved and enriched in the outer-membrane fraction, whereas the higher-affinity site ($K_d = 1 \times 10^{-9}$ M) was destroyed after removing the outer membrane with digitonin. Detergent fractionation of mitochondrial outer membranes saturated with MAP2 bound *in vitro* showed that MAPs are associated with membrane fragments which contain the pore-forming protein (porin). MAP2 also partially prevents the solubilization of porin from outer membrane, indicating a MAP-induced change in the membrane environment of porin. These observations demonstrate the presence of specific MAP-binding sites on the outer membrane, suggesting an association between porin and the membrane domain involved in the cross-linkage between microtubules and mitochondria.

INTRODUCTION

The intracellular distribution of mitochondria is correlated with the microtubule or with the interlinked microtubule-intermediate-filament networks [1–5]. In neurons, a constant flow of organelles occurs in both directions along axonal processes [4–7]. However, little is known about the proteins which participate in the interaction between mitochondria and the cytoskeletal network. The recent discovery that MAP1C is a dynein-like ATPase which mediates the migration of vesicles along microtubules *in vitro* [8] suggests that the axonal movement of organelles might involve a family of macromolecules including MAPs. This is supported by the observation that MAPs belong to the microtubular lattice which links cytoskeletal fibres and the membrane of transported organelles *in situ* [9,10].

In the present work we have studied the interactions *in vitro* of isolated rat brain mitochondria with purified microtubule proteins. The results show that specific binding sites exist for MAPs on the mitochondrial outer membrane and that binding of MAPs is associated with changes in the membrane environment of the outer-membrane pore-forming protein porin [11]. These studies provide, for the first time, the opportunity of defining the site where microtubules interact with mitochondria during the movement of the latter.

MATERIALS AND METHODS

Chemicals

Mes, ATP (vanadate-free, magnesium salt, from equine

muscle), GTP (type III S), colchicine and proteinase inhibitors were from Sigma. [γ - 32 P]ATP (triethylammonium salt; sp. radioactivity 3000 Ci/mmol) was from Amersham International. Percoll was from Pharmacia. Taxol (a plant alkaloid) was generously given by Dr. L. Guenard (Laboratoire de chimie des Substances Naturelles, Gif-sur-Yvette, France). All other reagents were from Merck.

Purification procedures

Rat brain mitochondria were purified as described in [12]. The method was adapted for the large-scale isolation of mitochondria from eight animals (Wistar, males, 150–180 g). All steps were carried out in STE buffer (0.32 M-sucrose/1 mM-EDTA/10 mM-Tris/HCl, pH 7.4). Proteinase inhibitors (see below) were added to all buffers used after the recovery of mitochondria from the Percoll gradients. The yield of mitochondria was 1.5 mg of protein/g of wet brain. Few synaptosomes were present (Fig. 1b).

Mitochondrial outer-membrane fragments were obtained by digitonin extraction (0.8 mg of digitonin/mg of mitochondrial protein) as published elsewhere [13].

Microtubules were purified from rat brain by two cycles of polymerization in reassembly buffer (0.1 M-Mes/1 mM-MgCl₂/1 mM-EGTA/1 mM-GTP, pH 6.8) as described in [14].

MAPs were purified from microtubules phosphorylated *in vitro*. Autophosphorylation of MAPs was performed by incubating the repolymerized microtubules

Abbreviations used: MAP(s), microtubule-associated protein(s); PAGE, polyacrylamide-gel electrophoresis; STE buffer, sucrose/Tris/EDTA buffer (full composition and pH are given in the text).

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Fig. 1. MAPs are associated with the outer membrane fraction of rat brain mitochondria

(a) Rat brain mitochondria were fractionated with digitonin (0.8 mg/mg of protein) into an outer-membrane and an inner-membrane (mitoplast) fraction. Aliquots (40 μg of protein) of unfractionated mitochondria (lane 1), outer membranes (lane 2) and inner membrane (lane 3) were resolved by SDS/PAGE on 7.5%-acrylamide gels. Gels were stained with Coomassie Blue (i) or were transferred to nitrocellulose for the immunodetection of MAPs (ii). Purified microtubules (4 μg of protein) were used as control (lane 4). The concentration of MAPs in mitochondria is less than one-tenth of that in microtubules. The positions of molecular-mass (*M*) standards are indicated by arrows at the left of the Figure. (b) Ultrastructure of mitochondria (i), outer membranes (ii) and inner membranes (iii) after fractionation with 0.8 mg of digitonin/mg of protein.

with 50 μM-[γ-³²P]ATP (sp. radioactivity 0.5 μCi/nmol) in the presence of 5 μM-cyclic AMP. The labelled preparation was separated from free nucleotides by centrifugation, and thermostable ³²P-MAPs were purified as described in [15]. ³²P-MAP2 was further separated from the Tau proteins by chromatography on Ultrogel ACA 34. The standard preparation of phosphorylated MAP2 had a specific radioactivity of 5–10 μCi/mg of protein and contained 1–2 mol of phosphate/mol incorporated *in vitro* by the microtubule-associated cyclic AMP-dependent protein kinase.

Purified tubulin was obtained by chromatography of dissociated microtubules (two cycles of polymerization) on phosphocellulose as described in [16].

Incubation *in vitro* of mitochondrial membranes with microtubule proteins

Microtubule proteins (2 mg/ml) were preincubated with 15 μM-Taxol or 50 μM-colchicine for 1 h at 30 °C in

reassembly buffer adjusted to 0.32 M-sucrose. The stabilized (Taxol) or dissociated (colchicine) preparations were mixed with mitochondria in STE buffer, and the mixture was incubated at 30 °C in a 50 μl volume containing proteinase inhibitors [*N*-*p*-tosyl-L-arginine methyl ester (0.1 mg/ml), aprotinin (0.05 unit/ml), 1 μM-pepstatin, 1 μM-leupeptin, 1 mM-phenylmethanesulphonyl fluoride, 0.1 mM-chloroquine and 10 nM-soybean trypsin inhibitor]. Mitochondria and microtubule proteins were separated by centrifugation at 12000 *g* for 4 min (20 °C) on 100 μl of 0.5 M-sucrose in STE buffer. Centrifugation was carried out in a 200 μl Eppendorf tube. The rotation angle was 90°.

Binding of ³²P-MAP2 to mitochondria was performed under the same conditions. When tubulin was added to the incubation mixture, 1 mM-GTP was also present to protect the molecule from denaturation and to allow its association in polymers with MAPs. GTP does not affect the binding of MAPs. The mitochondrial pellet con-

Fig. 2. MAP2, but not Tau, binds to mitochondria *in vitro*

Mitochondria (2 mg/ml) were incubated 15 min at 30 °C with 0.15 mg of ^{32}P -MAPs/ml before centrifugation on to a 0.5 M sucrose cushion. Samples were resolved by SDS/PAGE (7.5%-acrylamide) and protein bands revealed by autoradiography. Lane 1, 2.5 µg of thermostable MAPs isolated from ^{32}P -labelled microtubules; lane 2, 100 µg of mitochondrial pellet; lane 3, an aliquot of the supernatant showing the unbound MAPs; Tau was present only in the supernatant (unlabelled arrowheads).

taining bound ^{32}P -MAP2 and the supernatant fractions were recovered by centrifugation for 8 min, and radioactivity was counted. The protein composition of the two fractions was analysed on 7.5%-(w/v)-acrylamide/SDS slab gels as described in [17].

Binding of ^{32}P -MAPs to outer membranes was done as described above, with the exception that membranes were removed by sedimentation for 10 min at 170000 g on a 100 µl layer of 0.5 M-sucrose in STE buffer using a Beckman Airfuge at 20 °C. The pellet was recovered and radioactivity analysed. The properties of the MAP2-binding sites on mitochondrial membranes were analysed by using the affinity spectra calculation given in [18].

Extraction of the mitochondrial MAP-binding site

To characterize the MAP2-binding site(s), mitochondrial outer membranes containing bound ^{32}P -MAP2 were extracted with Triton X-100 and 1 M-NaCl by using a procedure for the isolation of mitochondrial porin [11]. The distribution of MAP2 was monitored by using porin as a marker. Solubilized fractions were centrifuged for 14 h at 300000 g (SW 50 rotor) on a 5 ml 5–15% (w/v)-sucrose linear gradient containing 10 mM-sodium phosphate, pH 7.0, Triton X-100 and NaCl as described elsewhere [19]. Fractions were collected, and porin was detected by Western blotting [20] by using a monospecific polyclonal antiserum raised against rat liver mitochondrial porin [21]. As previously reported [13], a proteolytic degradation product of porin is frequently observed in brain mitochondria. MAPs were detected either by counting ^{32}P in the phosphorylated molecules or by Western blotting using an antiserum raised against polymerized microtubules which was specific for MAP1

and MAP2. The bound primary antibody was revealed with a goat anti-rabbit IgG coupled to horseradish peroxidase [20].

Other procedures

Protein was measured by the Lowry method [22], with bovine serum albumin as a standard. Samples for electron microscopy were fixed for 45 min with 1% glutaraldehyde in STE buffer. They were post-fixed with 1% OsO_4 in the same buffer, and stained *en bloc* with 1% uranyl acetate in water before dehydration with ethanol and embedding in Spurr resin. Ultrathin sections were post-stained with 1% uranyl acetate in 50% ethanol/1% lead citrate.

RESULTS

Characterization of MAP binding

Isolated rat brain mitochondria contain trace amounts of both MAPs 1 and 2, as indicated by Western-blot analysis using anti-(rat brain MAPs) antibodies (Fig. 1a). Both polypeptides are enriched approx. 10-fold in the outer-membrane fraction (Fig. 1a, lane 2). This enrichment is consistent with the binding data presented below, and strongly indicates that MAPs are associated with the outer membrane. Localization of '*in-vitro*'-bound MAPs to the outer membrane of intact mitochondria by immuno-gold labelling confirmed this suggestion (results not shown).

The MAP-binding sites were characterized using ^{32}P -labelled MAPs purified from microtubules as described in [15]. Although the preparation contains MAP2 and Tau ($\approx 90\%$ and $\approx 10\%$ of the radioactivity respectively) (Fig. 2), only MAP2 binds to the mitochondrial membrane (Fig. 2).

To determine accurately the binding characteristics of MAP2, contaminating ^{32}P -Tau was removed by chromatography (see the Materials and methods section). The association of Tau-free MAP2 to mitochondria was rapid at 30 °C, and was stable for more than 30 min (Fig. 3a). Binding was saturable, indicating a limited number of sites for MAP2 (Fig. 3b). Pure tubulin inhibits only slightly the binding of MAPs to mitochondria (Figs. 3c and 3d). This indicates that the tubulin-binding domain of MAPs [23] do not participate in binding to mitochondria. As expected, unlabelled MAPs competed with ^{32}P -MAP2 for binding sites on intact mitochondria (Fig. 3c).

The mitochondrial binding sites for MAP2, with K_d values of approx. 1×10^{-9} M and 2×10^{-7} M, were found in three different experiments. A typical Scatchard plot is shown in Fig. 4(a). The estimated concentrations of the high- and low-affinity binding sites were 3 and 14 pmol of MAP2/mg of mitochondrial protein respectively. A single binding site ($K_d = 2 \times 10^{-7}$ M) is preserved on outer membranes purified by digitonin solubilization (Fig. 4b). The number of these binding sites is estimated to be 114 pmol of MAP2/mg of protein, which is consistent with the expected enrichment of the outer membranes (7% of the mitochondrial proteins). However, approx. 40% of the mitochondrial binding sites remained with the mitoplasts after digitonin fractionation. A similar distribution of porin and hexokinase was reported previously for brain mitochondria fractionated with digitonin and was suggested to reflect a unique domain of the mitochondrial outer membrane [13].

Fig. 3. Conditions of the binding of ^{32}P -MAPs to purified mitochondria

(a) MAPs (0.2 mg/ml) and mitochondria (0.4 mg/ml) were mixed at 4 °C and incubated at 30 °C for the times indicated. The samples were centrifuged on a layer of sucrose. An interval of 30 s separated the loading time and the initiation of the centrifugation. (b) Binding of MAPs to mitochondria (1 mg/ml) incubated 15 min at 30 °C in the presence of increasing concentrations of MAPs, showing a saturation of the bound radioactivity above 0.1 mg/ml. (c) Linear relationship between the bound MAPs (0.17 mg/ml) after 15 min at 30 °C and the amount of mitochondria of the sample, in the presence (○) or the absence (●) of tubulin (0.32 mg/ml). (d) Effect of increasing concentrations of tubulin (○) and unlabelled MAPs (●), added to the sample before the start of the incubation, on the binding of a fixed concentration of ^{32}P -labelled MAPs (0.2 mg/ml) to mitochondria (1.44 mg/ml) after 15 min at 30 °C. The radioactivity recovered in the pellet was expressed as a percentage of the value obtained in the control sample (radioactive MAPs and mitochondria alone).

Effects of microtubule stability on MAP binding

In order to investigate the mechanism of MAP binding, mitochondria were incubated with purified microtubules which were fully depolymerized by colchicine or were stabilized by Taxol, and then recovered by mild centrifugation under conditions which do not sediment microtubules. In both cases, MAPs are removed from the supernatant during centrifugation, whereas tubulin is not (Fig. 5). This indicates that MAP binding to mitochondria is independent of the state of polymerization of microtubules. Since the MAPs recovered in mitochondria were not degraded (Fig. 5, lanes P2), mitochondria appear to induce their release from Taxol-stabilized microtubules. The transfer of MAPs from microtubules

Fig. 4. Characterization of the MAP-binding sites on mitochondrial membranes

Binding of the ^{32}P -MAP2 to intact brain mitochondria (4.2 mg/ml) (a) and to outer-membrane fractions purified by digitonin solubilization (0.8 mg/ml) (b) was carried out as described in the Materials and methods section. (c) SDS/7.5%-PAGE of 2.5 μg of purified MAP2. Lane 1, Coomassie Blue staining; lane 2, autoradiograph.

to mitochondria probably involves a direct physical contact between the two organelles. Cross-bridges between the outer membrane of mitochondria and microtubules were observed by electron microscopy (results not shown).

Subfractionation of the MAP-binding domain

The membrane domain in which MAPs are located was characterized by extracting outer membranes containing pre-bound ^{32}P -MAP2 by using procedures designed for the purification of porin [11]. The fraction solubilized by Triton X-100 and 1M-NaCl [11] was centrifuged on a 5–15 %-sucrose gradient in the same buffer (Fig. 6). The bound MAP2 exhibited a bimodal distribution, appearing in fractions 15–20 and in the pellet fraction. In contrast, MAP2 centrifuged alone under the same conditions sedimented as a single peak of $\approx 4.5 \text{ S}$ [24] in the first third of the gradient (Fig. 6a, arrow). This demonstrates that the bound MAP2 was associated with components of the outer membrane. Fractions 15–20 contain a distinct set of polypeptides which includes porin (Fig. 6b). The pellet fraction contains, in addition to MAP2, primarily porin, as indicated by Coomassie Blue staining (Fig. 6b, top panels) and immunoblotting (Fig. 6b, lower panels).

MAP2 binding alters the physical environment of the outer membranes, as indicated by its effect on the

Fig. 5. Trapping of MAPs on mitochondria after incubation with microtubules

Mitochondria (20 mg/ml) were incubated in a final volume of 50 μ l (see the Materials and methods section) with microtubules (0.4 mg/ml) previously depolymerized with 50 μ M-colchicine (a) or stabilized by 15 μ M-Taxol (b). After incubation, the organelles were separated into supernatant (S) or pellet (P) fractions by centrifugation on a cushion of 0.5 M-sucrose. Samples were resolved by SDS/PAGE (7.5% acrylamide). MAPs in the supernatants were revealed by staining with Coomassie Blue, and those in the mitochondrial-containing pellet fractions were detected by Western blotting. Microtubules were incubated in the absence (lanes 1) or presence (lanes 2) of mitochondria. (c) Shows mitochondria incubated in the absence of microtubules.

Fig. 6. Sedimentation of outer-membrane-bound MAP2 on linear 5–15%-sucrose gradient after partial solubilization of the membrane with Triton X-100 and NaCl

32 P-MAPs (0.3 mg/ml) were bound to the outer membrane (4.1 mg/ml) for 15 min at 30 $^{\circ}$ C, followed by repurification of the membranes, which were then extracted with Triton X-100 + 1 M-NaCl as described in the Materials and methods section. The extract was centrifuged on a 5–15% linear sucrose gradient, and the pellet and soluble fractions 1–20 were collected. Samples were counted for radioactivity (a) or were resolved by SDS/PAGE (b). The gels were stained with Coomassie Blue (top panels) or were analysed for porin by Western blotting (lower panels). Free MAP2 centrifuged under the same conditions migrated as a single peak ($\approx$ 4.5 S) indicated by an arrow. Molecular-mass (*M*) markers were β -galactosidase, 116 kDa; albumin, 68 kDa; carbonic anhydrase, 29 kDa.

extraction of porin (Fig. 7). The total porin solubilized from outer membranes by Triton X-100 alone is decreased slightly in the presence of MAP2. A more significant influence of MAP2 was observed on the extraction of porin from the Triton X-100-insoluble

pellet. Extraction of this pellet with Triton X-100 plus 1 M-NaCl, the normal procedure for solubilizing porin [11], removes over 90% of the porin in the absence of MAP2 and only 65% in its presence (Fig. 7, fractions P2). The radioactivity in the pellet fraction was measured

Fig. 7. MAPs decrease the extractability of porin from mitochondria outer membranes

Outer membranes (3 mg/ml) were incubated in the absence (a) or presence (b) of MAP2 (75 μ g/ml) and then isolated by centrifugation through 0.5 M-sucrose (see the Materials and methods section). The sedimented membranes were extracted by a two-step procedure used for the isolation of porin [11]. First, a soluble fraction (S1) was obtained by extraction with 2% Triton X-100. The insoluble pellet was then extracted with 2% Triton X-100 + 1 M-NaCl to give a soluble fraction (S2) and a pellet (P2). All fractions were resolved on SDS/PAGE, and porin was detected by Western blotting. Purified liver porin (2 μ g) was used as a standard. The relative amount of porin in each fraction was obtained by densitometric scanning of photographic negatives. The lower band is a proteolytic product of porin which is easily generated in brain mitochondria [13].

and found in two separate experiments to be 45–55% of that bound to the membrane fraction (results not shown). Thus, MAP2 binding appears to make porin less susceptible to detergent extraction.

DISCUSSION

The location of specific MAP-binding sites on the outer membrane of brain mitochondria is supported by several lines of experimental evidence. First, endogenous MAPs are removed from mitochondria by digitonin under conditions which solubilize only the outer membrane [13], and MAPs are enriched in the outer membrane on a protein basis (Fig. 1a). Second, isolated outer membranes contain a high-affinity binding site for MAP2 which is enriched 10-fold over that in intact mitochondria. This is consistent with the purification of the outer membrane, assuming it represents only 7% of the total mitochondrial protein [11]. Furthermore, the MAP2/porin binding ratio was estimated to be 1:15 in both intact mitochondria and isolated outer membranes, suggesting co-purification of porin and the MAP2-binding site.

Binding studies *in vitro* revealed two high-affinity binding sites for MAPs on isolated mitochondria. One of these sites, which is located both in intact mitochondria and outer-membrane fragments, exhibits an affinity for MAP2 ($K_d = 2 \times 10^{-7}$ M) which is the same as that previously reported for neurofilaments [25] and could conceivably function in a similar manner. In addition, a higher affinity ($K_d = 10^{-9}$ M), low-capacity MAP2-binding site was observed in intact mitochondria. This site was destroyed during digitonin solubilization of the outer membranes, suggesting that it required an intact organelle.

Two results indicate that MAP2 and porin are located in the same domain of the outer membrane and might be physically associated with one another. First, sucrose-gradient centrifugation of outer membranes saturated with MAP2 and dissociated by Triton X-100 plus 1 M-NaCl shows that bound MAP2 distributes mainly into two fractions, both specifically enriched in porin (Fig. 6). Secondly, binding of exogenous MAP2 significantly reduces the amount of porin which is extracted by Triton X-100 under normal conditions used for purification of porin (Fig. 7). The possibility that porin and MAPs might interact physically could be tested in future experiments by using the purified polypeptides. In the meantime, however, it is uncertain whether this apparent association is physiologically relevant or merely fortuitous.

Binding experiments showed that MAP1 and MAP2 are selectively trapped on the mitochondrial membrane independent of their association with tubulin (Fig. 5). Since MAPs were released from Taxol-stabilized microtubules, where they are linked to the polymer [26], it is likely that transfer occurred via a domain on MAPs which is not in contact with the microtubule (i.e. the tubulin-polymerization domain) [23]. This assumption is strengthened by the lack of binding of Tau (Fig. 2) and by the finding that pure tubulin (competent for polymerization) inhibits only slightly the binding of MAP2 to mitochondria (Figs. 3c and 3d). The most plausible candidate for such an association is the arm-like projection of MAPs which is distal to the microtubule axis [23]. A physical cross-linkage between the two organelles consistent with this topology was observed in electron-microscopic investigations (results not shown). The partitioning of MAPs with mitochondria indicates that the affinity of MAPs is higher for mitochondria than for microtubules. A similar finding was reported for microtubule-neurofilament interactions [25].

A limitation of the present study is that information on the association of MAPs with mitochondria *in vitro* was obtained only for 32 P-labelled MAP2. Thus, in spite of the fact that both MAPs 1 and 2 are present in isolated mitochondria (Fig. 1a) and can bind *in vitro* to the organelle (Fig. 2), we do not know if they share a common binding site. In addition, it is possible that the phosphorylation of MAP2 [27,28], which is known to influence its interaction with tubulin [29] and actin [30], also modifies its binding to mitochondria.

The physiological significance of MAP-mediated interactions *in vitro* between microtubules and mitochondria remains to be determined. Among the three MAP1 and two MAP2 species, MAP1C is the only dynein-like ATPase capable of driving the translocation of vesicles along microtubules [8] and is a good candidate, together with kinesin [31,32], for a component of the 'molecular

motor' of the axonal flow of transported organelles. The precise function of MAP2 is not yet understood. One possibility is that it might act as a transitory anchoring site for the moving mitochondria, which are frequently attached to the microtubules through several cross-bridges [7]. This implies a mechanism of dissociation which could be studied *in vitro* by using the approach described here. In preliminary experiments performed with a mixture of microtubules and mitochondria (in the conditions of Fig. 5), the formation of an ATP-dependent network was observed that might reflect some of the dynamic properties of the interactions between the two constituents (M. Lindén & J.-F. Leterrier, unpublished work).

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