### Binding of exocytic vesicles from MDCK cells to microtubules in vitro

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#### Summary

Microtubules have been implicated in the transport of vesicles carrying newly synthesized proteins from the trans-Golgi network (TGN) to the cell surface. We have established a quantitative in vitro binding assay to investigate the putative interaction between these exocytic carrier vesicles and the microtubules at the molecular level. TGN-derived exocytic carrier vesicles, labeled with C<sub>6</sub>NBD-ceramide metabolites or viral glycoproteins, were obtained from polarized filter-grown MDCK II cells by perforation of the apical membrane with a nitrocellulose filter. These exocytic vesicles were incubated with taxol-polymerized tubulin and cytosol, layered on top of a 30% sucrose cushion and subjected to centrifugation. Quantitation of vesicles co-sedimenting with microtubules was done by measuring NBD-fluorescence of viral glycoproteins in the pellet and supernatant fractions. About 25 % of the label sedimented through the cushion in the presence of microtubules and cytosol. Both apically and basolaterally targetted carrier vesicles containing influenza virus HA2 or vesicular stomatitis virus G protein, respectively, associated with the microtubules. Only 2-5% NBD-fluorescence was obtained in the pellet when no cytosol or microtubules were added to the vesicles. Negative-stain electron microscopy of resuspended pellets showed distinct microtubule-vesicle complexes. Heat inactivation or treatment of cytosol with N-ethylmaleimide (NEM), or trypsinization of vesicles inhibited the binding of vesicles to microtubules. Furthermore, coating of microtubules with brain microtubuleassociated proteins abolished binding. These data suggest that NEM-sensitive cytosolic proteins are required for microtubule-vesicle association, and that the vesicles are bound via trypsin-sensitive receptor proteins on their surface.

Key words: epithelial cells, exocytic vesicles, microtubules.

#### Introduction

The plasma membrane of epithelial cells is differentiated into apical and basolateral domains that possess unique protein and lipid compositions (reviewed by Simons and Fuller, 1985). The maintenance of these distinct cell surface domains requires precise targeting and specific delivery of newly synthesized plasma membrane components. Polarized Madin Darby canine kidney (MDCK) cells infected with enveloped viruses have proven to be a useful model system in which to study transport to the two plasma membrane domains. The influenza virus glycoprotein hemagglutinin (HA) is inserted into the apical membrane, whereas the vesicular stomatitis virus glycoprotein (G) is delivered to the basolateral membrane (Rodriguez-Boulan, 1983; Sabatini et al. 1983). The trans-Golgi network (TGN) in MDCK cells appears to be the sorting station where segregation into carrier vesicles of proteins destined for the apical or

Journal of Cell Science 95, 545-553 (1990) Printed in Great Britain @ The Company of Biologists Limited 1990 basolateral membrane occurs (reviewed by Griffiths and Simons, 1986; Wandinger-Ness and Simons, 1990).

Two classes of TGN-derived exocytic carrier vesicles are implicated in the transport of newly synthesized membrane or secretory proteins to the apical or basolateral plasma membrane in epithelial cells (Bennett *et al.* 1988), and recent results show that they have different protein compositions (A. Wandinger-Ness, M. Bennett, C. Antony and K. Simons, unpublished data). Delivery of carrier vesicles to the apical cell surface appears to be mediated by microtubules (Quaroni *et al.* 1979; Rindler *et al.* 1987; Achler *et al.* 1989; Eilers *et al.* 1989; see Salas *et al.* 1986, for a dissenting view). It is still unclear, however, whether microtubules are also involved in the transport of carrier vesicles to the basolateral plasma membrane.

Microtubule-organelle binding assays have been used by Pratt (1986), as well as Mithieux and Rousset (1989) for identifying proteins that may be involved in the formation of microtubule-organelle complexes. We have developed a similar in vitro binding assay for studying the interaction of vesicles with microtubules. We have isolated apically and basolaterally targetted exocytic carrier vesicles from polarized MDCK cells perforated by nitrocellulose filter stripping. Exocytic vesicles are released from the TGN into the cell culture medium when perforated MDCK cells are incubated with ATP, and they can be isolated by sucrose gradient centrifugation (Bennett et al. 1988). The binding of these exocytic vesicles to microtubules has been assaved by co-sedimentation of the vesicles with the microtubules through a sucrose cushion. This microtubule-vesicle binding assay is rapid and sensitive. It allows the quantitation of the interaction between defined classes of vesicles with pure polymerized tubulin.

#### Materials and methods

#### Cell culture

MDCK II cells (Bennett *et al.* 1988), Vero cells, primary chicken embryo fibroblasts (CEF; Allan and Kreis, 1986) and human skin fibroblasts (HSF; Gieselmann *et al.* 1983) were cultured as described. NIH 3T3 cells were grown in DMEM containing 10% fetal calf serum, 100 units  $ml^{-1}$  penicillin and 100 mg ml<sup>-1</sup> streptomycin. HeLa spinner cell cultures were maintained in MEM supplemented with 2 mM glutamine, 10% new-born calf serum, 100 units  $ml^{-1}$  penicillin and 100 mg ml<sup>-1</sup> streptomycin.

#### Isolation of TGN-derived carrier vesicles

The protocols for viral infection, N-6-[7-nitro-2,1,3benzoxadiazol-4-yl]-aminocaproyl-sphingosine (C6NBD-ceramide) labeling and perforation of filter-grown MDCK II cells have been described (Bennett et al. 1988). TGN-derived exocytic carrier vesicles containing C6NBD-ceramide metabolites (C<sub>6</sub>NBD-sphingomyelin and C<sub>6</sub>NBD-glucosylceramide: NBD-labeled vesicles) were collected by centrifugation of the culture medium through 0.3 M sucrose in 10 mM Hepes, 2 mM EGTA, 1 mm dithiothreitol (DTT), pH 7.4, at 100 000 g for 3 h at 4°C. The vesicle pellets were resuspended in 10 mM Hepes, 1 M sucrose, pH 7.4, and either used immediately or frozen in aliquots in liquid nitrogen and stored at -80 °C. This fraction of vesicles was used as the 'exocytic vesicles' in the binding assays, unless indicated otherwise. In some experiments, vesicles were further purified by flotation (flotation-purified vesicles). Briefly, vesicles were resuspended in 1.5 M sucrose, 10 mM Hepes, pH7.4, overlaid with 1.2 M sucrose, 10 mM Hepes, pH 7.4, and subjected to centrifugation in a Beckman TLS 55 rotor at 255 000 g for 3 h at 4°C. Exocytic vesicles were recovered from the top layer.

#### Cytosol

Monolayer cell cultures (MDCK II, Vero, HSF (human skin fibroblast), CEF (chicken embryo fibroblast) and NIH 3T3) were grown on 24.5 cm×24.5 cm plates. Each plate was rinsed twice with phosphate-buffered saline (PBS), and the cells scraped into 15 ml 10 mM Hepes, 250 mM sucrose, pH 7.0 (MDCK II), or into 15 ml PBS (all other cells). The cells were pelleted at 1000 g for 5 min. The MDCK cell pellets were then resuspended in one volume (0.3 ml/plate) of 10 mM Hepes, 250 mM sucrose, pH 7.0, containing 10  $\mu$ g ml<sup>-1</sup> each of chymostatin, leupeptin, antipain and pepstatin, and homogenized by

passing six times through a 22 guage needle. The homogenate was subjected to centrifugation at 2000 g for 10 min to remove nuclei and large cell debris, and then at 100 000 g for 1 h. The resulting supernatant fraction (cytosol) had a protein concentration of  $15-20 \text{ mg ml}^{-1}$ . The other cell pellets were washed once in 100 mm-K-Pipes, 1 mm EGTA, 1 mm MgCl<sub>2</sub>, pH7.2 (PEM), resedimented and then resuspended in 10 volumes of 1 mм EGTA, 1 mм MgCl<sub>2</sub>, 2 mм DTT, 1 mм-phenylmethylsulphonyl fluoride (PMSF), pH 7.0. The cells were swollen for 4 min on ice and centrifuged at 500 g for 15 min. The supernatant was removed and 100 mM K-Pipes, and the following protease inhibitors: 5 mM benzamidine, 5 mg ml<sup>-1</sup> tosylamidophenylethyl chloromethyl ketone (TPCK),  $5 \text{ mg} \text{ ml}^{-1} p$ -tosyl-1-arginine-methyl ester (TAME),  $2 \text{ mg} \text{ ml}^{-1}$  pepstatin,  $1 \text{ mg} \text{ ml}^{-1}$  leupeptin,  $1 \text{ mg} \text{ ml}^{-1}$  chymostatin,  $1 \text{ mg} \text{ ml}^{-1}$  antipain and 5 mg ml<sup>-1</sup> soybean trypsin inhibitor (all final concentrations) were added. The swollen cells were homogenized with a tight-fitting Dounce homogenizer and centrifuged for 10 min at 43 000 g at 4°C. The supernatant was removed and recentrifuged for 90 min at 250 000 g at 4°C to obtain a cytosolic extract (cytosol). The cytosols prepared in this way contained 7-10 mg protein ml<sup>-1</sup>. Cytosol was also prepared from spinner HeLa cells using the same procedure as for the monolayer HeLa cells after harvesting the cells at 1000 g. Spinner HeLa cytosol had a protein concentration of 13-18 mg ml<sup>-1</sup>. Cytosols were divided into aliquots, frozen in liquid nitrogen, and stored at -80°C. Before use, frozen cytosol was rapidly thawed and precleared by centrifugation for 30 min at 250 000 g at 4°C.

#### Microtubules

Phosphocellulose-purified tubulin was prepared from bovine brain (Fig. 3B; and Kreis, 1987) and polymerized with  $20 \,\mu$ M taxol for 20 min at 37 °C. HeLa cell microtubules (Vallee, 1982) were polymerized with  $20 \,\mu$ M taxol for 20 min at 37 °C from once-cycled, salt-extracted microtubules prepared from a HeLa cell high-speed supernatant (Weatherbee *et al.* 1978). HeLa microtubules were frozen in aliquots in liquid nitrogen and stored at -80 °C. Heat-stable microtubule-associated proteins (MAPs) were prepared from twice-cycled bovine brain microtubules (Shelanski *et al.* 1973; Drubin and Kirschner, 1986), chromatographed on a PD10 column (Pharmacia) equilibrated with 50 mM K-Pipes, 50 mM potassium acetate, pH 7.3, and stored at -80 °C.

Microtubules were incubated in some experiments for 15 min in PEM in the presence of 20  $\mu$ M taxol (PEMT) with MAPs or antibody fragments at stoichiometric amounts for 15 min at room temperature. The mixture was then layered over a 30 % sucrose cushion in PEMT and centrifuged for 25 min at 40 000 g at 30 °C. The pellets were carefully resuspended in the appropriate buffer and used as 'MAP-' or 'Fab-coated' microtubules.

#### Organelle-microtubule binding assay

A 100  $\mu$ g sample of microtubules was incubated with NBDlabeled vesicles (obtained from ~0.5×10<sup>6</sup> MDCK II cells) in 50 mM K-Pipes, 50 mM-potassium acetate, 20  $\mu$ M taxol, pH 7.4 (KPAT), in a final volume of 300  $\mu$ l. After 15 min at 20°C the mixture was layered over 380  $\mu$ l of 30% sucrose (in KPAT) and centrifuged at 40 000 g for 25 min at 30°C in an SW50.1 rotor, using adaptors. Parallel experiments without vesicles were done to obtain the baseline fluorescence signals in pellet and supernatant fractions. NBD-fluorescence of supernatant and pellet fractions was measured on an SLM8000 spectrofluorometer (SLM Instruments, Urbana). Binding percentage was calculated after subtraction of baseline fluorescence using the formula Fp/(Fp+Fs), where Fp and Fs denote NBD-fluorescence signals in pellet and supernatant, respectively.

#### Electron microscopy

Microtubule-vesicle complexes were carefully resuspended in  $60 \,\mu$ l PEM containing 5 mM adenyl-imidodiphosphate (AMP-PNP), and  $20 \,\mu$ M taxol. Samples were incubated for 3 min on a glow-discharged Formvar-coated copper/palladium grid. Grids were rinsed once in this buffer, taken through three washes with water, stained for 1 min with 3 % uranyl acetate and air-dried.

#### Antibodies

Monoclonal antibodies against the carboxy terminus of  $\alpha$ tubulin (1A2, 3B6; Kreis, 1987) were raised in mice. Fabfragments were prepared as described (Kreis, 1986). Rabbit polyclonal antibodies against fowl plague virus HA and vesicular stomatitis virus G protein were prepared and affinity purified as described (Matlin *et al.* 1981).

#### Other methods

Pulse-chase labeling of MDCK II cells and immunoprecipitation of viral glycoproteins have been described (Bennett *et al.* 1988). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin or gamma globulin as standards. For gel electrophoresis, samples were either dissolved in sample buffer (5% glycerol, 2.5%  $\beta$ mercaptoethanol, 1.5% sodium dodecyl sulphate, 0.01% Bromophenol Blue, 25 mM Tris-HCl, pH 6.8) or mixed with an equal volume of 2× sample buffer, and resolved on 4% to 15% polyacrylamide gradient gels (Laemmli, 1970). Samples containing HA and G protein were analyzed as described (Bennett *et al.* 1988).

#### Results

#### Labeled exocytic vesicles

Three markers were used for labeling of the exocytic carrier vesicles: the fluorescent lipid analogue C6NBDceramide, influenza virus hemagglutinin (HA) and vesicular stomatitis virus glycoprotein (G). C<sub>6</sub>NBD-ceramide partitions into cellular membranes (Lipsky and Pagano, 1985), and its metabolites (predominantly C<sub>6</sub>NBD-sphingomyelin) accumulate in the TGN at 20°C (van Meer et al. 1987). HA and G also accumulate in the TGN of infected cells at 20°C. They were used as specific membrane protein markers for the apical and basolateral carrier vesicles, respectively. Upon increasing the incubation temperature to 37°C, vesicular membrane traffic between the TGN and the plasma membrane resumed, and exocytic carrier vesicles derived from the TGN, labeled at 20°C with C<sub>6</sub>NBD-ceramide metabolites or viral glycoproteins, could be obtained from perforated filter-grown MDCK II cells (Bennett et al. 1988). An enriched fraction of these exocytic vesicles was used in the binding assays. The three specific labels allowed quantitation of the interaction of exocytic vesicles with microtubules. The use of viral glycoproteins provided markers for differentiation between apical and basolateral carrier vesicles bound to microtubules (Bennett et al. 1988).

## In vitro assay for binding of exocytic carrier vesicles to microtubules

The binding assay that we devised depended on the cytosol-mediated sedimentation of microtubule-bound

vesicles under conditions where free vesicles would not sediment. Three components were used in this *in vitro* assay for analyzing binding of exocytic vesicles to microtubules: (1) cytosol from various cells; (2) labeled exocytic vesicles from polarized MDCK II cells; and (3) taxol-polymerized HeLa cell or bovine brain tubulin (microtubules). These three components were obtained separately (and from different sources), thus allowing analysis of factors mediating association of exocytic vesicles with microtubules. The three components were mixed together and, after incubation, sedimented through a 30% sucrose cushion. The centrifugation conditions chosen were such that microtubules (>85%) and the microtubule-associated exocytic vesicles sedimented through the cushion, but free vesicles remained in the supernatant. Pellet and supernatant fractions were then assayed for NBD-fluorescence, and viral glycoproteins were immunoprecipitated when infected cells were used. The amount of exocytic vesicle marker in the microtubule pellet was taken as a measure for the extent of binding of carrier vesicles to the microtubules.

Less than 2 or 5% of the NBD-labeled exocytic vesicles sedimented through the cushion in the absence of microtubules or cytosol, respectively (Fig. 1A). Addition of either MDCK or HeLa cytosol induced co-sedimentation of labeled exocytic vesicles in a concentration-dependent and saturable way. A plateau for binding was reached at about 20-25% of total vesicle input (about 5-fold stimulation of binding) with cytosol concentrations of  $3-5 \text{ mg ml}^{-1}$  (Fig. 1B). We used HeLa cytosol in all subsequent binding assays at a concentration of  $3 \text{ mg ml}^{-1}$  because it was easier to prepare in quantity than MDCK cytosol. These data suggested that MDCK and HeLa cytosol contain factors that mediate binding of exocytic vesicles to microtubules.

Similar sedimentation efficiencies were obtained with microtubules polymerized with taxol from either HeLa or bovine brain tubulin (data not shown). Bovine brain tubulin was used in all subsequent binding assays for the same reasons as we used HeLa cytosol.

The specificity of cytosol-dependent binding of exocytic vesicles to microtubules was further tested. Cosedimentation of NBD-labeled exocytic vesicles with microtubules was not simply due to entrapment in a meshwork formed by microtubules and cytosol, since less than 4% of the NBD fluorescence was measured in the pellets when the 75% of the initial input vesicles remaining in the supernatant were reincubated with fresh cytosol and microtubules, and resedimented (data not shown). Thus, at least ~25% of the input exocytic vesicles are competent for binding specifically to microtubules.

#### Cytosol from epithelial cells is required for binding

Since the assay system is heterologous in its components (exocytic vesicles from MDCK II cells, cytosol from HeLa cells, microtubules from bovine brain tubulin), we investigated the question of how conserved the cytosolic factors mediating the interaction between microtubules and vesicles were. We compared cytosols from epithelial cells, MDCK and HeLa (epithelial in the sense that they



Fig. 1. Cytosol-dependent binding of NBD-labeled TGN-derived exocytic carrier vesicles to microtubules. A. NBD-labeled exocytic vesicles were isolated from filter-grown MDCK II cells as described in Materials and methods. Exocytic vesicles were incubated with or without microtubules or HeLa cytosol, respectively, and separated by centrifugation through 30% sucrose. NBD-fluorescence was measured in pellet and supernatant fractions as described in Materials and methods. Parallel control experiments were run without vesicles to assess reagent fluorescence. B. Binding of exocytic vesicles to microtubules is dependent on the cytosol concentration. Microtubules and labeled vesicles were incubated with increasing concentrations of HeLa cytosol and microtubule-bound fluorescence determined as described in A. The values indicated represent % of total fluorescence input.

express the typical cytokeratin pattern; see, for example, Franke *et al.* 1981), with cytosols obtained from primary fibroblast cultures of human skin (HSF) and chicken embryos (CEF), and the established fibroblastic cell lines NIH 3T3 and Vero. As shown before, HeLa cytosol could substitute for MDCK II cytosol (Fig. 2). Cytosols from all the fibroblastic cells tested, however, were incapable in mediating binding of vesicles to microtubules (Fig. 2).

## Heat-stable brain microtubule-associated proteins inhibit binding of exocytic vesicles to microtubules

The effects of MAPs and antibodies against tubulin were investigated in an attempt to abolish specifically binding of NBD-labeled exocytic vesicles to microtubules coated with microtubule binding proteins (Fig. 3). Microtubules were incubated with saturating amounts of Fabfragments of a monoclonal antibody against tubulin (1A2-Fabs; Kreis, 1987). 1A2-Fabs bound to tubulin



**Fig. 2.** Epithelial but not fibroblastic cytosol induces binding of carrier vesicles to microtubules. Comparison of the binding of NBD-labeled exocytic vesicles to microtubules in the presence of epithelial (HeLa and MDCK) or fibroblastic (CEF, HSF, NIH-3T3 and Vero) cytosol. The different cytosols were used at a protein concentration of 3 mg ml<sup>-1</sup>. Incubation, centrifugation, and quantitation was done as described in the legend to Fig. 1.

ing of exocytic vesicles to microtubules (Fig. 3A). Heatstable bovine brain MAPs (not present in the HeLa or MDCK cytosol; see also Lewis et al. 1986), however, reduced binding of exocytic vesicles to microtubules to background levels (~7%, Fig. 3A). Densitometric scanning of Coomassie Blue-stained gels (Fig. 3B) revealed that this MAP preparation contained equal amounts of MAP-2 and tau protein. We estimated that one MAP-2 or tau molecule was bound per 15 tubulin dimers. Previous electron-microscopic analysis had shown that MAP-2 binds at regular intervals to microtubules and forms distinct projections off the microtubule wall (Kim et al. 1979). Recently it was reported that MAP-2 also inhibited kinesin-induced gliding of microtubules on glass (von Massow et al. 1989). Thus, MAP-2 might prevent binding of the exocytic carrier vesicles to microtubules by steric hindrance. Alternatively, MAP-2 and tau protein might compete with the cytosolic factors for the same binding domain on the microtubules.

with 1:1 stoichiometry did not significantly reduce bind-

### NEM-sensitive cytosolic proteins stimulate binding of exocytic vesicles to microtubules

Cytosol was NEM- or heat-treated to characterize further the components that mediated association of the NBDlabeled exocytic vesicles with microtubules (Fig. 4). Treatment of cytosol with 1 mM NEM during 15 min on ice, followed by 15 min on ice with 2 mM DTT to quench the NEM, reduced binding of exocytic vesicles to microtubules to background levels. For heat-treatment, cytosol was incubated for 5 min at 70°C. After immediate cooling on ice and removal of particulate material, it was tested in the binding assay. Binding activity in the resulting supernatant fraction was reduced to background levels. Furthermore, when cytosol was replaced by gel-filtered cytosol (all identical concentrations), NBD-labeled exocytic vesicle binding activity remained unchanged (not shown), suggesting that low molecular weight components present in the cytosolic extracts do not signifi-



Fig. 3. Brain microtubule-associated proteins (MAPs) inhibit binding of exocytic vesicles to microtubules. A. Microtubules were coated with stoichiometric amounts of either heat-stable bovine brain MAPs, or Fab fragments of a monoclonal antibody against tubulin (1A2), and separated from unbound protein by centrifugation through 30 % sucrose as described in Materials and methods. The pellets containing coated microtubules were resuspended and incubated with NBDlabeled exocytic vesicle and HeLa cytosol (3 mg ml<sup>-1</sup>). NBDfluorescence in pellets and supernatants was measured and analyzed as described in the legend to Fig. 1. Microtubules coated with MAPs but not with Fab-fragments did not bind exocytic vesicles. B. SDS-polyacrylamide gel electrophoresis of taxol-polymerized purified bovine brain tubulin (the microtubules used in the binding assays), boiled bovine brain MAPs, and microtubules coated with boiled MAPs. The molecular weight standards  $(\times 10^{-3})$  indicated are (from top to bottom): 200, 116.2, 92.5, 66.2 and 45.



Fig. 4. Binding of NBD-labeled exocytic vesicles to microtubules depends on NEM-sensitive cytosolic proteins. HeLa cytosol was treated with 1 mm NEM for 15 min on ice or heat-treated as described in Materials and methods before it was used in the binding assay. The binding assay was then performed and analyzed as described in the legend to Fig. 1.

cantly contribute to binding. These data suggest that NEM-sensitive cytosolic protein(s) mediate(s) binding of exocytic carrier vesicles to microtubules.

#### Binding of exocytic vesicles to microtubules is mediated by trypsin-sensitive membrane proteins

NBD-labeled exocytic vesicle fractions were pre-incubated with 1 µg TPCK-treated trypsin per ml of cytosol to test whether membrane-associated receptor proteins are required for cytosol-dependent binding of exocytic vesicles to microtubules. After 15 min at 37°C,  $10 \,\mu g \, ml^{-1}$  soybean trypsin inhibitor was added and the incubation continued for 15 min on ice. It has been shown previously that this trypsinization procedure does not affect the functional integrity of the TGN-derived carrier vesicles (Bennett et al. 1988). Trypsin treatment of the exocytic vesicle fraction reduced their binding to microtubules to background (Fig. 5). Cytosol could not restore binding, and no increase in binding was observed in the absence of cytosol. Furthermore, NEM treatment of exocytic vesicles did not reduce their binding to microtubules. These data suggest involvement of vesicular protein(s) in exocytic carrier vesicle-microtubule interaction. This is in agreement with earlier findings where it has been shown that movement of squid organelles along microtubules in vitro may be inhibited by treatment of the organelles with trypsin (Gilbert and Sloboda, 1984; Schroer et al. 1985; Vale et al. 1985a).

### Binding of exocytic vesicles to microtubules does not depend on nucleotides

NBD-labeled vesicles were incubated with either cytosol depleted of ATP, cytosol supplemented with 1 mM ATP and an ATP-regenerating system (10 mM creatine phosphate,  $80 \,\mu g \, ml^{-1}$  creatine kinase), or cytosol containing 1 mM GTP, to analyze the nucleotide requirements for their binding to microtubules (Fig. 6). ATP was depleted from the cytosol by preincubation for 4 min at room temperature with 30 units ml<sup>-1</sup> of hexokinase and 6 mM-

Exocytic vesicle binding to microtubules 549



Fig. 5. Binding of NBD-labeled exocytic vesicles to microtubules depends on trypsin-sensitive vesicle-associated proteins. Exocytic vesicles were digested for 15 min with  $1 \,\mu g \, ml^{-1}$  TPCK-treated trypsin at 37 °C. Protease activity was then blocked with soybean trypsin inhibitor ( $10 \,\mu g \, ml^{-1}$ ). NEM treatment of vesicles was done as described in the legend to Fig. 4. The binding assay with treated exocytic vesicles was performed and analyzed as described in the legend to Fig. 1.



**Fig. 6.** Effect of nucleotides on the binding of NBD-labeled exocytic vesicles to microtubules. Cytosol was depleted from ATP by preincubation during 4 min with 30 units ml<sup>-1</sup> of hexokinase and 6 mM D-glucose (-ATP) before addition of microtubules and NBD-labeled exocytic vesicles. Cytosol was supplemented with ATP (+ATP) by addition of 1 mM ATP and an ATP-regenerating system (10 mM creatine phosphate and 80  $\mu$ g ml<sup>-1</sup> creatine kinase). GTP was added to the cytosol at a concentration of 1 mM (+GTP). The binding assay was then performed and analyzed as described in the legend to Fig. 1.

D-glucose before microtubules and exocytic vesicles were added. Binding of exocytic vesicles to microtubules was independent of ATP or GTP (Fig. 6). Clearly, however, further experiments will be necessary to determine whether the microtubule-based motor proteins kinesin and cytoplasmic dynein are involved in the binding of exocytic vesicles to microtubules *in vitro*.



Fig. 7. Floatation-purified NBD-labeled apical and basolateral exocytic vesicles carrying the viral glycoproteins HA2 or G, respectively, bind to microtubules. Triple-labeled vesicles were prepared as described in Materials and methods and incubated with microtubules in the presence or absence of MDCK cytosol  $(3 \text{ mg ml}^{-1})$ . NBD-fluorescence was measured in the pellet and supernatant fractions. The viral glycoproteins were immunoprecipitated from the same samples and quantitated. The values indicate % of total input.

## Exocytic vesicles en route to the apical and basolateral plasma membrane bind to microtubules

NBD-labeled exocytic vesicles containing viral glycoproteins (HA or G) were prepared as described previously (Bennett et al. 1988). Both HA2- or G-carrying exocytic vesicles co-sediment with microtubules, and MDCK (Fig. 7) as well as HeLa (data not shown) cytosol induce association of these carrier vesicles with microtubules. Co-sedimentation of exocytic vesicles carrying viral glycoproteins with microtubules was similar to NBD-labeled exocytic vesicles. The amounts of viral glycoproteins present in supernatant and pellet fractions were determined by immunoprecipitation with specific antibodies (Fig. 7). The cleaved form of HA (HA2) was quantitated, since it represents a late transport marker (Bennett et al. 1988). NBD-fluorescence was also quantitated in these same fractions. The amounts of both viral markers in the microtubule-exocytic vesicle pellets were three- to fivefold above background (i.e. no cytosol added) and consistently lower than the amount of NBDlabeled exocytic vesicles that was bound. Thus, association of exocytic vesicles with microtubules was observed independent of the marker used to label the exocytic pathway, and both apically and basolaterally targetted vesicles bound to microtubules.

### Electron-microscopic analysis of microtubule-vesicle complexes

Resuspended microtubule-vesicle pellets were analyzed by negative-stain electron microscopy (Fig. 8). Single microtubules with apparently tightly attached vesicles could be detected. At higher magnification (Fig. 8B-D) it is clear that the bound vesicles are membrane-delineated and very closely associated to the microtubules. The tightness of the microtubule-vesicle interaction may be appreciated by the apparent flattening of the region of



Fig. 8. Electron-microscopical analysis of microtubule-exocytic vesicle complexes. Microtubule-vesicle pellets were processed for negative-stain electron microscopy as described in Materials and methods. Micrograph A shows an overview (microtubule-vesicle complexes are indicated by arrows; bar,  $1 \mu m$ ) and micrographs B-C show higher magnifications of microtubule-vesicle complexes (bar, 100 nm). The microtubule-vesicle complex shown in D is the lower one shown in A. Microtubule-bound vesicles appear flattened at the site of interaction with the microtubules.

the vesicular membrane in contact with the filament. The diameter of the microtubule-bound vesicles was about 80-150 nm, a size that may be expected for this class of vesicle (C. Antony, unpublished observation). None of these membrane structures was seen in control experiments in which microtubules were sedimented either with NBD-labeled exocytic vesicles or cytosol alone.

#### Discussion

We have established an *in vitro* system for assaying cytosol-dependent binding of TGN-derived exocytic vesicles to microtubules. The obvious advantage of such an *in vitro* assay is the better definition of the components used and the ease of experimental manipulation. Binding of exocytic carrier vesicles to microtubules was assayed by co-sedimentation of the vesicles with microtubules through a sucrose cushion under conditions where the vesicles alone do not sediment. The validity of the assay and the specificity of the interaction of the carrier vesicles with microtubules has been controled by several independent criteria. (1) Interaction of vesicles with microtubules is cytosol-dependent and the 'binding' activity in the cytosol is saturable. (2) Co-sedimentation of vesicles with microtubules cannot be attributed to random entrapment of membranous organelles in the microtubule network, since virtually no vesicles competent for binding to microtubules remained in the supernatant after the first round of incubation. (3) Coating of microtubules with MAPs abolished the binding of vesicles. (4) Treatment of the cytosol with NEM or trypsinization of the vesicles abolished microtubulevesicle interaction. Thus, microtubules, vesicles and cytosol can all be inactivated independently to abolish microtubule-vesicle complex formation.

The labeled TGN-derived exocytic carrier vesicles that were used in the binding assay have been characterized previously (Bennett et al. 1988). The exocytic vesicles were obtained by perforation (no homogenization) of MDCK cells and subsequent addition of ATP to enhance release of exocytic vesicles from the TGN. Thus, the vesicles were isolated under extremely gentle conditions. This may have been critical with respect to the preservation of their functional properties (e.g. their binding to microtubules). The exocytic vesicle fraction contains about 2-10% contamination from Golgi elements (Bennett et al. 1988). These low amounts of (NBD-labeled) Golgi membrane contaminants cannot, however, account for the fluorescence signals obtained by co-sedimentation with microtubules in our assays (20-30% of the total input). We conclude therefore, that the amount of microtubule-associated fluorescence corresponds mostly to the exocytic carrier vesicles. This was corroborated by electron microscopy of the vesicle-microtubule pellets.

We have observed that maximally 20-30% of the marker in the exocytic vesicle fraction co-sediments with microtubules in the presence of cytosol. Several factors may contribute to these binding efficiencies. It is likely that not all of the total input label is associated with the exocytic carrier vesicles (see above). It is also conceivable that some of the vesicles have lost essential components of the microtubule-vesicle linker complex during the process of their isolation and in the binding assay. Furthermore, binding may be regulated by the presence of releasing and promoting factors. Mithieux and Rousset (1989) have identified a membrane protein that may mediate stable association of lysosomes with microtubules. For this study they used an in vitro assay for binding of brain microtubules (containing all microtubule-associated proteins) to purified thyroid lysosomes (Mithieux et al. 1988). This protein-mediated interaction of lysosomes with microtubules is regulated by Mg<sup>2+</sup> and ATP (Mithieux and Rousset, 1988). Analogous factors may be involved in stabilizing TGN-derived exocytic vesicle binding to microtubules. In contrast to the purified thyroid lysosomes, however, ATP and GTP did not significantly effect the association of MDCK cellderived exocytic carrier vesicles with microtubules.

Previous studies with microtubule-depolymerizing drugs have suggested that microtubules are involved in vesicular transport of newly synthesized proteins to the apical plasma membrane in polarized epithelial cells (Quaroni et al. 1979; Rindler et al. 1987; Achler et al. 1989; Eilers et al. 1989). It is unclear, however, whether microtubules also mediated transport of vesicles to the basolateral plasma membrane. The exocytic vesicles obtained from virus-infected, NBD-labeled MDCK cells consist of the two populations of vesicular carriers involved in transport to both the apical and basolateral domains (Bennett et al. 1988). Our data suggest that both apical and basolateral carrier vesicles bind to similar extents to microtubules. This result suggests that microtubules are also involved in the transport of carrier vesicles to the basolateral plasma membrane. This observation is in contrast with previous data obtained in MDCK (Salas et al. 1986; Rindler et al. 1987) and Caco-2 (Eilers et al. 1989) cells in vivo, where transport of membrane components to the basolateral domain remained unaffected by microtubule-specific drug treatment of the cells. The conclusions of these latter studies obviously relied on the assumption that all microtubules had been depolymerized by the nocodazole or by the other pharmacological ag ...ts. In fact, Eilers et al. (1989) reported that they were not able to disrupt the microtubules completely with nocodazole in Caco-2 cells. It is also very difficult to induce complete microtubule depolymerization with nocodazole in filter-grown MDCK cells (our own unpublished data). Thus, it cannot be excluded that stabilized microtubules may be involved in facilitating transport of basolaterally targetted vesicles. Our data are in favour of a mechanism by which microtubules are involved in transport of both classes of vesicles.

It should be pointed out that the organization of the microtubules in polarized MDCK cells is quite different from that in fibroblasts. Most microtubules are longitudinally arranged, with their minus ends apical and their plus ends basal (Bacallao et al. 1989). The centrosomes are close to the apical membrane and have lost their microtubule-nucleating capacity (Bré et al. 1987). This arrangement of the microtubules in MDCK cells poses interesting possibilities for the movement of the two classes of carrier vesicles to the polarized plasma membrane. One intriguing possibility is that apical and basolateral carrier vesicles use motors of opposite polarities to move from the TGN to their respective destinations. Kinesin, a microtubule plus end-directed motor (Vale et al. 1985b; Porter et al. 1987), might associate with the basal carrier vesicles, and cytoplasmic dynein, a microtubule minus end-directed motor (Paschal et al. 1987; Schroer et al. 1989), with the apical vesicles. Carrier vesicles with cargo to the basolateral plasma membrane would therefore resemble the exocytic vesicles in fibroblasts, whereas the apical vesicles would exhibit features distinct from the fibroblastic carrier vesicles. Further work with in vitro reconstituted exocytic carrier vesicle movement along microtubules will be necessary to elucidate the nature of the molecular motors associated with apical and basal carrier vesicles.

In conclusion, we have developed a quantitative assay to investigate binding of defined classes of vesicles to microtubules. Both NEM-sensitive cytosolic factors and trypsin-sensitive vesicle-associated proteins are involved in the binding of TGN-derived exocytic vesicles to microtubules. It is not clear yet whether this binding occurs via microtubule-based motor proteins, or whether it reflects tethering of the exocytic vesicles to microtubules via other proteins when the motors are not working. With this binding assay it should now be possible to identify and characterize the proteins involved in this interaction. The molecular analysis of these proteins will help to understand better the role of microtubules in the targeting and transport of exocytic carrier vesicles to the polarized surface of epithelial cells.

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Exocytic vesicle binding to microtubules 553