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Studies on the Interaction Between Mitochondria and the Cytoskeleton

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Abstract

Mitochondrial movements and morphology are regulated through interactions with the cytoskeletal system, in particular the microtubules. An interaction between the microtubule-associated proteins (MAPs) and the outer surface of rat brain mitochondria has been demonstrated *in vitro* and *in situ*. One of the MAPs, MAP2, binds to specific high-affinity sites on the outer membrane. Upon binding, MAP2 is released from microtubules, and it induces a physical alteration in the outer membrane which is characterized by a tighter association of porin with the membrane. It is concluded that MAP2 either binds to porin or to a domain of the outer membrane which alters the membrane environment of porin. The possibility is raised that this domain participates in mitochondrial mobility *in situ*.

Key Words: Mitochondria; microtubules; microtubule-associated proteins (MAPs); cytoskeleton; porin; Voltage-dependent anion-selective channels.

Introduction

A large body of evidence has accumulated indicating that mitochondria are closely associated with the cellular cytoskeletal system (Smith *et al.*, 1975; Hirokawa, 1982; Hirokawa *et al.*, 1985; see Chen, 1988, 1989 for reviews). This evidence ranges from morphological changes in mitochondria upon disruption of the microtubules with colchicine (Heggeness *et al.*, 1978; Ball and Singer, 1982; Summerhayes *et al.*, 1983), to direct measurements of organelle movements along microtubules *in vitro* (Martz *et al.*, 1984; Vale *et al.*, 1985a; Allen *et al.*, 1985; Bridgman *et al.*, 1986). It seems likely that

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mitochondrial interactions with the cytoskeleton fulfill a physiologic function in many, if not all, cell types (Chen, 1989). However, the most impressive evidence for this has accumulated for nerve cells in which axonal transport of mitochondria along microtubules is well documented (Griffin *et al.*, 1983; Miller and Lasek, 1985).

Practically nothing is known about the molecular mechanism through which mitochondria and cytoskeletal proteins interact. Movement of brain organelles along microtubules *in vitro* is apparently supported by at least two polypeptides which activate vesicle translocation in opposite directions (Vale *et al.*, 1986). One of these has been identified as kinesin (Vale *et al.*, 1985b, Scholey *et al.*, 1989) and the other is dynein (Vallee *et al.*, 1989; Schnapp and Reese, 1989), and is equivalent to the microtubule-associated protein, MAP 1C (Paschal *et al.*, 1987; Vallee *et al.*, 1989). No other cytoskeletal components necessary for mitochondrial movement have so far been identified. However, morphological studies have revealed short, thin cross-links between microtubules and mitochondria which appear structurally similar to microtubule-associated proteins (MAPs) (Hirokawa, 1982). In addition, studies by Schnapp and Reese (1989) showed that the structures which cross-link and translocate microtubules and organelles probably require proteins other than kinesin and dynein.

In order to better understand the interaction between mitochondria and the cytoskeleton, we initiated experiments to elucidate the participating components of both organelles (Lindén *et al.*, 1989). We have concentrated our efforts on the outer mitochondrial membrane on the assumption that the mitochondrial surface must contain the sites of attachment to the cytoskeletal system. The purpose of this paper is to review the progress we have made in this effort and to describe new immunocytochemical results.

Materials and Methods

Preparation of rat brain mitochondria, digitonin fractionation of outer membranes, preparation of rat brain microtubules and MAPs and MAP2, autophosphorylation of MAPs, *in vitro* binding of ^{32}P-MAPs to mitochondria, and extraction of bound MAPs from mitochondria were done as described by Lindén *et al.* (1989). Neurofilaments were purified according to Leterrier and Eyer (1987).

Tissues used for immunoelectron microscopic analysis were prepared as described by Tokuyashu (1984), and labeling was done as described by Slot and Geuze (1984). Localization of MAPs on the mitochondrial outer membrane by immunoelectron microscopy was done as follows. Mitochondria incubated with MAP2 for 15 min at 30°C were centrifuged through 0.5 M

sucrose, and the pellet was suspended in STE buffer (0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4). Samples were deposited on carbon–Formvar coated copper grids (300 mesh). The grids were successively transferred to STE buffer and then to STE buffer containing 1% BSA and 0.1% non-immune sheep IgG to saturate nonspecific sites. Grids were then incubated with antisera against rat brain MAPs (diluted 1/500) and decorated with anti-rabbit IgG labeled with 20 nm colloidal gold (Janssen Pharmaceutical, Beerse, Belgium). The grids were stained with 1% uranyl acetate.

Results

Association of MAPs with Brain Mitochondria in situ and in vitro

The suggestion from structural studies that microtubule-associated proteins (MAPs) might mediate the cross-link between mitochondria and microtubules *in situ* (Hirokawa, 1982; Hirokawa *et al.*, 1985) was analyzed by immunocytochemical methods. Frozen ultrathin sections of rat brain tissue were incubated with MAPs antibodies followed by a second antibody labeled with 15-nm gold particles. MAPs were found, as expected, on microtubules, but also in close association with the mitochondrial outer membrane (Fig. 1, closed arrow). The distribution pattern of MAPs strongly suggests that these structures are in contact with the mitochondrial surface *in situ*. This is consistent with our earlier Western blot analysis showing the association of MAPs with isolated nonsynaptic rat brain mitochondria (Lindén *et al.*, 1989). Furthermore, digitonin fractionation of these mitochondria into inner and outer membrane fractions resulted in an approximately 10-fold enrichment of MAPs in the outer membrane (Lindén *et al.*, 1989), as would be expected from the enrichment of an outer membrane polypeptide. Thus, data from both fractionation and *in situ* immunochemical analysis support the idea that MAPs are associated with the surface of brain mitochondria.

To obtain further evidence for a MAPs/mitochondrial interaction, MAP2 were bound to nonsynaptic rat brain mitochondria *in vitro*, and negatively stained preparations were analyzed using immunogold labeling. These studies (Fig. 2) indicate that bound MAP2 is located on the outer mitochondrial membrane, and appears to be clustered in domains which are separated from the inner membrane during the washing procedure (mitochondria were not fixed before staining). Controls performed using mitochondria incubated without MAP2 and processed identically were not stained (not shown). The above data provide strong morphological evidence that MAPs are specifically associated with the outer membrane of nonsynaptic rat brain mitochondria both *in situ* and *in vitro*.

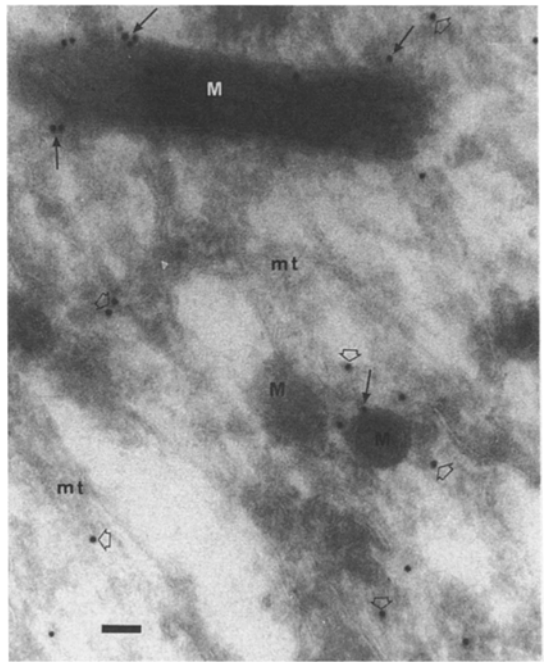

Fig. 1. Detection of MAP2 on the outer surface of rat brain mitochondria *in situ*. Immunoelectron micrograph of a nerve cell in the cortex of the rat brain. The slice is labeled with anti-rabbit IgG–gold complex to demonstrate the presence of MAPs using MAP antibody. MAPs are found on the surface (closed arrows) of mitochondria (M). Note also specific labeling (open arrows) of microtubules (mt). The bar represents 0.1 μm.

Characterization of MAP2 Binding Sites on Mitochondria and Outer Membranes

The above data and *in situ* observations by others (Hirokawa, 1982) are consistent with the idea that MAPs are involved in linking microtubules to mitochondria. It was therefore of interest to attempt to reconstitute such cross-links *in vitro*. This was performed by incubating nonsynaptic rat brain mitochondria and polymerized microtubules (containing endogenous MAPs) from rat brain (Lindén *et al.*, 1989) in an isotonic buffer at several concentrations of both organelles. In these samples the mitochondrial membrane is often aligned along microtubules, with a constant space being preserved between the two structures where cross-links are observed (Fig. 3, closed arrows). The cross-links consist of lateral projections of MAPs (which also decorate the length of microtubules) (Voter and Erikson, 1982) and electron-dense structures from the mitochondrial membrane. These mitochondria/microtubule complexes are stable during prolonged incubation *in vitro* in the presence of protease inhibitors (over several hours at 25°C as found by

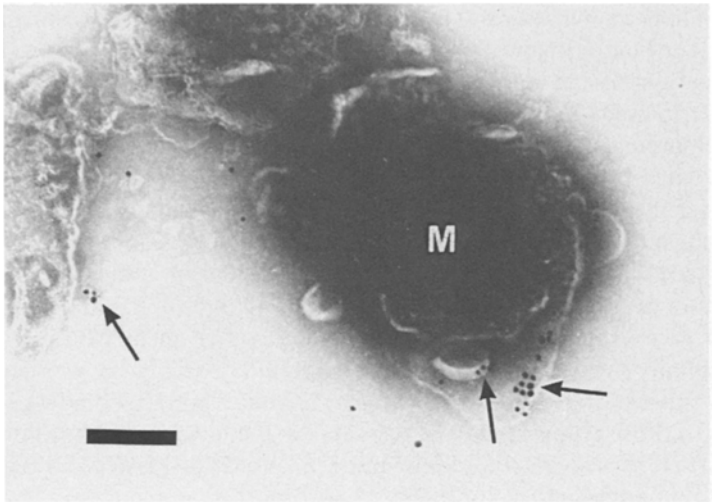

Fig. 2. MAP2 bound *in vitro* to rat brain mitochondria are located on the outer membrane. MAP2-saturated rat brain mitochondria were prepared as described (Linden *et al.*, 1989). Samples attached to Formvar-carbon coated copper grids were treated with anti-MAP2 antibodies and then decorated with a second antibody labeled with 20-nm colloidal gold particles (Janssen Pharmaceutical, Beerse, Belgium). Clusters of gold grains are observed in areas of the outer membrane which are detached from the inner membrane. The bar represents 0.2 μm.

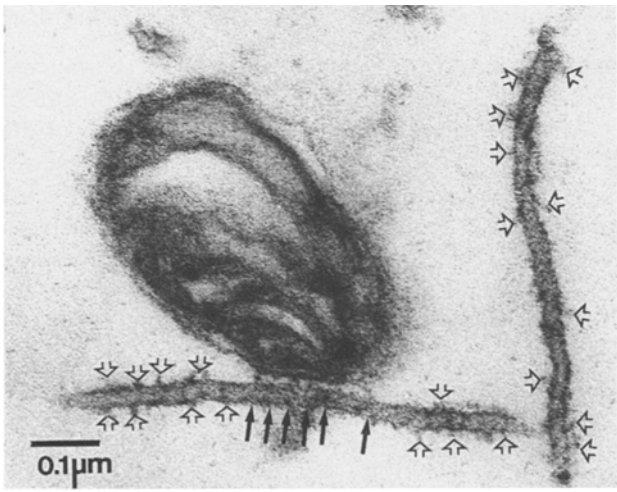

Fig. 3. Reconstitution of mitochondrial/microtubule cross-bridges *in vitro*. Nonsynaptic rat brain mitochondria and microtubules were incubated 1 hour at 30°C and fixed for electron microscopy. Cross-links between mitochondria and microtubules are indicated by the closed arrows, and the MAPs decorating the microtubules are indicated by open arrows. Each cross-link between the microtubule and the mitochondrial membrane is about 10 nm in length. The point of attachment covers a distance of 100 nm and includes several cross-linked sites.

video-enhanced microscopy, unpublished observations), suggesting a relatively tight binding between the interacting components.

Analysis of the binding properties of one of the MAP polypeptides (MAP2) to mitochondria has been completed (Lindén *et al.*, 1989). The specific binding site for MAP2 was studied using purified ^{32}P-labeled MAP2 and isolated nonsynaptic brain mitochondria and purified outer mitochondrial membranes (Lindén *et al.*, 1989). MAP2 binding was found to be rapid and saturable, indicating a limited number of binding sites. In accordance with this, two binding sites were found on intact mitochondria (Lindén *et al.*, 1989); one high-affinity ($K_d = 1 \times 10^{-9}$ M) and one lower-affinity ($K_d = 2 \times 10^{-7}$ M). In fractionated outer membranes, only the lower-affinity site was preserved. The high-affinity binding site was destroyed by the digitonin fractionation. The K_d for the mitochondrial binding sites are similar to those reported ($2–4 \times 10^{-7}$ M) for the binding of phosphorylated MAPs (Leterrier *et al.*, 1982; Heimann *et al.*, 1985) or MAP2 (Miyata *et al.*, 1986) to a neurofilament protein.

The number of high- and low-affinity binding sites found were 3 and 14 pmol MAP2/mg mitochondrial protein, respectively. The number of binding sites on isolated outer membranes is estimated to be 114 pmol MAP2/mg protein, a figure which is consistent with that expected from the enrichment of the outer membrane (Lindén *et al.*, 1989).

Mechanism of MAP2 Interaction with Mitochondria and Microtubules

An important but totally untouched problem is the mechanism by which MAPs mediate interaction between mitochondria and microtubules. Relatively little information is available on the function of the individual MAP polypeptides. MAP1C has a dynein-like ATPase activity (Paschal *et al.*, 1987). Dynein is apparently part of the "motor" which drives retrograde organelle translocation along microtubules toward the cell body (Schnapp and Reese, 1989; Vallee *et al.*, 1989). A second protein, kinesin, has also been implicated in the directional translocation of organelles (Vale *et al.*, 1986; Scholey *et al.*, 1989). It translocates organelles in the opposite direction, i.e., toward the nerve terminal.

Although the function of MAP2 is not understood, the molecular basis for its interaction with microtubules is becoming known. MAP2 has a carboxy terminal 18 amino acid repeat which contains the microtubule binding motif (Sally *et al.*, 1988). The microtubule binding domain is also present in the TAU protein (Sally *et al.*, 1988). That the microtubule binding domains on MAP2 and TAU do not react with the mitochondrial surface is suggested from the finding that TAU proteins do not bind to mitochondria (Lindén *et al.*, 1989) and from the morphology of the cross-links.

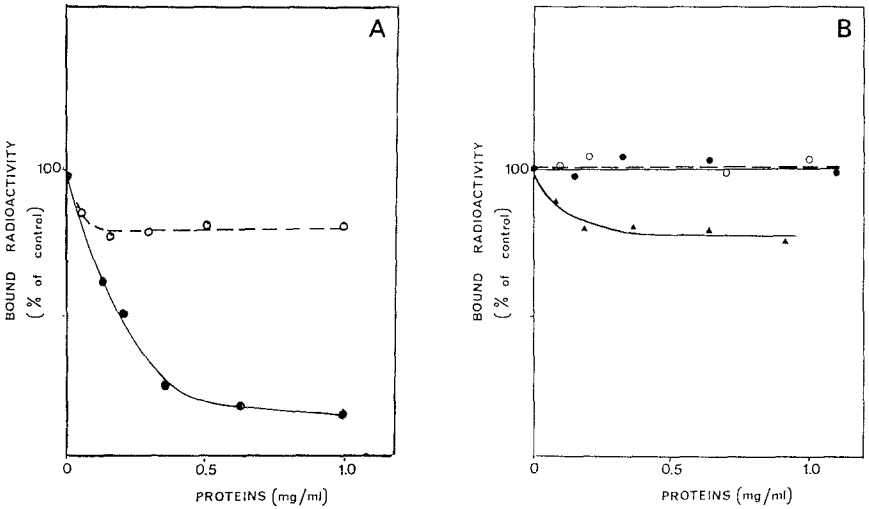

Fig. 4. Tubulin does not release MAP2 from isolated rat brain mitochondria. Isolated non-synaptic brain mitochondria were incubated with ^{32}P-labeled MAP2 at 30°C. At the zero time point (A), and after 15 min (B), the following additions were made and the incubation was continued for 15 min: (A) tubulin (○) or unlabeled MAP2 (●); (B) tubulin (○), unlabeled MAP2 (▲), or bovine serum albumin (●). Control radioactivity is that bound during incubation of mitochondria with ^{32}P-labeled MAP2 alone for 30 min at 30°C.

To further test this, experiments were conducted to measure the effect of purified tubulin on MAP2 binding (Fig. 4). Approximately 80% of the ^{32}P-labeled MAP2 bound during a 15-min incubation can be readily displaced by unlabeled MAP2, but only 20% of the MAPs is released by tubulin (Fig. 4A). Thus, tubulin does not release MAPs from their mitochondrial binding site(s). However, the MAP binding site seems to change its character with time, since only 20% of the molecules bound after 30 min of incubation were exchanged by unlabeled MAP2, and tubulin or BSA had no effect (Fig. 4B). This is discussed below.

A second important observation is that the MAPs associated with Taxol-stabilized microtubules can be detached by, and transferred to, mitochondria (Lindén *et al.*, 1989). Microtubules remain in the soluble fraction and MAPs are removed with mitochondria by centrifugation. A reasonable interpretation of this result is that attachment of MAPs to the mitochondrial surface result in a weakening of the MAP/microtubule binding domain. The mitochondrial binding site on MAPs probably involves the long side-arm projecting from the microtubule axis (Voter and Erikson, 1982).

Structural changes in MAPs also lead to their release from mitochondria. This is shown by digestion of prebound ^{32}P-labeled microtubules (the phosphorylated proteins include MAP2 and TAU) with the endogenous

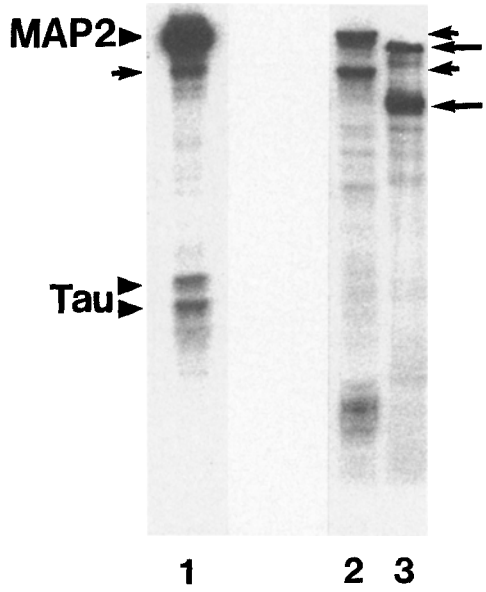

Fig. 5. Proteolytic digestion releases MAP2 from the mitochondrial membrane. Purified, autophosphorylated microtubules (lane 1) were incubated *in vitro* with nonsynaptic rat brain mitochondria for 30 min at 30°C in the absence of protease inhibitor. The sample was separated into a mitochondrial pellet (lane 2) and a soluble fraction (lane 3). MAP2 is digested by an endogenous mitochondrial protease, and two fragments (265 and 190 kDa) are released to the soluble fraction (lane 3). Undigested, intact MAP2 (MW 280 kDa), together with an unidentified phosphorylated protein present in microtubules (lane 1), remains bound to mitochondria (lane 2).

proteases of the isolated mitochondria (Fig. 5). MAP2 is split and released from mitochondria (Fig. 5, lane 3). However, an additional unidentified phosphorylated polypeptide in microtubules (Fig. 5, lane 1) is not digested, and remains with the membrane fraction (Fig. 5, lane 2). This proteolytic activity is present in nonsynaptic brain mitochondria but not in synaptic mitochondria or synaptic plasma membranes (unpublished results). Thus, one might speculate that proteolytic digestion is part of the physiological mechanism of MAPs binding and release during axonal transport of mitochondria.

Components of the Outer Membrane Which React with MAP2

A second important question about which very little is presently known concerns the components of the mitochondrial outer membrane which participate in MAPs binding. Lindén *et al.* (1989) reported two lines of evidence indicating that MAP2 and the outer mitochondrial channel forming protein (porin, VDAC) are located in the same, or closely related, domains of the

outer membrane. For the first, MAP2 and porin were coextracted from outer membranes containing prebound MAP2, using the extraction procedure for porin purification (Lindén *et al.*, 1982). Upon sucrose gradient centrifugation of the extract, two populations of bound MAP2 were separated, both of which were highly enriched in porin.

A second line of evidence indicates that, in addition to cofractionation of MAPs and porin, MAPs binding to outer membranes changes the extractability of porin (Lindén *et al.*, 1989). Less porin is extracted under standard conditions of Triton X-100 + 1 M NaCl (Lindén *et al.*, 1982) if MAP2 is prebound (Lindén *et al.*, 1989). It was concluded from these experiments that MAP2 alters the physical environment of the outer membrane and that porin and MAP2 might interact physically (Lindén *et al.*, 1989). A MAPs-induced reorganization of the outer membrane is also consistent with the data in Fig. 4. In these experiments the MAPs bound after 15 min of incubation with mitochondria were readily exchanged for unlabeled MAPs, whereas that bound after 30 min was not. This suggests a time-dependent change in the MAP binding site which could result from a reorganization of the outer membrane domain.

In order to further identify the outer membrane polypeptides which interact with MAPs, the same detergent extraction of outer membranes with or without prebound MAP2 was performed as described by Lindén *et al.* (1989). The fractions were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. By an overlay binding method (Heimann *et al.*, 1985) using ^{32}P-labeled MAP2 as the probe, three major outer membrane polypeptides with apparent molecular weights of 68, 36, and 30 kDa were found to bind MAP2 (Fig. 6, lane 2). A similar result was obtained upon binding the intact, phosphorylated MAP complex (Rendon *et al.*, 1987). As shown by our experiments MAP2 is probably the binding species.

The 30-kDa polypeptide is identified as porin, since it has the same mobility as porin and since isolated porin was able to bind ^{32}P-labeled MAP2 on the same blot (Fig. 6, lane 9). In addition, immunoblotting of the same fractions revealed that the 30-kDa polypeptide reacted with porin antibodies (not shown). The remaining polypeptides are not identified.

The results in Fig. 6 also show, in accordance with previous findings for porin (Lindén *et al.*, 1989), that MAP2 alters the Triton X-100 extraction pattern of the 30-kDa MAP2-binding protein. The 30- and 36-kDa polypeptides were only partially removed from the pellet fraction (cf. lanes 5 and 8), indicating a change in the organization of the outer membrane, and probably specifically in porin. Thus, several observations support the idea that physical changes occur in the outer membrane upon binding of MAP2, and that these involve membrane domains which either contain porin or which affect the distribution of porin within the membrane.

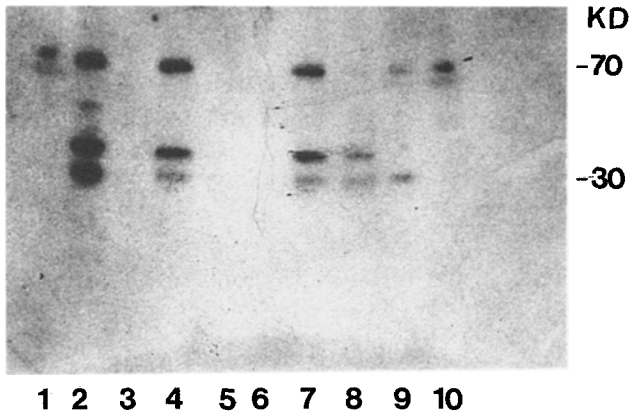

Fig. 6. Detection of MAP2 binding proteins in the outer mitochondrial membrane. Outer membranes were incubated in the absence (lanes 2–5) or presence (lanes 6–8) of MAP2, and then extracted by a two-step procedure used for the isolation of porin (Lindén *et al.*, 1982). The first extraction with 2% Triton X-100 gives a porin-free soluble fraction (lanes 3 and 6) and a pellet. The pellet, upon extraction with 2% Triton X-100 + 1 M NaCl, yields a porin-containing, soluble fraction (lanes 4 and 7) and a pellet (lanes 5 and 8) which is normally free of porin (Lindén *et al.*, 1982). All fractions were resolved on SDS-PAGE and transferred to nitrocellulose. The latter was incubated with ^{32}P-labeled MAP2 as described (Heimann *et al.*, 1985). An autoradiograph is shown. Porin from nonsynaptosomal rat brain mitochondria (lane 9) and purified neurofilaments (lanes 1 and 10) were used as standards.

Summary

That mitochondria and microtubules are closely associated *in situ*, and that this association is necessary for organelle organization and movement, is well documented. However, the mechanism through which microtubules interact with mitochondria is not understood. Our studies, reviewed here, allow the following conclusions:

1. Microtubule-associated proteins (MAPs) are associated with mitochondria surfaces both *in situ* and *in vitro*, and bind to specific binding sites on the outer membrane of rat brain mitochondria *in vitro*.
2. Binding involves the armlike projection of MAP2 and not the tubulin binding site.
3. Porin is either located in MAP2 binding domains, or its distribution within the membrane is influenced by bound MAPs.
4. The mechanism of attachment of MAPs to mitochondria requires structurally intact MAP2.

The physiological relevance of these observations is far from understood. We do not know, for example, if similar binding sites for MAPs exist

on mitochondria from tissues other than brain. Even for neural tissue, where axonal transport of mitochondria via microtubule associations is best documented, our results raise many questions. Since most of the MAPs associated with microtubules purified by the polymerization/depolymerization procedure used in our work contain few if any MAP1C or kinesin (Vallee *et al.*, 1989), it is unlikely that the MAP binding sites studied here participate directly in mitochondrial movements. Thus, MAP2 participates either indirectly in movement (perhaps in a complex with kinesin and/or MAP1C), or it has other functions. One such function might be that it participates in the arrested state of mitochondria on microtubules *in situ*. This state is visualized by the presence of stable cross-bridges (Smith *et al.*, 1975). Dissociation of these cross bridges appears to be required for translocation of mitochondria, and might be involved in the mitochondrial-associated protease reaction described in the present work.

Acknowledgments

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