

Microtubule-associated Protein-dependent Binding of Phagosomes to Microtubules*

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In macrophages, phagosome movement is microtubule-dependent. Microtubules are a prerequisite for phagosome maturation because they facilitate interactions between phagosomes and organelles of the endocytic pathway. We have established an *in vitro* assay that measures the binding of purified phagosomes to microtubules. This binding depends on the presence of membrane proteins, most likely integral to the surface of phagosomes, and on macrophage cytosol. The cytosolic binding factor can interact with microtubules prior to the addition of phagosomes to the assay, suggesting that it is a microtubule-associated protein (MAP). Consistent with this, depletion of MAPs from the cytosol by microtubule affinity removes all binding activity. Microtubule motor proteins show no binding activity, whereas a crude MAP preparation is sufficient to support binding and to restore full binding activity to MAP-depleted cytosol. We show that the activating MAP factor is a heat-sensitive protein(s) that migrates at around 150 kDa by gel filtration.

Phagocytosis is a process whereby a cell forms a new membrane compartment, the phagosome, to engulf particles that are too large to be internalized by endocytosis. This organelle subsequently matures into a phagolysosome, by a complex series of interactions with the endocytic pathway (Pitt *et al.*, 1992a; Pitt *et al.*, 1992b; Desjardins *et al.*, 1994a; Jahraus *et al.*, 1994). While it is well established that the actin cytoskeleton is important for the earliest steps of phagocytosis (Silverstein *et al.*, 1989), the available evidence argues that later transport events require microtubules (D'Arcy Hart *et al.*, 1983; D'Arcy Hart *et al.*, 1987; Toyohara and Inaba, 1989; Knapp and Swanson, 1990; Desjardins *et al.*, 1994a; Jahraus *et al.*, 1994).

The microtubule cytoskeleton is important for the positioning and function of many organelles (Kelly, 1990), including the endoplasmic reticulum (Terasaki *et al.*, 1984; Mizuno and Singer, 1994; Terasaki *et al.*, 1986) and the Golgi complex (Wehland and Willingham, 1983; Sandoval *et al.*, 1984; Ho *et al.*, 1989; Scheel *et al.*, 1990). Movement of transport vesicles along microtubules is also necessary for directed secretion (Achler *et al.*, 1989; Kreis *et al.*, 1989; Lafont *et al.*, 1994), and the transport of internalized material from early to late endosomes (Matteoni and Kreis, 1987; Swanson *et al.*, 1987; Gru-

enberg *et al.*, 1989; Bomsel *et al.*, 1990; Hollenbeck and Swanson, 1990; Young *et al.*, 1990; Aniento *et al.*, 1993).

In the present study we have focused on the interaction of phagolysosomes with microtubules. For this, we used 1- μ m latex beads as a convenient marker for phagocytosis (Wetzel and Korn, 1969; Stossel *et al.*, 1971; Muller *et al.*, 1980). The attraction of these beads is the ease with which they allow the subsequent purification of phagolysosomes on a simple flotation gradient, from J774 mouse macrophages that have internalized them (Desjardins *et al.*, 1994a; Desjardins *et al.*, 1994b). For convenience, we will refer to all the organelles purified in this manner, irrespective of their maturation state, as phagosomes.

Previous video analysis from our group has shown that in macrophages, endosomes and lysosomes containing endocytosed colloidal gold and phagosomes containing latex beads move within the cell, interacting with one another multiple times (Desjardins *et al.*, 1994a). Late organelles of the endocytic pathway are known to move along microtubules (Kreis *et al.*, 1989; Hollenbeck and Swanson, 1990). Phagosome movements are best observed within the first hours following bead internalization. Movement is mainly, but not always, centripetal, leading to a gradual accumulation of phagosomes around the nucleus, near the microtubule organizing center. When these cells are treated with the microtubule-depolymerizing drug nocodazole (Noc),¹ phagosome movement ceases.² These results suggest that phagosomes move within the cell along microtubules.

The microtubule motor proteins cytoplasmic dynein and kinesin were the first identified molecules that could account for organelle-microtubule interactions (Vale *et al.*, 1985; Schroer *et al.*, 1989). That these motors can interact with membrane organelles is now well established (Neighbors *et al.*, 1988; Hollenbeck, 1989; Pfister *et al.*, 1989; Hirokawa *et al.*, 1990; Hirokawa *et al.*, 1991; Lacey and Haimo, 1992; Leopold *et al.*, 1992; Lin and Collins, 1992; Yu *et al.*, 1992; Morin *et al.*, 1993). However, recent data argue that motors alone are insufficient to mediate motile interactions of organelles with microtubules (Schroer *et al.*, 1988; Schroer and Sheetz, 1991; Gill *et al.*, 1991; Burkhardt *et al.*, 1993). In addition, evidence has been provided that motor proteins are not the major cytosolic factors involved in the static binding of organelles to microtubules (Scheel and Kreis, 1991). Indeed, in studies where the static binding of membrane organelles to microtubules has been examined, binding has been attributed to the activity of a MAP, in one

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¹ The abbreviations used are: Noc, nocodazole; AMP-PNP, 5'-adenylylimidodiphosphate; ATP γ S, adenosine 5'-3-O-(thio)triphosphate; GTP γ S, guanosine 5'-3-O-(thio)triphosphate; CLIP, cytoplasmic linker protein; MAP, microtubule-associated protein; PI, protease inhibitor; MES, 4-morpholineethanesulfonic acid.

² A. Blocker *et al.*, manuscript in preparation.

case to MAP2 (Severin *et al.*, 1991) and in the other to a novel MAP, CLIP-170 (Pierre *et al.*, 1992).

MATERIALS AND METHODS

Cell Culture—J774A.1 macrophages were obtained from American Type Tissue Collection and maintained as described previously (Desjardins *et al.*, 1994a), except with 10% newborn calf serum (Seromed). Cells were grown adherent in a 5% CO₂ atmosphere and passaged by vigorous pipetting. For cytosol preparations, cells were grown as spinner cultures to 5×10^5 /ml in Joklik's modified Eagle's medium (Life Technologies, Inc.) containing 2 g/liter NaHCO₃ and supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin (both from Life Technologies, Inc.).

Preparation of Latex Beads—Naked polystyrene latex beads, either untreated or carboxylate-modified, were found to have high intrinsic nonspecific binding to microtubules. Therefore, to block the bead surface for binding studies, carboxylate-modified, orange fluorescent latex beads, 1 µm in diameter (Molecular Probes) were incubated with 1 mg/ml fish skin gelatin (Sigma) in 50 mM MES buffer, pH 6.7, and covalently coupled using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, according to the manufacturer's recommendations.

Phagosome Purification—Phagosomes were isolated using the high buoyant density of latex, as described previously (Desjardins *et al.*, 1994a), except that cells were broken using several passes through a 22-gauge syringe needle, and the sucrose gradient was simplified to three steps: the postnuclear supernatant adjusted to 40% sucrose, and steps of 25 and 10% sucrose. Protease inhibitors (PIs) in the form of 1 µg/ml pepstatin A, 0.5 µg/ml *N*-tosyl-L-phenylalanine, 0.5 µg/ml leupeptin, and 4 µg/ml aprotinin (all from Sigma), were included throughout the isolation procedure. Phagosomes were collected from the 10%/25% sucrose interface and frozen in liquid nitrogen.

Where indicated, digestion of the phagosome membrane with proteases and mock treatment was performed at 37 °C for 15 min. After inactivation of the enzyme with 3,4-dichloroisocoumarin (Boehringer Mannheim), phagosomes were repurified using a small scale version of the sucrose gradient above. Treatment of phagosomes with 1 M NaCl was performed for 30 min at 4 °C, and the salt was removed by flotation.

Measurement of Fusion between Phagosomes and Late Compartments of the Endocytic Pathway—Macrophages grown on 15-cm dishes were allowed to internalize horseradish peroxidase (Sigma) at 2 mg/ml for 20 min at 37 °C in internalization medium (MEM; 10 mM Hepes, 5 mM D-glucose, pH 7.4). Cells were washed twice at 4 °C with PBS, twice with PBS containing 5 mg/ml bovine serum albumin, and chased for 1 h at 37 °C in growth medium. Cells were then pulsed for 20 min at 37 °C with latex beads at a 0.05% solids suspension in growth medium, returned to 4 °C, and washed several times with cold PBS. Noc (Sigma) was then added to 10 µM, and cells were returned to 37 °C for the indicated times of chase. At the end of 120 min of chase, Noc was washed out with PBS, and the cells were returned to normal medium for an additional 120-min chase period. At the end of each chase time, cells were transferred to ice, phagosomes were isolated as described above, and horseradish peroxidase activity in each sample was determined as described by Gruenberg *et al.* (1989). To normalize samples for varying recovery of phagosomes, the optical density of the latex beads at 600 nm was determined. The data are expressed as arbitrary units of horseradish peroxidase activity, corrected for bead content of each time point.

Preparation of Cytosol—Cells were collected by centrifugation and washed twice in cold PBS, resuspended in PMEE/sucrose (35 mM Pipes buffered with KOH to pH 7.4, 5 mM MgSO₄, 1 mM EGTA, 0.5 mM EDTA, 0.25 M sucrose), and pelleted at $2,500 \times g$. Cells were resuspended using 0.9 volume of PMEE/sucrose with PIs and 1 mM dithiothreitol (DTT, Sigma) and homogenized using several passages through a 22-gauge syringe needle. A postnuclear supernatant was generated by centrifugation at $6,000 \times g$ for 15 min at 4 °C and centrifuged at $200,000 \times g$ for 30 min at 4 °C to remove particulates. The top lipid layer was discarded, and the underlying clear cytosol fraction was collected and frozen in liquid nitrogen. For larger scale preparations, cells were first concentrated 10-fold using a Millipore cell concentrator and then collected by centrifugation, washed three times in PBS, and broken using several strokes in a Dounce homogenizer fitted with a B pestle. Both methods reproducibly produced cytosol at 30–50 mg/ml protein.

Depletion of MAPs and Motors by Microtubule Affinity—This procedure is derived from modifications of methods published by Vallee (1982), Vale *et al.* (1985), Paschal *et al.* (1987), Schroer *et al.* (1989), and Scheel and Kreis (1991). Microtubules were polymerized from phosphocellulose-purified tubulin obtained from bovine brain (Mitchison and

Kirschner, 1984) in PMEE containing 1 mM GTP (Boehringer Mannheim) and 20 µM taxol (Sigma) for 20 min at 37 °C and collected by centrifugation either in an airfuge at 25 p.s.i. or in a conventional ultracentrifuge at $70,000 \times g$ for 5 min at 20 °C. To those cytosol samples that were to be depleted of either MAPs (sample 1) or MAPs and motors (sample 2), $\frac{1}{10}$ protein concentration of polymerized microtubules resuspended in PMEE, 20 mM GTP, and 200 µM taxol was added; to the mock sample (sample 3) only PMEE was added. To sample 1 $\frac{1}{20}$ volume of an ATP-regenerating system, freshly prepared by mixing a 1:1:1 volume of stock solutions of 100 mM ATP, pH 7, with HCl, 800 mM creatine phosphate, and 4 mg/ml creatine phosphokinase at 800 units/ml in 50% glycerol (both from Boehringer Mannheim), was added; sample 2 was brought to 2 mM AMP-PNP (Sigma) and received $\frac{1}{10}$ volume of an ATP depletion system, freshly prepared from a 1:1 mix of stock solutions of hexokinase (Boehringer Mannheim; 1500 units/ml in 50% glycerol) and 1 M D-glucose; to sample 3 an equivalent amount of PMEE was added. All samples were incubated at 37 °C for 15 min and then centrifuged at $70,000 \times g$ for 20 min at 20 °C to pellet microtubules and associated proteins. The supernatants were collected and were desalted into PMEE containing PIs and 1 mM DTT on PD-10 columns (Pharmacia Biotech Inc.), which were quenched for protein binding by pretreatment in 5 mg/ml casein in PMEE, PIs, 1 mM DTT. These samples were frozen in liquid nitrogen.

Immunodepletions of Motor Proteins—Cytosol was depleted of kinesin with the SUK4 monoclonal IgG bound to protein A-Sepharose beads as described by Ingold *et al.* (1988). Another monoclonal IgG (P5D4), raised against a viral antigen (Kreis, 1986), was used as a control antibody. The same procedure (Bomsel *et al.*, 1990; Aniento *et al.*, 1993b) was used to deplete cytoplasmic dynein with the 70.1 monoclonal IgM (Sigma) coupled to goat anti-mouse agarose beads. Monoclonal IgM F13, raised as an anti-idiotypic antibody to a viral antigen (Vaux *et al.*, 1988) served as a matched control.

Preparation of J774 MAPs and Motors—To generate a MAP preparation, macrophage cytosol was treated initially as above for MAP depletion (sample 1). To generate a motor preparation, macrophage cytosol was treated as for MAP and motor depletion (sample 2). In each case the microtubules were pelleted for 20 min at 20 °C and $75,000 \times g$ through a sucrose gradient containing steps of 15 and 25% sucrose, in PMEE, PIs, 1 mM DTT, 10 µM taxol. Microtubule pellets were resuspended in a large volume of PMEE, PIs, 1 mM DTT with 10 µM taxol and pelleted again over the same sucrose gradient. The microtubule pellets were then resuspended in PMEE, PIs, 1 mM DTT, 10 mM taxol, 10 mM ATP and incubated at room temperature for 30 min to remove any contaminating motor proteins in the case of sample 1 and to elute the motor proteins in the case of sample 2. The microtubules were pelleted at $22,000 \times g$ for 15 min at 20 °C, and the supernatant of the pellet from sample 1 was discarded, whereas the supernatant of sample 2, the motor preparation, was collected. The microtubule pellet of sample 1 was then resuspended in PMEE, PIs, 1 mM DTT, 10 µM taxol containing 0.5 M NaCl and incubated at room temperature for 30 min to elute the MAPs. The microtubules were removed by centrifugation as above, and the supernatant, the MAP fraction, was collected. Both fractions were desalted into PMEE, PIs, 1 mM DTT using a fast desalting PC 3.2/10 column on a SMART system (Pharmacia) and frozen in liquid nitrogen.

Fractionation of cytosol was performed on a Superose 12 column on a SMART system; 50-µl fractions were collected. Heat treatment of MAPs was performed according to Kuznetsov *et al.* (1978) and Kim *et al.* (1979). MAPs were adjusted to 1 M NaCl and heated to 95 °C for 5 min. A mock sample was adjusted to 1 M NaCl and left on ice. Samples were cooled on ice and centrifuged at $300,000 \times g$ for 20 min at 4 °C. The supernatant was desalted as directly above.

Binding Assay—Microscope chambers were built from a glass microscope slide and two pieces of double sided tape (Scotch) onto which an 11-mm circular glass coverslip (Menzel) was sealed, forming a 3-µl chamber. Chambers were perfused with rhodamine polarity-marked microtubules, prepared as in Howard and Hyman (1993), in PMEE containing 10 mM taxol. A dense lawn of these microtubules adhered to the glass coverslip within a short time. The excess microtubules were washed away by perfusion of 2 volumes of assay buffer (PMEE, PIs, 1 mM DTT, 10 µM taxol, 250 µg/ml casein) containing antifade in the form of 0.1 mg/ml glucose oxidase, 0.1 mg/ml catalase (both from Sigma), and 10 mM glucose. Where indicated, coverslips were precoated with poly-L-ornithine prior to addition of the microtubules as described by Urrutia *et al.* (1993). This results in a lawn of strongly bound microtubules, which are not dislodged by the activity of microtubule motor proteins. Any free poly-L-ornithine was neutralized by a wash with 10 mg/ml aspartic acid at pH 7 followed by a wash in assay buffer. The chambers were then perfused with 2 volumes of the phagosome-binding reaction

containing 1–2 μ l of purified phagosomes diluted into assay buffer and cytosol where indicated, and the organelles were allowed to bind to the microtubules in humidified chambers for 20 min at room temperature. Unbound phagosomes were washed away with 2 volumes of assay buffer with antifade. Chambers were observed using a Zeiss photomicroscope with a 10 \times ocular and a Zeiss Planapo 63 \times lens (field surface area of 35,000 μ m²). The bound phagosomes were counted by eye, and in each experiment values from at least 10 fields were averaged. The errors reported are the population standard deviations from at least two separate but identical reactions.

Electron Microscopy—Phagosomes were mixed in assay buffer with taxol-stabilized microtubules in the presence or absence of 2 mg/ml macrophage cytosol and allowed to bind in solution for 30 min at room temperature. Samples were then mixed with 1 volume of 62% sucrose, 3 mM imidazole, pH 7.4, and repurified in the presence of taxol using a small version of the sucrose gradient used for phagosome purification. Floated phagosomes and any bound microtubules were collected, mixed with an excess of 1% glutaraldehyde, 0.2 M sodium cacodylate, pH 7.2, and pelleted at 15,000 \times g. Pellets were postfixed in osmium tetroxide, dehydrated, and embedded in epon according to standard procedures. Thin sections were contrasted with lead citrate and uranyl acetate.

Antibodies and Electrophoresis—SUK4 (Ingold *et al.*, 1988) was obtained from Developmental Studies Hybridoma Bank, and 70.1 (Steuer *et al.*, 1990) was purchased from Sigma. KMTBX, a rabbit affinity-purified polyclonal antibody raised against a peptide of the motor domain of the *Xenopus* kinesin-like protein Eg5, was a gift of Drs. I. Vernos and E. Karsenti (Vernos *et al.*, 1995). The Db1 rabbit affinity-purified polyclonal antibody was a gift from Dr. E. A. Vaisberg (Vaisberg *et al.*, 1993). Professor T. E. Kreis donated α 55, an affinity-purified rabbit polyclonal to CLIP-170 (Rickard and Kreis, 1990) and P5D4. Dr. S. Fuller gave us F13, and Dr. J. Olmsted gave us IF5.2.2, a rat monoclonal antibody against mouse MAP4.³ SDS-polyacrylamide gel electrophoresis was performed as by Laemmli (1970), and blots were performed using the ECL detection system (Amersham Corp.).

RESULTS

Microtubules Facilitate Exchanges of Material between Phagosomes and Late Endocytic Compartments—Desjardins *et al.* (1994a) showed that treatment of macrophages having internalized latex beads with microtubule-depolymerizing drugs inhibits the acquisition by the phagosomes of two lysosomal glycoproteins, Lamp1 and Lamp2, membrane markers of late endocytic/lysosomal compartments (Kornfeld and Mellman, 1989). To further assess the role of microtubule-based movement in phagosome-lysosome interactions, J774 cells were fed the fluid phase marker horseradish peroxidase under conditions that selectively load late endocytic compartments (Desjardins *et al.*, 1994a). The cells were then pulsed with latex beads and chased in the presence or absence of Noc. At each chase time, the phagosomes were purified, and the quantity of horseradish peroxidase transferred to the phagosome compartment was determined. As shown in Fig. 1, Noc treatment inhibited the transfer of horseradish peroxidase into the phagosomes by nearly 70% over a 2-h chase period of the latex beads. The effects of nocodazole were reversible. When the treated cells were returned to nocodazole-free medium and the microtubule cytoskeleton was allowed to reform, horseradish peroxidase was transferred to the phagosomes at a rate that was comparable with, if slightly slower than, the rate found in control cells. This result shows that late endocytic organelles and phagosomes interact with microtubules in intact cells and that this interaction is important for facilitating the exchange of material between them.

Reconstitution of Phagosome-microtubule Binding in Vitro—To probe the molecular details of phagosome-microtubule interactions, we established an *in vitro* phagosome-microtubule binding assay. Latex bead-containing phagosomes were isolated by flotation in a discontinuous sucrose gradient, a procedure that yields phagosomes of high purity, with a defined

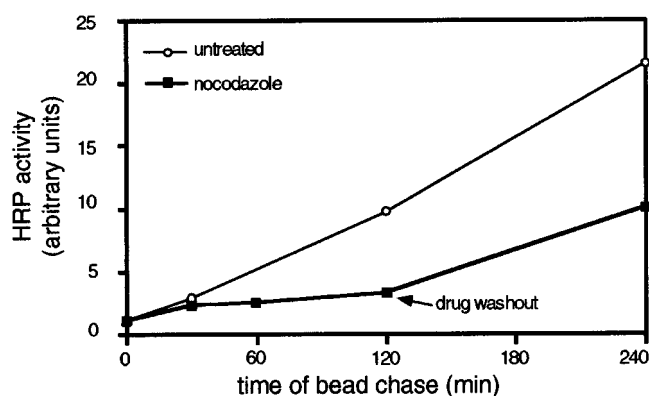


FIG. 1. **Transfer of horseradish peroxidase from late endosomes/lysosomes to phagosomes in intact cells requires microtubules.** J774A.1 cells were fed horseradish peroxidase for 20 min and, after thorough washing, chased for 1 h. At this point latex beads were pulsed for 20 min, and the cells were chased in the presence or absence of nocodazole for up to an additional 120 min. At this time the drug was washed out, and the cells were chased for a subsequent 120 min. At each time point phagosomes were purified, and horseradish peroxidase was quantified. The data are expressed as arbitrary units of horseradish peroxidase activity, corrected for bead content of each time point. This experiment was repeated 3 times and gave similar results, though the final values could not be merged to produce error bars.

protein composition (Desjardins *et al.*, 1994a; Desjardins *et al.*, 1994b; Burkhardt *et al.*, in press). Unless otherwise stated, macrophages were allowed to internalize beads for 1 h and were chased for 1 h prior to phagosome purification. The ability of the phagosomes to bind to microtubules was tested using a modification of the assay developed by Sorger *et al.* (1994) to measure kinetochore-microtubule interactions. For this, dimly labeled rhodamine microtubules, stabilized with taxol, were perfused into a small microscopy chamber and allowed to adsorb to the coverslip (Fig. 2A). After washing away excess microtubules, phagosomes containing rhodamine labeled latex beads were added in the presence or absence of macrophage cytosol and allowed to bind. The phagosomes that remained bound after a wash and were in the focal plane of the microtubule lawn were counted by direct observation. Binding of phagosomes to microtubules was minimal in the absence of cytosol, but when macrophage cytosol was added, numerous phagosomes bound (Fig. 2, B and C).

Visualization of the Phagosome-Microtubule Complex—To visualize the phagosome-microtubule interaction at higher resolution, the complex was analyzed by electron microscopy. For this, phagosomes were incubated in solution with microtubules in the presence or absence of cytosol under standard binding assay conditions. The phagosomes and bound microtubules were separated from unbound material by flotation, collected, and processed for electron microscopy. When cytosol was omitted from the reaction, no microtubules were floated with the organelles (data not shown). When cytosol was included in the reaction, microtubules were observed tangentially associated with the phagosomes and in close apposition (2–10 nm) to their enclosing membranes (Fig. 2D). In some cases dense material was observed in the intervening space.

Binding Is Dependent on Cytosol and Phagosome Membrane Proteins—The absolute number of phagosomes bound per field in our light microscopy assay varied essentially linearly with the number of input phagosomes (data not shown), but the stimulation by cytosol remained a constant, typically 5–10-fold (Fig. 3, A and B). Binding was stimulated by cytosol concentrations up to approximately 2 mg/ml in the assay (Fig. 3A). At higher concentrations of cytosol, however, binding was inhibited by 50% or more. Therefore, all subsequent assays were performed at 2 mg/ml cytosol. Phagosome binding absolutely

³ J. Olmsted, unpublished data.

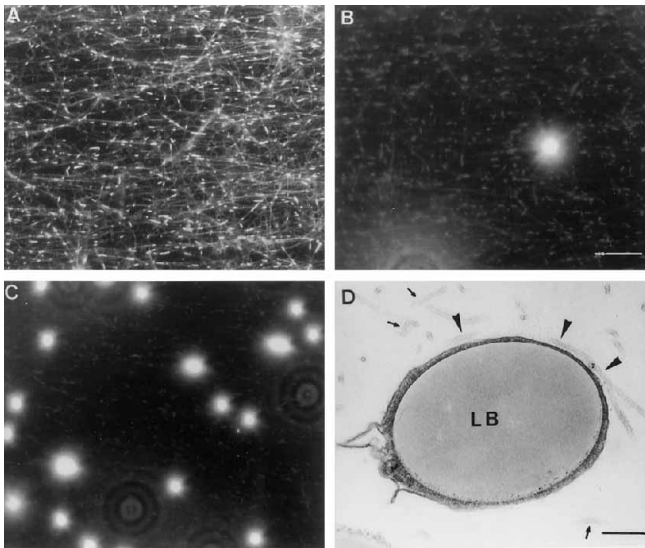


FIG. 2. Visualization of the binding assay and of the phagosome-microtubule complex. Typical fields of binding assays incubated in the absence of phagosomes to allow visualization of the microtubule lawn (A), in the absence of cytosol but with phagosomes (bar = 10 μ m) (B), and in the presence of 2 mg/ml cytosol and phagosomes (C) are shown. D, electron micrograph of the floated phagosome-microtubule complex (bar = 0.2 μ m). Phagosomes were incubated with taxol-stabilized microtubules in the presence of 2 mg/ml macrophage cytosol. Phagosomes were then repurified by flotation and processed for thin section electron microscopy. Only in the presence of cytosol do microtubules bind to the phagosomes and become copurified. Multiple microtubules are seen in association with the phagosome membrane (arrowheads). In addition, a network of microtubules is floated with the organelles (small arrows).

required the presence of microtubules on the coverslip (Fig. 3B), was time-dependent, saturating at 20–30 min of incubation, and showed little difference whether performed at 25 or 37 °C (data not shown).

The ability of the phagosomes to bind to microtubules is a property of their surrounding membranes, since uninternalized latex beads coupled to fish skin gelatin showed very little tendency to bind to microtubules, even in the presence of cytosol (Fig. 3B). Moreover, phagosome-microtubule interaction was abolished by heating the phagosomes to 80 °C for 10 min (data not shown) and by digestion of the phagosome membrane with trypsin (Fig. 3B). Phagosome-microtubule interaction was also inhibited by digestion of the phagosomes with chymotrypsin or proteinase K but not by V8 protease.⁴ Stripping of phagosomes with 1 M NaCl diminished subsequent binding in the presence of cytosol only slightly (78.0 ± 7.1 for mock-treated phagosomes, versus 52.3 ± 1.8 for salt-washed phagosomes). This indicates that if peripheral membrane proteins are required for binding, these can be supplied by the added cytosol. Taken together, these results indicate that the binding requires the activity of one or more specific, and probably integral, membrane proteins on the phagosome surface.

Previous studies have shown that the protein composition of phagosomes changes with time, as these organelles fuse with endocytic compartments to become progressively more late endosome/lysosome-like (Pitt *et al.*, 1992a; Pitt *et al.*, 1992b; Desjardins *et al.*, 1994a; Desjardins *et al.*, 1994b). We therefore asked whether the protein machinery responsible for microtubule binding competence also changes with time. Phagosomes were prepared at various times after bead internalization and tested for their ability to bind microtubules. As shown in Fig. 3C, the ability of phagosomes to bind microtubules was highest

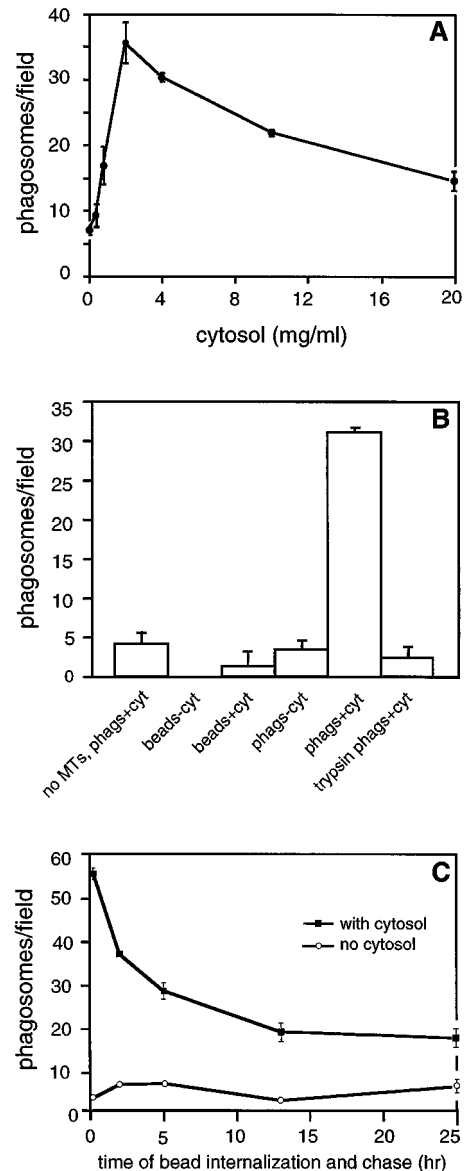


FIG. 3. Binding requires microtubules, cytosol, and proteins on the phagosome membrane. A, phagosome binding was tested in the presence of the indicated final cytosol concentrations. Optimal binding reproducibly occurred at 2 mg/ml cytosol. At higher concentrations inhibition was observed. B, binding to the microtubule lawn of uninternalized fish skin gelatin beads (beads) and phagosomes purified after fish skin gelatin bead internalization (1-h pulse, 1-h chase, phags) was tested in the absence (– cyt) or presence (+ cyt) of 2 mg/ml cytosol. Only phagosomes displayed cytosol-dependent binding, suggesting that both cytosolic and phagosome membrane factors are required. Background binding of phagosomes to the chamber in the absence of microtubules (no MTs, phags + cyt) was minimal. Treatment of phagosomes with 100 μ g/ml trypsin as described under “Materials and Methods” completely abolished their cytosol-dependent microtubule binding (trypsin phags + cyt), suggesting that membrane proteins are required. In each case, samples were adjusted for difference in bead content. C, phagosome binding activity is modulated by maturation of the organelle. Phagosomes were prepared after a 20-min pulse or after a 1-h pulse followed by 4, 12, or 24 h of chase. Phagosomes adjusted to the same bead concentration were tested for their ability to bind to microtubules in the presence or absence of 2 mg/ml cytosol.

at the earliest times after internalization, decreasing steadily with time to about $\frac{1}{3}$ of its original value. The ability to bind microtubules in the presence of cytosol was never lost but reached a stable low level after 12 h that persisted for 24 h after internalization.

The Binding Factor Can Interact with Microtubules in the Absence of Phagosomes—To probe the mode of interaction

⁴ J. K. Burkhardt, unpublished data.

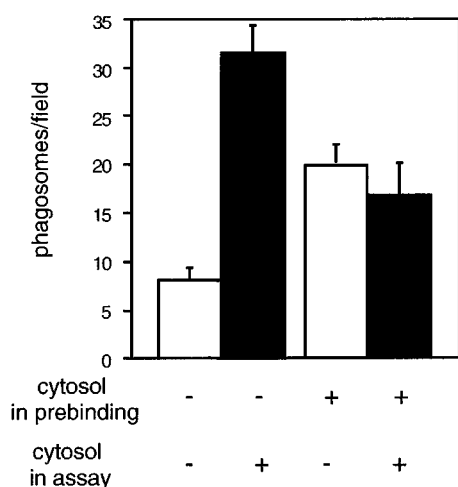


FIG. 4. The phagosome-microtubule binding factor can interact with microtubules independently of phagosomes. Assay chambers containing microtubules were preincubated for 20 min at room temperature with assay buffer or cytosol at 2 mg/ml (without phagosomes). The buffer or cytosol was washed away. Phagosomes were then perfused in the presence or absence of fresh cytosol at 2 mg/ml, incubated, washed, and counted as usual.

among phagosomes, microtubules, and the cytosolic binding factor, order-of-addition experiments were performed. In the absence of microtubules, phagosomes were incubated with cytosol for 20 min, floated, and then tested in the binding assay in the presence or absence of additional cytosol. A 2-fold stimulation of binding was sometimes apparent after preincubation with cytosol, but this was not reproducible ($n = 6$, with a stimulation of binding seen in about 50% of trials; data not shown). We conclude from this that under the conditions tested, the cytosolic binding factor interacts only weakly with the phagosome membrane or that, in the absence of microtubules, it binds in a partially inactive form.

To examine whether the binding factor could independently interact with microtubules, the microtubule lawn was preincubated with cytosol at 2 mg/ml in the absence of phagosomes. Cytosol was then washed out of the chamber using assay buffer, and phagosomes were added in the presence or absence of 2 mg/ml additional cytosol. As shown in Fig. 4, when microtubules were preincubated with cytosol, phagosomes bound in the absence of additional cytosol. The number of phagosomes bound was not as high as under standard assay conditions (*i.e.* simultaneous addition of phagosomes, microtubules, and cytosol), presumably because of the intervening wash step, but it was significantly higher than in the control reaction lacking cytosol altogether. This indicates that the binding activity can interact first with microtubules and then with the phagosome membrane. The fact that the binding factor can interact independently with microtubules suggests that it could be a MAP.

The Cytosolic Binding Activity Behaves Like a MAP—As expected of a MAP or a motor, binding was linearly sensitive to an increasing addition of salt (Fig. 5A), with a complete abolition of binding occurring at 150 mM KCl. In order to determine whether the activity is that of a motor or a true MAP, we examined the ability of phagosomes to bind to microtubules in the presence of a variety of nucleotides and nucleotide analogs. As shown in Fig. 5B, phagosome-microtubule binding was not greatly affected by depletion of endogenous cytosolic ATP, by the addition of ATP, by the nonhydrolyzable ATP analogs AMP-PNP and ATP γ S, or by GTP or its nonhydrolyzable analog GTP γ S. These results suggest that the majority of phagosome binding in this assay is not mediated by motor proteins, as motors are expected to bind more strongly (in the rigor state)

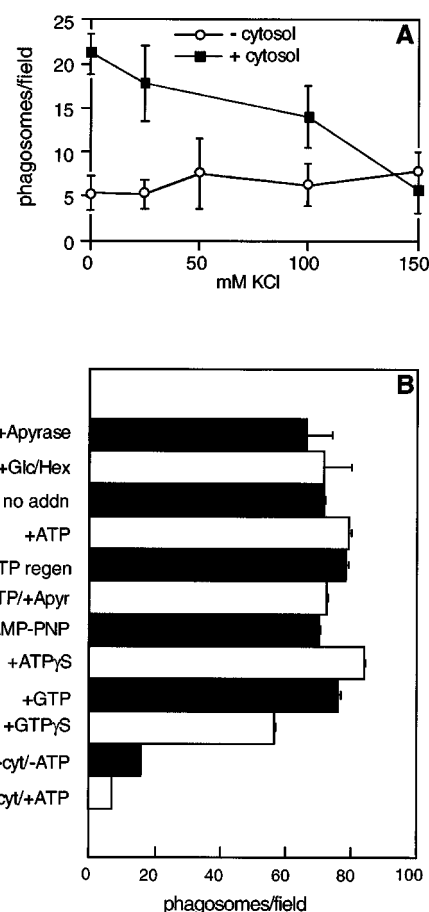


FIG. 5. A, the binding is highly sensitive to increasing salt concentrations. KCl was titrated into the usual assay using 2 mg/ml cytosol. B, binding is insensitive to nucleotides or nonhydrolyzable nucleotide analogs. The ATP-depleting agents apyrase and glucose/hexokinase and the ATP-regenerating system ATP/creatine phosphate/creatine phosphokinase were added exactly as in Bomsel *et al.* (1990). Phagosomes were mixed in the presence or absence of 2 mg/ml cytosol with the indicated nucleotides or analogs at 2 mM and perfused into chambers coated with poly-L-ornithine prior to the addition of the microtubules. This method was used to minimize the loss of microtubules from the lawn due to force exerted by cytosolic microtubule motor proteins in the presence of hydrolyzable nucleotides. Poly-L-ornithine slightly increased background binding without affecting the stimulation normally observed in the presence of cytosol.

in the absence of ATP or in the presence of nonhydrolyzable ATP analogs.

MAPs Are Necessary and Sufficient for Binding—To assess more directly the role of MAPs and motors, microtubule affinity depletion was used to remove these proteins from the macrophage cytosol. Excess exogenous taxol-stabilized microtubules were added to the macrophage cytosol, and the proteins that bound to these microtubules were removed by pelleting. If this is performed in the presence of ATP, MAPs are depleted but motors remain. If an ATP depletion system and AMP-PNP are added, both MAPs and motor proteins are depleted. Western blotting of both types of depleted cytosols confirms the specific depletion of representative MAP and motor proteins (Fig. 6A). As shown in Fig. 7A, the MAP-depleted cytosol failed to support phagosome binding, whether or not the motor proteins were removed. Since by microtubule affinity it is not possible to selectively deplete only the motor proteins, these were removed by immunodepletion (Fig. 6, C and D). As shown in Fig. 7B, immunodepletion of either cytoplasmic dynein or kinesin (the only motors for which adequate reagents were available) had no effect on binding.

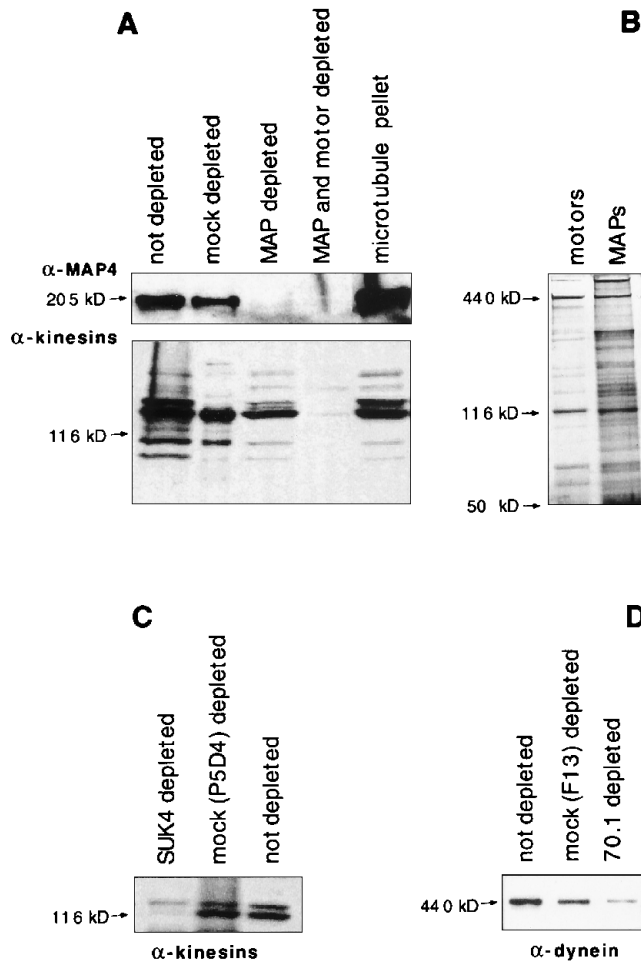


FIG. 6. A, cytosols depleted of MAPs or of MAPs and motor proteins as described under "Materials and Methods" were blotted with IF5.2.2, an anti-MAP4 antibody, or KMTBX, an antibody that recognizes the motor domain of multiple kinesin-like proteins. B, silver-stained 6% SDS-polyacrylamide gel of ATP-eluted motors and NaCl-eluted MAPs. C, kinesin was depleted with SUK4 anti-kinesin heavy chain and blotted with KMTBX; the mock sample was incubated with P5D4, an isotype-matched control antibody. Note that the 116-kDa kinesin heavy chain is removed while other kinesin-related proteins remain. D, cytoplasmic dynein was immunodepleted with 70.1 anti-dynein intermediate chain and blotted with rabbit anti-dynein heavy chain; the mock sample was treated with matched control antibody F13.

Motor proteins and MAPs can be eluted from the microtubule pellets using ATP or high salt, respectively, as shown in Fig. 6B. Readdition of the MAP preparation to MAP-depleted cytosol restored binding activity (Fig. 7C), while readdition of motor proteins had little effect (data not shown). These results indicate that motor proteins do not represent the predominant cytosolic microtubule binding activity measured in our assay. Instead, one or more MAP proteins are absolutely required for phagosome-microtubule binding.

To determine whether the proteins removed by microtubule affinity are sufficient to mediate the binding of phagosomes to microtubules in the absence of other cytosolic activities, the eluted MAP and motor preparations were tested alone in the assay. Fig. 7D shows that only the MAP preparation was able to support any significant binding. The low level of binding supported by the motor fraction was not ATP-sensitive (data not shown); hence we attribute it to contaminating MAPs.

To determine whether the MAP factor could interact directly with the phagosome membrane, we tested the ability of the MAP preparation (50 μ g/ml) to mediate the interaction of 1 M NaCl-stripped phagosomes with microtubules. The MAPs me-

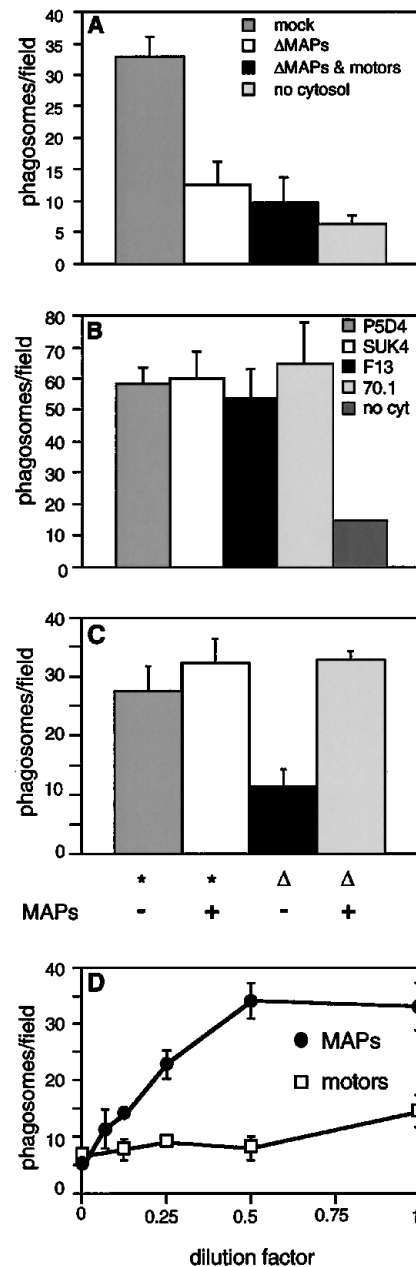


FIG. 7. MAPs, but not known microtubule motors, are necessary and sufficient for binding. A, phagosomes were tested for their ability to bind to microtubules in the presence of 2 mg/ml cytosol depleted of MAPs or of MAPs and motors by microtubule affinity (A). The cytosol solely depleted of MAPs but where motors remained had lost all binding activity. B, binding was tested in the presence of 2 mg/ml cytosol immunodepleted of cytoplasmic dynein with 70.1 or kinesin with SUK4; these depletions had no effect on the binding. Mock immunodepletion with isotype-matched control antibodies F13 and P5D4 (blots shown in Fig. 6, C and D) also had no effect. C, macrophage MAPs (20 μ g/ml in the assay) are able to restore activity to MAP-depleted cytosol (Δ) to the same levels as mock-depleted cytosol (*). D, the cytosolic binding activity is recovered in the MAP but not in the motor fraction. Crude fractions of MAPs and motors eluted sequentially from the same microtubule pellet (shown in Fig. 6B) were titrated by sequential 2-fold dilution for their ability to support phagosome binding in the absence of cytosol. A dilution factor of 1 represents the maximal amount of MAPs or motors that can be added to the assay, representing 140 μ g/ml protein for MAPs and 40 μ g/ml protein for motors.

diated this interaction to the same level as 2 mg/ml cytosol (67.2 ± 4.9 for mock-treated phagosomes *versus* 52.8 ± 8.9 for salt-washed phagosomes), suggesting that all the required soluble components are present in this preparation.

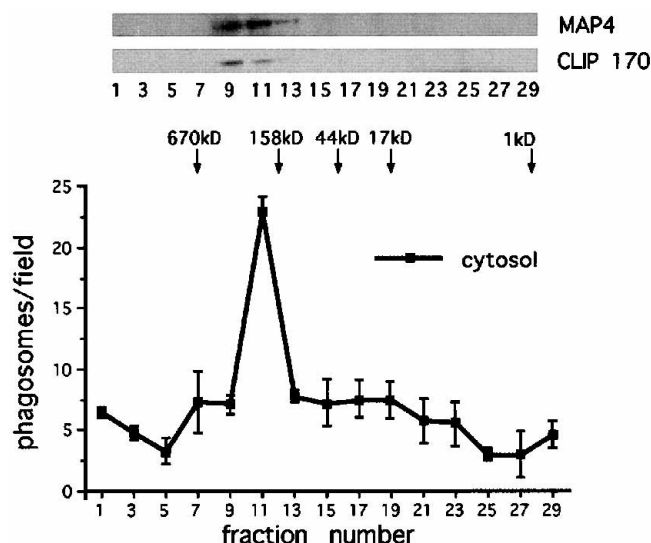


FIG. 8. The cytosolic binding factor behaves as a 150-kDa globular protein upon fractionation on Superose 12. Cytosol was fractionated on Superose 12 (V_0 is found at fraction 3, V_t at fraction 36). Fractions were pooled sequentially two-by-two and tested for binding activity. Fractionation of the cytosol yielded a single activity peak around 150 kDa. Note that when active fractions were assayed individually the 150-kDa peak was found to be contained mainly in fraction 12. Blotting of pooled cytosol fractions with $\alpha 55$ anti-CLIP-170 and IF5.2.2 anti-MAP4 shows that the profiles of these proteins do not correspond with the binding activity.

The Cytosolic Binding Factor Is a Heat-sensitive MAP in the Range of 150 kDa—As shown in Fig. 8, fractionation of macrophage cytosol on a Superose 12 column yields a single peak of binding activity. As with the activity in whole cytosol (Fig. 3C), the active fractions preferentially supported the binding of newly formed *versus* late phagosomes (data not shown). Though it is still unclear whether the activity represents a single protein, the active fraction corresponds to a globular protein around 150 kDa. Close to this size fall two previously identified MAPs, which were candidate proteins to mediate this binding. These are CLIP-170, identified as a MAP that mediates binding of endocytic vesicles to microtubules (Pierre *et al.*, 1992), and MAP4, which shares structural features with neuronal MAP2, itself identified as the mediator of chromaffin granule-microtubule binding (Severin *et al.*, 1991). Western blotting of column fractions from whole cytosol shows that despite some fractions containing these two proteins showing binding activity, the peak of activity does not exactly co-migrate with either protein; both MAP4 and CLIP-170 run slightly faster than the peak of activity (Fig. 8). This suggests that our factor is likely to represent either a new MAP or a known MAP to which no such organelle-binding function has yet been assigned.

The finding that a number of MAPs, including MAP4, are heat-stable facilitated their purification. We therefore tested the thermoresistance of the phagosome-binding factor. All binding activity was lost following brief heating of the MAP fraction to 95 °C (41.6 ± 1 for the mock-treated fraction *versus* 15.5 ± 5 for the heat-treated fraction). Thus, the binding factor is heat-sensitive.

DISCUSSION

Although considerable progress has been made toward understanding the molecular mechanisms of microtubule motors, our knowledge of motor-membrane interactions remains rudimentary. Still less is known about the possible role in membrane traffic of the heterogeneous family of proteins operationally classified as MAPs. Slow progress in this field is due

largely to technical difficulties in purifying a suitable membrane organelle in biologically active form. This is a prerequisite for any attempts to identify the molecules essential both for stable binding and for motility of membrane organelles along microtubules.

Latex bead-enclosing phagosomes are a powerful model system to study the interactions of defined membrane organelles with microtubules. Phagosomes move along microtubules *in vivo*,² and this movement is required for their interaction with compartments of the endocytic pathway (Desjardins *et al.*, 1994a; Jahraus *et al.*, 1994). As phagosomes are generated by engulfment of individual latex beads, they are large, discrete, and labeled organelles. Moreover, the buoyancy characteristics of latex make phagosomes remarkably easy to purify. These properties facilitate *in vivo* and *in vitro* analysis. Finally, since phagosomes are formed *de novo*, one can study their biogenesis and how this relates to their binding and motility along microtubules.

We describe here a novel *in vitro* light microscopy assay to measure the binding of phagosomes to microtubules. This assay requires minimal amounts of biological material and it is simple, fast, and reproducible in quantitative terms. Using this assay, we show that the binding of phagosomes to microtubules depends on both membrane proteins, probably integral to the surface of phagosomes, and exogenous cytosol. Sensitivity of phagosome-microtubule binding to proteases exhibiting different specificities suggests that a phagosome protein(s) functions as a receptor on the phagosome surface for the cytosolic microtubule binding factor. The fact that significantly more phagosomes bound, in the presence of the same cytosol preparation, when they were isolated at earlier rather than later times after their internalization indicates that the presence or activity of the membrane receptor is regulated. We are now pursuing the identification of this receptor activity using its protease sensitivity profile and established two-dimensional gel maps of the phagosome preparation (Desjardins *et al.*, 1994b; Burkhardt *et al.*, in press).

The cytosolic factor responsible for phagosome-microtubule binding is a heat-sensitive MAP(s) with a molecular weight in the range of 150 kDa. Classification of this factor as a MAP is based on several criteria. First, it is able to bind to microtubules in the absence of phagosomes. Second, its binding to microtubules is sensitive to moderate salt concentration but not to nucleotides. Third, it is removed from cytosol under conditions where MAPs are removed. Finally, it is present in a crude MAP preparation. Our data lead us to conclude that two proteins previously recognized as mediators of membrane organelle-microtubule interactions are probably not involved in the interaction we observe; the gel filtration profiles of CLIP-170 and MAP4 (some properties of which resemble neuronal MAP2; Olmsted (1993) and Walden (1993)) are different from, albeit overlapping, that of the activity we measure. Our factor is heat-sensitive, which provides evidence that it is not MAP2/MAP4 which are known to belong to the family of heat-stable MAPs (Herzog and Weber, 1978; Parysek *et al.*, 1984). CLIP-170 demonstrates nucleotide- and phosphorylation-sensitive binding to microtubules, whereas the phagosome-microtubule binding factor does not (Rickard and Kreis, 1991; Scheel and Kreis, 1991). It therefore appears that the activating MAP factor is most likely a novel MAP or a previously identified MAP for which no such role has yet been demonstrated.

At first glance, the MAP activity we describe seems redundant to the activity of a motor, which must itself mediate some form of organelle-microtubule binding. Although there is substantial evidence that purified motors can interact with organelles, there is so far no evidence that organelles carrying

only bound motors can interact with microtubules. In fact, there is evidence that motors alone are insufficient to mediate motile interactions of organelles with microtubules (Schroer *et al.*, 1988; Gill *et al.*, 1991; Schroer and Sheetz, 1991; Burkhardt *et al.*, 1993). As was found previously by Scheel and Kreis (1991) for endocytic vesicles, we find that immunodepletion of conventional kinesin and cytoplasmic dynein from cytosol has no effect on the binding of phagosomes to microtubules. Moreover, we tested the entire subset of proteins that bind to microtubules in an ATP-sensitive manner and found that this microtubule motor preparation, which contains cytoplasmic dynein, kinesin, and at least several kinesin-like proteins (see blot of microtubule pellet with a pan-kinesin antibody in Fig. 7A), was also unable to support binding. We therefore conclude either that microtubule motors are unable to support organelle-microtubule binding on their own or that the binding they support is too weak to be detected in our assay. Together with the work of Scheel and Kreis (1991), Severin *et al.* (1991) and Pierre *et al.* (1992), our results show that interaction of three different kinds of organelles with microtubules requires a type of MAP.

What functional role might these MAPs play? It has been previously suggested that the position of organelles such as the Golgi complex reflects an equilibrium between plus and minus end-directed motors. This is consistent with the work of others (Heuser, 1989; Parton *et al.*, 1991; Lin and Collins, 1992; Feiguin *et al.*, 1994). Yet the weight of recent evidence suggests that MAPs must also play a role in this process. We propose that the function of the MAP factor is to create a high affinity static link between the organelle and the microtubule, a function that a motor may not be able to perform. Specific MAP linker type molecules could function to define the position of each organelle relative to microtubules within cells, perhaps by antagonizing the action of a motor. Alternatively, or additionally, these proteins could facilitate motor-driven movements by creating a high affinity microtubule-organelle link, which may be required to initiate lower affinity motor interactions. The MAP may be lost from the organelle-motor complex when the organelle begins to move, or motors could just loosen this static link and perhaps use it to stabilize their transient interactions with microtubules while moving.

Any of these options requires a form of regulation of the linker MAP, to allow the switch between tethering and movement. We have already seen that the activity we describe is regulated on the membrane side as the phagosome matures. Rickard and Kreis (1991) have shown that CLIP-170 is released from microtubules by phosphorylation. We have been unable to modulate the activity of the binding factor by manipulating the nucleotide or phosphorylation state of the cytosol. However, a possibility for regulation in our system comes from our observation that at above 2 mg/ml cytosol, the MAP binding activity is inhibited. Such an inhibitory activity was not observed in other binding studies (van der Sluijs *et al.*, 1990; Scheel and Kreis, 1991; Severin *et al.*, 1991) partly, we think, because cytosol above 5 mg/ml was not tested. We have evidence that the binding and inhibitory activities are distinct and that the inhibitor is also a MAP.⁵ Since we have been unable to modulate the activity of the binding factor, we are investigating the possibility that it is the activity of the inhibitor which is regulated.

Clearly, the most direct approach to understanding the nature of the binding/motility switch is to study tethering and movement in parallel. Toward this goal we have established a second *in vitro* assay, very similar to the one described here,

which reconstitutes the movement of purified phagosomes along polarity-marked microtubules in macrophage cytosol.² In this assay phagosomes move bidirectionally along microtubules, although mainly toward the minus end. Used in tandem, the two assays provide an excellent model to identify the molecules that mediate anchoring and movement of organelles along microtubules and to study how they function coordinately.

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⁵ A. Blocker, unpublished data.

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