Motor Protein Independent Binding of Endocytic Carrier Vesicles to Microtubules in Vitro*

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We have established an in vitro assay to characterize the binding of endocytic carrier vesicles to microtubules. Magnetic beads coated with microtubules were used as an affinity matrix. A fraction from nocodazole-treated cells enriched in endocytic carrier vesicles, labeled with internalized horseradish peroxidase, was used in the binding experiments. Binding of the endocytic carrier vesicles to microtubules in vitro was cytosol-dependent. This activity of cytosolic factors was saturable, heat-sensitive, and insensitive to N-ethylmaleimide. Binding was sensitive to GTP and ATP. Addition of neuronal microtubule-associated proteins completely abolished binding of the endocytic organelles to microtubules. This binding was independent of the cytosolic microtubule-based motor proteins kinesin and cytoplasmic dynein, since cytosol depleted of these proteins remained fully active. Microtubule-binding proteins from HeLa cells, however, stimulated the interaction of endocytic carrier vesicles with microtubules. Trypsinized vesicles could no longer bind to microtubules in the presence of cytosol. These results suggest that cytosolic microtubule-binding proteins, other than the known microtubule-based motor proteins, as well as membrane proteins are involved in the nucleotide-dependent interaction of endocytic carrier vesicles with microtubules.

Soluble and membrane proteins internalized by animal cells are delivered to an early endosomal compartment, from which they are routed to late endosomes and lysosomes or recycled back to the cell surface (for reviews see Willem et al., 1986; Goldstein et al., 1985; Hopkins, 1986; Hubbard, 1989; Gruenberg and Howell, 1989; Kornfeld and Mellman, 1989). Molecules destined for degradation in lysosomes appear first in tubulovesicular early endosomes located at the cell periphery and then in late endosomes and lysosomes in the perinuclear region of the cell (Geuze et al., 1985; Hopkins and Towbridge, 1983; Pastan and Willingham, 1985; Schmid et al., 1988; Gruenberg et al., 1989). Early and late endosomes are morphologically and functionally distinct (Wall et al., 1986; Geuze et al., 1983; Hopkins and Towbridge, 1983; Wall and Hubbard, 1985; Marsh et al., 1986; Griffiths et al., 1988, Gruenberg et al., 1989; Woods et al., 1989), and they differ in acidification properties and protein composition (Baenziger and Fietz, 1986; Yamashiro and Maxfield, 1987; Schmid et al., 1988). Carrier vesicles mediating transport between early and late endosomes have recently been identified (Gruenberg et al., 1989).

Morphological and biochemical studies have revealed that the microtubule network is required for efficient transport of internalized molecules to late endosomes and lysosomes. Microtubules are involved in the clustering of late endosomes and lysosomes in the perinuclear region of the microtubule organizing center (Matteoni and Kreis, 1987; Swanson et al., 1987; Scheel et al., 1986), and depolymerization of microtubules slows down degradation of internalized proteins (Oka and Weigel, 1985; Caron et al., 1985; Gruenberg et al., 1989). In addition, transport of endocytic vesicles between the cell periphery and the perinuclear region has been shown to occur along microtubules in vivo (Matteoni and Kreis, 1987; DeBrabander et al., 1988). The precise molecular basis of the interactions of endocytic organelles with microtubules is unknown, so far.

Recently, cell-free assays have been developed to study the interaction of cytoplasmic organelles with microtubules. Kinesins (Vale et al., 1985a) and cytoplasmic dyneins (Paschal and Vallee, 1987), two mechanochemical enzymes that are involved in movement of vesicles along microtubules, have been characterized in vitro motility assays (for recent reviews see McIntosh and Porter, 1989; Vale, 1990; Vallee and Shpetner, 1990). Kinesins are required for the formation of tubular extensions of lysosomes in vivo (Hollenbeck and Swanson, 1990). Moreover, it has been shown that movement of organelles along microtubules in vitro requires other cytosolic proteins in addition to kinesin (Schroer et al., 1988) and cytoplasmic dynein (Schroer et al., 1989). On the other hand, microtubule-organelle binding assays have been developed to identify soluble and membrane proteins mediating the interaction of microtubules with organelles. Cytosolic proteins have been shown to be involved in the binding of axoplasmic (Pratt, 1986) and exocytic trans-Golgi-derived carrier vesicles (van der Slujs et al., 1990) to microtubules. Furthermore, a lysosomal membrane protein was identified that mediates nucleotide-dependent binding of lysosomes to microtubules (Mithieux and Rouset, 1989).

Accumulation of internalized markers in the endocytic carrier vesicles after depolymerization of microtubules (Gruenberg et al., 1989) indicates that an interaction with microtubules is required at this stage for proceeding in the endocytic pathway. Fusion of endocytic carrier vesicles presumably with late endosomes was facilitated by the presence of microtubules in a cell-free assay (Bomsel et al., 1990). We have used a novel in vitro binding assay to study the interaction of these endocytic carrier vesicles with microtubules. Efficient binding of the endocytic carrier vesicles to microtubules required cytosolic proteins, was nucleotide-dependent, and involved endosomal membrane proteins. Kinesin and cytoplasmic dynein were not required for binding, indicating that this interaction of endocytic carrier vesicles with microtubules depends on...
cytosolic proteins other than the microtubule-based motor proteins.

**EXPERIMENTAL PROCEDURES**

Cell Culture—Baby hamster kidney (BHK-21) cells were grown in Glasgow minimum essential medium supplemented with 5% fetal calf serum, 10% tryptose phosphate broth, and 2 mM glutamine as described (Gruenberg et al., 1989). 10 μM nocodazole was added to the culture medium for 60 min at 37 °C to depolymerize microtubules in BHK cells. HeLa spinner cells were maintained in Joklik-minimum essential medium supplemented with 2 mM glutamine, 5% newborn calf serum at cell densities of 1–6 × 10^6/ml at 37 °C (Rickard and Kreis, 1990).

**Antibodies**—A monoclonal antibody against the carboxyl terminus of α-tubulin (1A2; Kreis, 1987) and a monoclonal antibody against the cytoplasmic tail of the glycoprotein of vesicular stomatitis virus (P5D4; Kreis, 1986) were affinity purified from ascites, and Fab fragments of these antibodies were prepared as described (Kreis, 1986). The monoclonal antibody against sea urchin kinesin (SU4; Ingold et al., 1988) was a gift of Dr. J. M. Scholey (University of California, Davis), and the monoclonal antibody against the intermediate chain of chicken cytoplasmic dynein (70.1; Steuer et al., 1990) was a gift from Drs. E. R. Steuer and M. P. Sheetz (Duke University, Durham, NC).

Microtubules—Tubulin was purified from bovine brain and polyvinyl acetate was added to as “microtubules” (in this work) as described (Rickard and Kreis, 1990). Heat-stable MAPs from bovine brain and HeLa cell microtubules were prepared as described (van der Sluijs et al., 1990). Tubulin, microtubules, and MAPs were stored in liquid nitrogen for up to 6 months. To label microtubules with Europium, microtubules from bovine brain (1 mg/ml) were incubated with biotinylated Fab fragments of 1A2 (~1 Fab fragment/250 tubulin dimers) for 20 min at 25 °C in PEMT (0.1 M K-Pipes, 1 mM EGTA, 1 mM MgSO₄, 20 μM taxol, pH 6.8). The mixture was layered over a cushion of 10% sucrose in KPAT (50 mM K-Pipes, 50 mM KOAc, 20 μM taxol), pH 7.2) and centrifuged at 40,000 × g for 25 min at 25 °C. The supernatant with KPAT in the pellet was resuspended in 1 ml of 1M europium-streptavidin (prepared by I. Hemmilä, Wallac Oy, Turku, Finland) was added to 10 μg/ml, incubated for 15 min at 25 °C and the mixture centrifuged as above. The pellet was resuspended in KPAT to a final concentration of 1 mg/ml.

**Coupling of Microtubules to Magnetic Beads**—Monodisperse magnetic beads were chemically activated with tosylchloride, and a linker protein-depleted cytosol, and the microtubule-binding proteins were removed by treatment of HeLa cytosol with 20 μM taxol for 15 min at 37 °C and sedimented for 5 min at 200,000 × g. The fraction of endocytic carrier vesicles was always prepared fresh on the day of the binding experiment.

**Endocytosis—**Cytosol from HeLa spinner cells and MDCK II cells was prepared as described (van der Sluijs et al., 1990) except that 1 mM DTT and 1 μM cytochalasin D were present in all the solutions used. HeLa cytosol containing 1 mM MgATP, 0.1 mM vanadate (+5 oxidation state) was irradiated at 366 nm for 1 h on ice using a UV lamp (MinUVa, Desaga, Heidelberg, Federal Republic of Germany) to cleave cytoplasmic dynein. Vanadate was subsequently reduced and inactivated by incubation with 5 mM norepinephrine for 20 min on ice (Gibbons et al., 1987). Cytosol was depleted of kinesin with SUK4. An IgG fraction of SUK4 ascites, obtained by precipitation of ascites with 5% ammonium sulfate, was dialyzed against PBS and incubated at 30°C with protamine sulfate to inactivate cytoplasmic dynein. Following this, cytosol was prepared by brief centrifugation in 0.2 ml of Enhancer solution (Wallac Oy), and Europium was released from the fraction with 0.2 ml of Enhancer solution (Wallac Oy), and Europium fluorescence was measured in an ARCS/DELFIA time-resolved fluorometer (Wallac Oy).

**Endocytic Carrier Vesicles**—After depolymerization of microtubules with 1 μM nocodazole for 1 h at 37 °C, microtubules were allowed to internalize horseradish peroxidase (5 mg/ml in Glasgow minimum essential medium containing 10 mM glucose) for 10 min at 37 °C followed by a 25-min chase in marker-free medium (Gruenberg et al., 1989). The following steps were done on ice: cells were washed three times with PBS after internalization of horseradish peroxidase, scraped with a rubber policeman into 3 ml of PBS/10% calf serum, centrifuged for 5 min at 200 × g, and washed once in homogenization buffer (5 mM imidazole, 250 mM sucrose, pH 7.1). The pellet was resuspended in homogenization buffer (0.2 ml/10-cm dish) supplemented with 1 μM cytochalasin D, 1 mM DTT, 2 mM EGTA, and protease inhibitors (van der Sluijs et al., 1990), homogenized by eight passages through a 1 ml-Gilon pipette tip, and centrifuged at 1000 × g for 10 min at 4 °C. The postnuclear supernatant was centrifuged again under the same conditions, brought to 40.6% sucrose in a final volume of 1 ml, and overlayed subsequently with 1 ml of 35% sucrose, 1 ml of 25% sucrose, and 0.5 ml of 12% sucrose (all sucrose solutions contained 5 mM imidazole, pH 7.1) in a 3.5 ml polycarbonate tube (Beckman). The 25% to 12% sucrose interphase was collected after 1-h centrifugation at 165,000 × g at 4 °C. The fraction of endocytic carrier vesicles was always prepared fresh on the day of the binding experiment.

**Endocytosis—**Cytosol from HeLa spinner cells and MDCK II cells was prepared as described (van der Sluijs et al., 1990) except that 1 mM DTT and 1 μM cytochalasin D were present in all the solutions used. HeLa cytosol containing 1 mM MgATP, 0.1 mM vanadate (+5 oxidation state) was irradiated at 366 nm for 1 h on ice using a UV lamp (MinUVa, Desaga, Heidelberg, Federal Republic of Germany) to cleave cytoplasmic dynein. Vanadate was subsequently reduced and inactivated by incubation with 5 mM norepinephrine for 20 min on ice (Gibbons et al., 1987). Cytosol was depleted of kinesin with SUK4. An IgG fraction of SUK4 ascites, obtained by precipitation of ascites with 5% ammonium sulfate, was dialyzed against PBS and incubated at 30°C with protamine sulfate to inactivate cytoplasmic dynein. Following this, cytosol was prepared by brief centrifugation (5 min, 10,000 × g, 4 °C) to obtain the kinesin-depleted cytosol. Cytoplasmic dynein-depleted cytosol was obtained by the same procedure, using the 0.1 antibody coupled to goat anti-mouse-agarose (Sigma Chemie GmbH, Deisenhofen, Federal Republic of Germany).

**Microtubule-binding Proteins**—Endogenous microtubules were proliferated in HeLa cytosol with 0.05 μg/ml taxol for 15 min at 37 °C and removed by centrifugation at 40,000 × g for 25 min at 20 °C. The supernatant (taxol-treated cytosol) was dialyzed against PEM (PIMENT without taxol) for 6 h. Alternatively, microtubule-binding proteins were removed by treatment of HeLa cytosol with 20 μM taxol, 0.5 mg/ml taxol microtubules from bovine brain, 25 units/ml hexokinase (Sigma Chemie GmbH), 10 mM glucose and 1 mM AMP-PNP, followed by centrifugation as described above. Microtubule-binding proteins were eluted from the microtubule pellet by incubation with 0.8 M NaCl in PEMT at 25 °C for 10 min. Microtubule-binding protein-depleted cytosol, and the microtubule-binding protein-depleted cytosol were dialyzed against PEMT for 6 h.

**Organelle-microtubule Binding Assay**—In a standard binding assay, 0.2-mg magnetic beads coated with microtubules from bovine brain were incubated with 15 μl of the endosome fraction (corresponding to ~0.4 μg of protein) in PE buffer (80 mM K-Pipes, 1 mM MgSO₄, 1 mM EGTA, 1 μM cytochalasin D, 1 mM DTT, 2 μM taxol, pH 7.0) in a final volume of 100 μl. The mixture was incubated at 23 °C for 50 min before retrieval of the magnetic beads with the magnet. The unbound fraction was removed, and Triton X-100 was added to 1%. The beads were resuspended in 100 μl of PE buffer containing 1% Triton X-100. Both unbound and bound fractions were vortexed, centrifuged at 13,000 × g for 5 min, and aliquots of the supernatants were analyzed for horseradish peroxidase activity. For incubations in the presence of trinitrophenylated, creatine phosphate (10 mM) and creatine kinase (5 μg/ml) were used to regenerate ATP, and acetyl phosphate (10 mM) and acetyl kinase (0.05 unit/ml) were used to regenerate GTP.

**Electron Microscopy**—Microtubule beads and organelle fractions were fixed with 1% glutaraldehyde in 0.2 M cacodylate, pH 7.2.
were labeled with Europium (see "Experimental Procedures") and tubules polymerized with taxol from purified bovine brain tubulin. Bound microtubules were retrieved on a magnet, and binding was quantitated by incubation with increasing amounts of Europium-labeled microtubules (final concentration 0.5 mg/ml) were incubated with magnetic beads carrying 1A2 (Fig. 1A and B), whereas no microtubules were observed on control beads (Fig. 2C). The surface of the beads was covered with microtubules extending into the medium and providing long flexible arms to which organelles could bind to (Fig. 2B).

**RESULTS**

**Coupling of Microtubules to Magnetic Beads**—The efficiency of binding of microtubules to magnetic beads was quantitated using Europium-labeled microtubules. Binding of labeled microtubules to the beads was dependent on the presence of an anti-tubulin antibody (1A2) on the bead surface (Fig. 1A). Binding of microtubules to the beads could be competed with unlabeled monomeric tubulin (Fig. 1A) and was saturable (Fig. 1B). No significant dissociation of microtubules from the beads was observed during at least 1.5 h of incubation in the presence of 2 μM taxol (data not shown). Electron microscopical analysis of the beads showed that microtubules were attached to the bead surface carrying 1A2 (Fig. 2A and B), whereas no microtubules were observed on control beads (Fig. 2C). The surface of the beads was covered with microtubules specific for horseradish peroxidase, a postnuclear supernatant from nocodazole-treated horseradish peroxidase-labeled cells was fractionated by floatation on a sucrose step gradient, similar to the floatation gradient described by Gorvel et al. (1991) to separate early endosomes from endocytic organelles of later stages in the pathway (see "Experimental Procedures"). ~60% of the horseradish peroxidase activity of the postnuclear supernatant was recovered from the 25/12% sucrose interphase, and the specific horseradish peroxidase activity in this fraction was ~15–20-fold higher than in the postnuclear supernatant (data not shown). Electron micrographs of the 25/12% sucrose interphase showed horseradish peroxidase-positive structures exhibiting the typical spherical and multivesicular appearance of endocytic carrier vesicles (Fig. 3; see Gruenberg et al., 1989; McDowall et al., 1989). In contrast, less than 5% of the early endosomes, labeled after 5 min of internalization of horseradish peroxidase, were present in the 25/12% sucrose interphase. Since the 25/12% sucrose interphase was depleted of early endosomes, but enriched in endocytic carrier vesicles specifically labeled with horseradish peroxidase, we used this fraction in the *in vitro* binding assays.

**Preparation of Endocytic Carrier Vesicles**—Horseradish peroxidase internalized in nocodazole-treated BHK cells for up to 45 min at 37°C accumulates in a distinct and rather uniform population of endocytic carrier vesicles without being delivered to late endosomes (Gruenberg et al., 1989). To obtain an organelle fraction enriched in these vesicles containing horseradish peroxidase, a postnuclear supernatant from nocodazole-treated horseradish peroxidase-labeled cells was fractionated by floatation on a sucrose step gradient, similar to the floatation gradient described by Gorvel et al. (1991) to separate early endosomes from endocytic organelles of later stages in the pathway (see "Experimental Procedures"). ~60% of the horseradish peroxidase activity of the postnuclear supernatant was recovered from the 25/12% sucrose interphase, and the specific horseradish peroxidase activity in this fraction was ~15–20-fold higher than in the postnuclear supernatant (data not shown). Electron micrographs of the 25/12% sucrose interphase showed horseradish peroxidase-positive structures exhibiting the typical spherical and multivesicular appearance of endocytic carrier vesicles (Fig. 3; see Gruenberg et al., 1989; McDowall et al., 1989). In contrast, less than 5% of the early endosomes, labeled after 5 min of internalization of horseradish peroxidase, were present in the 25/12% sucrose interphase. Since the 25/12% sucrose interphase was depleted of early endosomes, but enriched in endocytic carrier vesicles specifically labeled with horseradish peroxidase, we used this fraction in the *in vitro* binding assays.

**Cytosol-dependent Binding of Endocytic Carrier vesicles to Microtubules in Vitro**—The interaction of endocytic carrier

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**Fig. 1. Coupling of microtubules to magnetic beads.** Microtubules polymerized with taxol from purified bovine brain tubulin were labeled with Europium (see "Experimental Procedures") and incubated with magnetic beads for 20 min at 25°C. The beads with bound microtubules were retrieved on a magnet, and binding was quantitated by measuring Europium fluorescence. A, Europium-labeled microtubules (final concentration 0.5 mg/ml) were incubated with magnetic beads with either an antibody against tubulin (1A2) or a control antibody (P5D4) coupled to the surface. Binding of labeled microtubules to the beads was competed by preincubation of the beads with unlabeled tubulin (5 mg/ml) for 30 min on ice. B, binding of microtubules to magnetic beads carrying 1A2 was titrated by incubation with increasing amounts of Europium-labeled microtubules.

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**Fig. 2. Morphology of microtubule beads.** Microtubules were incubated with magnetic beads coated with 1A2 (A and B) or control antibody P5D4 (C). Beads were then washed, fixed, and processed for cryosectioning and labeling with the 1A2 antibody and protein A-gold. The density of microtubules bound to the magnetic beads is revealed best in sections cut through the top of a bead (arrow). Bar, 1 μm (A and C) and 0.2 μm (B).
vesicles with microtubules was assayed by quantitating the binding of the horseradish peroxidase containing vesicles to microtubule beads using a procedure similar to the immunosolation of organelles with magnetic beads (Howell et al., 1988). Organelles bound to magnetic microtubule-beads were separated from unbound organelles by retrieving the beads with a magnet, removing the unbound fraction, and measuring the activity of the internalized marker enzyme, horseradish peroxidase, in the fractions. Each of the three components, cytosol, microtubules, and endocytic carrier vesicles, could be analyzed separately in this in vitro binding assay.

In the absence of cytosol, less than 5% of labeled endocytic carrier vesicles bound to the microtubule beads after incubation for 50 min at 23 °C (Fig. 4). Upon addition of HeLa cytosol, binding of endocytic carrier vesicles to microtubule beads was stimulated about 10-fold (Fig. 4). The time course of binding showed that binding increased almost linearly during the incubation and reached a plateau after ~45 min (data not shown). The stimulatory effect of cytosol on the binding of endocytic carrier vesicles to microtubule beads was saturable and reached the maximal effect at about 2 mg/ml of HeLa cytosol (Fig. 4). At this concentration, ~35% of total horseradish peroxidase present in the fraction was bound to the microtubule beads. Cytosol from MDCK cells and turkey liver also stimulated binding of BHK endocytic carrier vesicles to microtubules to a similar extent. Less than 10% of the bound activity could be removed from the beads by subsequent washing steps; beads with bound vesicles were therefore routinely not washed. The horseradish peroxidase activity recovered on microtubule beads was membrane enclosed, since no activity was bound when soluble horseradish peroxidase was added together with cytosol and unlabeled organelles (Fig. 4). More than 99% of the soluble horseradish peroxidase was recovered in the supernatant (Fig. 4), indicating that unbound material was quantitatively separated from bound vesicles.

The HeLa cytosol was pretreated in various ways to study the cytosolic factor(s) involved in the binding assay. Binding could be abolished by heating the cytosol for 5 min to 95 °C (Fig. 5). Furthermore, the factors mediating the interaction of endocytic carrier vesicles with microtubules eluted in the void volume of a Sepharose G25 column and could be precipitated with ammonium sulfate (data not shown). Pretreatment of cytosol with 1 mM NEM did not affect the binding assay (Fig. 5). These data suggest that cytosolic protein(s) insensitive to NEM are involved in the interaction of endocytic carrier vesicles with microtubules.

Cytosol-dependent binding of endocytic carrier vesicles to microtubule-beads was competed efficiently by addition of free microtubules (Fig. 6A). Only background values of horseradish peroxidase in endocytic carrier vesicles were observed on the beads when microtubules were omitted. In these control experiments the magnetic beads carried 1A2 but no pre-bound
Binding of Endocytic Carrier Vesicles to Microtubules

The specificity of cytosol-dependent binding of endocytic carrier vesicles was demonstrated by the inhibition of binding by specific competition with binding sites or to steric hindrance (see also van der Sluijs et al., 1990). These results clearly demonstrate that the endocytic carrier vesicle proteins with NEM did not alter their properties exposed on the cytoplasmic surface of the organelles which were present in the untreated fraction of endocytic carrier vesicles, were removed by this treatment (data not shown). An involvement of extractable membrane associated proteins in the interaction of the vesicles with microtubules can, however, not be completely excluded in these experiments, since these proteins could rebind and be repurposed by the cytosol during the incubation.

The specificity of cytosol-dependent binding of endocytic carrier vesicles to microtubules was further tested by titration of the organelles in the assay. The binding of endocytic carrier vesicles was saturable when high concentrations of organelles were used (Fig. 6B). As the endocytic carrier vesicles were routinely used at a protein concentration of ~4 mg/ml, microtubules were present in excess. Cytosol-dependent binding of horseradish peroxidase-labeled endocytic carrier vesicles to microtubules was also observed when a crude postnuclear supernatant was used instead of the endocytic carrier vesicle fraction (data not shown).

**Binding of Endocytic Carrier Vesicles to Microtubules Is Nucleotide-sensitive**—The effect of various nucleotides and their analogs was tested on the binding of endocytic carrier vesicles to microtubules (Table 1). Binding was completely inhibited by 1 mM GTP, whereas 1 mM GDP or the GTP analogs GTP S or GMP-PNP had no significant effect. ATP reduced binding to ~50%, but no complete inhibition was obtained even at high concentrations of ATP (up to 5 mM). ADP and the ATP analogues ATP S and AMP-PNP had no effect on the binding of endocytic carrier vesicles to microtubules. Efficient binding of endocytic carrier vesicles to microtubules also occurred in the absence of nucleotides, using Sephadex G25 gel-filtered cytosol or after ATP depletion with 10 mM glucose and 25 units/ml hexokinase (data not shown). Vanadate (0.2 mM), which alone had no effect, abolished the inhibitory effect of ATP. Thus, binding of endocytic carrier vesicles to microtubules was affected by the presence of hydrolyzable nucleotides.

**Trypsin-sensitive Membrane Proteins Mediate Binding of Endocytic Carrier Vesicles to Microtubules**—The membranes of the endocytic carrier vesicles were treated in various ways to test whether membrane proteins were required for the interaction of endocytic carrier vesicles with microtubules. None of the treatments used influenced the floatation behavior of the endocytic carrier vesicles on sucrose gradients, although the yield of salt-extracted vesicles was lower than in the control. Treatment of endocytic carrier vesicles with trypsin (50 μg/ml TPCK-treated trypsin, for 25 min at 30 °C) completely abolished binding, confirming the requirement of proteins exposed on the cytoplasmic surface of the organelles for interaction with microtubules (Fig. 7). Alkylation of endocytic carrier vesicle proteins with NEM did not alter their cytosol-mediated binding to microtubules (Fig. 7). Neither did pretreatment of organelles with 1 mM NaCl, to remove peripheral membrane proteins, have any effect on the binding of endocytic carrier vesicles to microtubules (Fig. 7). The microtubule-based motor proteins kinesin and cytoplasmic dynein, which were present in the untreated fraction of endocytic carrier vesicles, were removed by this treatment (data not shown). An involvement of extractable membrane associated proteins in the interaction of the vesicles with microtubules can, however, not be completely excluded in these experiments, since these proteins could rebind and be repurposed by the cytosol during the incubation.

**Cytosolic Factors Different from Kinesin or Cytoplasmic Dynein Are Involved in Binding of Endocytic Carrier Vesicles to Microtubules in Vitro**—The microtubule-based motor proteins cytoplasmic dynein and kinesin can mediate movement of organelles along microtubules (for reviews see McIntosh and Porter, 1989; Vale 1990; Vallee and Shpetner, 1990). To

![Graph A](image)

**FIG. 6.** Biochemical characterization of the binding of endocytic carrier vesicles to microtubules. A, competition with free microtubules. The endocytic carrier vesicle fraction (4 μg protein/ml final concentration) was incubated with 2 mg/ml of HeLa cytosol and microtubule beads, and increasing concentrations of free taxol microtubules from bovine brain were added at the beginning of the incubation (opolitan). Heat-stable MAPs from bovine brain were added to 50 μg/ml at the beginning of the incubation without addition of free microtubules (滨江). B, titration of organelles. Increasing concentrations of endocytic carrier vesicles (final protein concentrations are indicated) were incubated with microtubule beads and 2.5 mg/ml of HeLa cytosol. Binding was analyzed as described in the legend of Fig. 4.

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plasmic dynein have been used to demonstrate motor protein dependend movement of organelles, microtubules, or latex beads (Vale et al., 1985b; Schroer et al., 1988; Schnapp and Reese, 1989; Euteneuer et al., 1989; Bomsel et al., 1990).

Since removal of the two microtubule-based motor proteins from the cytosol had no effect in the in vitro binding assay, we concluded that they are not required for efficient binding of endocytic carrier vesicles to microtubules. Repeating the experiments with lower concentrations of motor protein depleted cytosols (0.5–1.5 mg/ml), allowing a more sensitive detection of changes in the stimulatory activity, revealed no differences to untreated cytosol (data not shown). Since salt-treated endocytic carrier vesicles were used in all these binding experiments, no organelle-associated motor proteins were present. Thus, the cytosolic activity stimulating the interaction of endocytic carrier vesicles with microtubules is different from kinesin or cytoplasmic dynein.

**Binding of Endocytic Carrier Vesicles to Microtubules Is Mediated by Microtubule-binding Proteins—Microtubule-binding proteins were retrieved from the cytosol by their affinity to microtubules to investigate their potential role in the in vitro binding assay. Removal of endogenous microtubules by taxol alone (see “Experimental Procedures”) did not reduce the cytosolic activity in the binding assay (Fig. 9). To increase the efficiency of the microtubule affinity step, cytosol

![Figure 7](image_url)

**Fig. 7.** Membrane proteins of the endocytic carrier vesicles are required for cytosol-dependent binding to microtubules. The organelles recovered from the 25/12% interphase of the sucrose step gradient were digested with 50 μg/ml TPCK-treated trypsin for 25 min at 30°C before inhibition of the protease by addition of soybean trypsin inhibitor to 0.1 mg/ml and phenylmethylsulfonyl fluoride to 2 mM (trypsin). Treatment of vesicles with NEM (NEM) was done as described for the alkylation of cytosolic proteins (see legend to Fig. 5). Organelles were extracted with salt by adding 1 M NaCl to the postnuclear supernatant before floatation on the sucrose gradient (salt). Untreated organelles were used as a control (+). HeLa cytosol was present at 2 mg/ml.

![Figure 8](image_url)

**Fig. 8.** Kinesin or cytoplasmic dynein are not required for binding of endocytic carrier vesicles to microtubules. A, photobleavage of cytoplasmic dynein. HeLa cytosol was irradiated with UV light in the presence of ATP and vanadate. Cleavage was analyzed by electrophoresis using 4% polyacrylamide gels and silver staining. One band with a molecular mass of ≥400 kDa (CDH) was cleaved after UV irradiation (UV), giving rise to two cleavage products of ~240 kDa (H(UV)) and ~190 kDa (L(UV)). Only the relevant part of the gel is shown. Untreated cytosol is shown for comparison (control). B–D, immunodepletion of cytosol from kinesin and cytoplasmic dynein. HeLa cytosol was incubated with beads coupled to an antibody against kinesin (SU4K), an antibody against cytoplasmic dynein (70.1), or a control antibody (PSD4; see “Experimental Procedures”). The beads and bound proteins were removed by centrifugation, and the supernatants were analyzed by electrophoresis using 8% polyacrylamide gels and immunoblots with an antibody against kinesin (B), 70.1 (C), or with 4% gels and silver staining as described in A (D). Only the relevant part of the gel and the immunoblots is shown, and the positions of kinesin heavy chain (KH), cytoplasmic dynein intermediate chain (CDI), and heavy chain (CDH) are indicated. The effects of depletion of cytosol from microtubule-based motor proteins on the binding of endocytic carrier vesicles to microtubules are shown in E. Binding of salt-treated endocytic carrier vesicles to microtubules was analyzed with (+) or without (−) HeLa cytosol, using UV irradiated cytosol (UV; see A) or cytosols immunodepleted of motor proteins (see B–D). The concentration of cytosol used in the binding assay was 2 mg/ml.
was treated with taxol in the presence of exogenous microtubules from bovine brain and under conditions of ATP depletion (see also Rickard and Kreis, 1990). When this cytosol depleted of microtubule-binding proteins was tested in the in vitro binding assay, binding was reduced more than 5-fold (Fig. 9). This suggests that cytosolic proteins mediating the binding of endocytic carrier vesicles to microtubules bind to microtubules under these conditions. This was confirmed by a significant rescue of binding when these proteins were added back to cytosol depleted of microtubule-binding proteins. Microtubule-binding proteins alone also stimulated binding of endocytic carrier vesicles to microtubules (Fig. 9), suggesting that cytosolic microtubule-binding proteins promote the binding of endocytic carrier vesicles to microtubules in the in vitro assay.

**DISCUSSION**

The microtubules play a key role in the spatial arrangement of cytoplasmic organelles including endosomes in eukaryotic cells (for reviews see for example Pastan and Willingham, 1985; Hopkins, 1986; Kreis, 1990). Microtubule-based motor proteins have been identified and biochemically characterized with in vitro motility assays (Vale et al., 1985a, 1985b; Brady, 1985; Paschal and Vallee, 1987; Exte neur et al., 1989), and their role in movement of cytoplasmic organelles has been established (Vale et al., 1985a, 1985b; Schroer et al., 1988, 1989; Schnapp and Reese, 1989). It has been suggested that kinesin is required for the formation of tubular extensions of lysosomes in vitro (Hollenbeck and Swanson, 1990) and that efficient fusion of apical and basolateral endocytic carrier vesicles from MDCK cells in vitro depends on the microtubule-based motor proteins kinesin and cytoplasmic dynein (Bomsel et al., 1990).

In contrast to movement along microtubules, little is known about those proteins that mediate binding of cytoplasmic organelles to microtubules. Here, we initially characterize the interaction of cytoplasmic organelles with microtubules using a novel in vitro binding assay. We began studying the interaction of endocytic carrier vesicles with microtubules, since the accumulation of these vesicles in nocodazole-treated cells suggested that an interaction with microtubules is required at this stage for proceeding in the endocytic pathway (Gruenberg et al., 1989; Bomsel et al., 1990). Our in vitro assay is designed to allow the molecular analysis of the components involved in the cytosol-dependent binding of endocytic carrier vesicles to microtubules, i.e. microtubules, vesicle-associated proteins, and cytosolic factors. Each of these three components was required to form microtubule-vesicle complexes, and each of these three components was either saturable or comparable in the assay. Binding of endocytic carrier vesicles to microtubules was specifically inhibited by addition of heat-stable MAPs from bovine brain. Furthermore, binding was nucleotide-sensitive. Inhibition or inactivation of each of the three components corroborated the validity of the assay.

Binding of lysosomes purified from thyroid cells to microtubules has been demonstrated previously, and a 50-kDa protein mediating this interaction has been identified (Mithieux and Rousset, 1989). Binding of thyroid lysosomes to microtubules is independent of cytosolic proteins and the presence of MAPs, whereas binding of the endocytic carrier vesicles to microtubules depends on cytosolic factors and can be completely abolished by addition of MAPs. Additionally, the interaction of lysosomes with microtubules is, in contrast to the endocytic carrier vesicles, sensitive to NEM, magnesium ions, and adenine nucleotides other than ATP (Mithieux and Rousset, 1988). Binding of endocytic carrier vesicles to microtubules is, therefore, clearly different from the binding of lysosomes to microtubules and is thus likely to be mediated by proteins other than the 50-kDa lysosomal membrane protein.

Observations in vivo have indicated that cytoplasmic organelles, which normally move along microtubules in the retrograde direction, interact with microtubules by various mechanisms, probably involving different proteins. Acidification of the cytosol, for example, reversibly changes the microtubule-dependent location of late endosomes and lysosomes from the perinuclear region to the cell periphery (Heuser, 1989; Parton et al., 1991). The Golgi apparatus, however, the location of which is also dependent on microtubules (Kreis, 1990) and elements of which can move along microtubules in vivo (Ho et al., 1989), is not affected by lowered cytoplasmic pH (Heuser, 1989). This differential sensitivity to pH of the factors which are involved in the interaction of endosomes and elements of the Golgi apparatus with microtubules implies that organelle-specific factors are involved in their interaction with microtubules.

It is unclear, so far, whether movement along and binding of cytoplasmic organelles to microtubules is mediated by the same proteins, namely the microtubule-based motors and their associated proteins. Since removal of kinesin and cytoplasmic dynein from cytosol and the organelle surface had no effect on the cytosol-dependent binding of endocytic carrier vesicles to microtubules in the in vitro binding assay, we concluded that a novel class of proteins, probably unrelated to the microtubule-based motor proteins, is involved in linking endocytic carrier vesicles to microtubules. These proteins may belong to a class of proteins involved in docking of the endocytic carrier vesicles to the microtubules before movement along microtubules occurs, or alternatively, be involved in tethering of these organelles to microtubules when the motor proteins are inactive or once they have reached the appropriate region in the cytoplasm. This interaction of endosomes with microtubules may be dynamic; it could also be regulated by ATP and GTP, since it is inhibited in the presence of these nucleotides. The nucleotides may be required for phosphorylation of factors involved in the interac-
tion of the endocytic carrier vesicles with microtubules and, for example, release the tethering activity and allow or induce the motor proteins to move the endocytic carrier vesicles along microtubules. Proteins mediating endocytic carrier vesicle-microtubule interaction co sediment with taxol microtubules when cytosol has been depleted from ATP. pp170 may be a possible candidate for such an activity, since it is not depleted from taxol-treated cytosol but is enriched in the fraction of microtubule-binding proteins (Rickard and Kreis, 1990). Furthermore, binding of pp170 to microtubules is regulated by phosphorylation. We are currently testing a possible role of pp170 in the binding of endocytic carrier vesicles to microtubules.

The assay system which we have established is simple, relatively rapid, and quantitative. It will be useful for further screening and fractionation of the cytosolic and membraneassociated factors involved in the binding of the endocytic carrier vesicles to microtubules. It may also be used for testing putative inhibitory effects of specific antibodies in this interaction. Thus, this binding assay will be instrumental for identifying and further characterizing the proteins involved in the cytosol dependent interaction of endocytic carrier vesicles with microtubules on a molecular level.

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