

A Microtubule-binding Protein Associated with Membranes of the Golgi Apparatus

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Abstract. A monoclonal antibody (M3A5), raised against microtubule-associated protein 2 (MAP-2), recognized an antigen associated with the Golgi complex in a variety of non-neuronal tissue culture cells. In double immunofluorescence studies M3A5 staining was very similar to that of specific Golgi markers, even after disruption of the Golgi apparatus organization with monensin or nocodazole. M3A5 recognized one band of $M_r \sim 110,000$ in immunoblots of culture cell extracts; this protein, designated 110K, was enriched in Golgi stack fractions prepared from rat liver. The 110K protein has been shown to partition into the aqueous phase by Triton X-114 extraction of a Golgi-enriched fraction and was eluted after pH 11.0 carbonate wash-

ing. It is therefore likely to be a peripheral membrane protein. Proteinase K treatment of an isolated Golgi stack fraction resulted in complete digestion of the 110K protein, both in the presence and absence of Triton X-100. As the 110K protein is accessible to protease in intact vesicles *in vitro*, it is presumably located on the cytoplasmic face of the Golgi membrane *in vivo*. The 110K protein was able to interact specifically with taxol-polymerized microtubules *in vitro*. These results suggest that the 110K protein may serve to link the Golgi apparatus to the microtubule network and so may belong to a novel class of proteins: the microtubule-binding proteins.

THE Golgi apparatus is the organelle which is the processing and sorting center for proteins en route to the plasma membrane or lysosomes, and also for secretory proteins (for review see Farquhar and Palade, 1981). The Golgi apparatus consists of well-ordered stacks of membrane cisternae which are usually found in the perinuclear region of the cell. Although many of the enzymes involved in the posttranslational modification of proteins passing through the Golgi region are characterized, no proteins have yet been identified that have a role in maintaining Golgi apparatus structure or position within the cell.

There is considerable evidence that the perinuclear location of the Golgi apparatus relies on the presence of the cytoplasmic microtubule network. During mitosis, for example, the cytoplasmic microtubules are depolymerized and the Golgi apparatus becomes scattered and fragmented (e.g., Robbins and Gonatas, 1964; Burke et al., 1982). Drugs which induce depolymerization of microtubules cause similar disruption of the Golgi apparatus' perinuclear organization (Robbins and Gonatas, 1964; Moskalewski et al., 1975; Reaven and Reaven, 1980; Rogalski and Singer, 1984). Microinjection of anti-tubulin antibodies in sufficient quantities to disorganize the microtubule network also results in scattering of the Golgi complex throughout the cytoplasm (Wehland and Willingham, 1983; Wehland et al., 1983).

Consistent with microtubules playing a part in maintaining the position of the Golgi apparatus within the cell, the

microtubule-organizing center (MTOC)¹ has often been observed in the same perinuclear region by immunofluorescence studies (Kupfer et al., 1982). Electron microscopic data have shown that the MTOC is frequently found in the vicinity of the *trans* Golgi cisternae (Robbins and Gonatas, 1964; Farquhar and Palade, 1981). In addition, Tassin et al. (1985) observed that during myogenesis the microtubule-initiating sites reorganized to the nuclear membrane and also that the Golgi apparatus redistributed to surround the nuclei completely. It has also been shown in motile cells, such as fibroblasts or macrophages, that mobility results in a relocation of both the MTOC and the Golgi apparatus to the side of the nucleus nearest to the cell's leading edge (Couchman and Rees, 1982; Kupfer et al., 1982; Nemere et al., 1985). This polarization of the Golgi apparatus and the cytoskeleton in the motile cell may direct the transport of new membrane material from the Golgi apparatus to the leading edge of the cell (Bergmann et al., 1983). There is conflicting evidence, however, regarding the importance of the microtubule network in directing polarized transport of plasma membrane components from the Golgi apparatus to the plasma membrane (compare Rogalski et al., 1984, with Salas et al., 1986).

Microtubule-associated proteins (MAPs) are proteins that

1. *Abbreviations used in this paper:* CEF, chicken embryo fibroblast; MAP, microtubule-associated protein; MTOC, microtubule-organizing center; PB, polymerizing buffer; VSV-G, vesicular stomatitis virus glycoprotein.

co-polymerize with tubulin and are found associated with microtubules *in vivo*. As some MAPs project from the microtubule surface, it has been suggested that they are involved in linking organelles to microtubules or in cross-linking microtubules to neurofilaments (for reviews see Vallee, 1984; Vallee and Bloom, 1984; Wiche, 1985). Even though there is strong evidence to suggest the linkage of the Golgi complex to the microtubule network, including the MTOC, nothing is known about the protein(s) which are involved in this interaction. Likewise, it is not understood how Golgi cisternae are held together in a stack. The proteins performing such functions must obviously be accessible to the cytoplasm. Until now, only two cytoplasmically oriented Golgi membrane proteins have been identified (Chicheportiche et al., 1984) and their functions are unknown. In this paper, we describe for the first time a protein of M_r 110,000, designated 110K, which is associated with the cytoplasmic face of the Golgi complex in tissue culture cells. This 110K protein is of particular interest since it is immunologically related to MAP-2. Like MAP-2, it interacts with taxol-polymerized microtubules *in vitro*. It is therefore a candidate for linking the Golgi apparatus to the microtubule network.

Materials and Methods

Cell Culture

Vero cells (African green monkey kidney cells, ATCC CCL 81) were maintained in MEM containing 5% FCS, 1% non-essential amino acids, and 1% L-glutamine. Madin-Darby canine kidney (MDCK) type II cells were grown in MEM containing 10% FCS, 10 mM HEPES, and 1% glutamine. PtK₂ cells (Potoroo kidney epithelial cells, ATCC CCL 56) were grown in MEM containing 10% FCS, 1% non-essential amino acids, and 1% L-glutamine. All cells were maintained in humidified CO₂ incubators (7% CO₂, 93% air) at 37°C (or 31°C or 39.5°C where stated).

Chicken embryo fibroblasts (CEFs) were prepared from 11-d-old embryos and passaged twice before infection with the temperature-sensitive vesicular stomatitis virus (VSV) mutant, ts-045. CEF cells were grown on 20-mm² glass coverslips and then infected with 2×10^7 plaque-forming units of ts-045 VSV as described by Kreis (1986). 3.5 h after infection at 39.5°C (nonpermissive temperature), the infected cells were shifted to 31°C (permissive temperature) for 30 min and then used for immunofluorescence as described below. Chicken neuronal cells were prepared from 8-d-old chicken embryos as described by Pettmann et al. (1979).

To test the effects of two drugs on the Golgi apparatus, 10 μ M monensin (Calbiochem-Behring Corp., La Jolla, CA) or 10 μ M nocodazole (Sigma Chemical Co., Taufkirchen, FRG) was added to the culture medium for 15 or 45 min, respectively, before processing the cells for immunofluorescence.

Monoclonal Antibody Production

Phosphocellulose chromatographically purified MAPs were prepared from goose brains using the method of Fellous et al. (1977). BALB/c mice were immunized with 200 μ g MAPs in Freund's complete adjuvant intraperitoneally and intradermally and then boosted 3 wk later intraperitoneally with 100 μ g protein in Freund's complete adjuvant. A further intraperitoneal booster injection of 100 μ g protein in PBS was given 7 wk later, with a final intravenous boost of 100 μ g protein in PBS, given the next day. Spleens were removed from two immunized mice 3 d later, and the spleen cells were fused with P3X63-Ag8.653 myeloma cells as described by Köhler and Milstein (1975). Positive clones were identified by immunofluorescence screening on PtK₂ cells. Briefly, PtK₂ cells were grown on 25 \times 60-mm No. 1 glass coverslips and fixed with methanol and acetone at -20°C, as described below, and air dried. A grid of 24 drops of test culture supernatants was placed on parafilm and the coverslip was inverted onto the drops, using spacers to limit the spread of the supernatants. The location of the supernatants were marked on the coverslip with waterproof ink. Indirect immunofluorescence staining was performed as described below, and the coverslips were mounted on glass slides using Mowiol (E. O. Thomas Chemikalien, Heidel-

berg, FRG). The pen marks were then copied onto the slide. Pen marks on the coverslips were removed before observation under an oil immersion objective, as the ink was highly fluorescent. Positive clones were subcloned twice by the method of limiting dilution. The monoclonal antibody, M3A5, was shown to be a member of the IgG₁ subclass by double immunodiffusion (Ouchterlony and Nilsson, 1978) and by dot blot analysis (data not shown).

Indirect Immunofluorescence

Cells grown on glass coverslips were fixed by immersion in methanol at -20°C for 10 min followed by 1 min in acetone at -20°C. Fixed cells were incubated with M3A5 ascites fluid either alone or with anti-galactosyltransferase or anti-VSV-G for double staining. Rabbit anti-galactosyltransferase (Roth and Berger, 1982) and anti-VSV-G antibodies were kind gifts from E. Berger (University of Bern, Switzerland) and K. Simons (European Molecular Biology Laboratory, Heidelberg, FRG), respectively. A rat monoclonal anti-tubulin antibody YL1/2 (Kilmartin et al., 1982) was a kind gift from J. Kilmartin (Medical Research Council, Cambridge, UK). Goat anti-mouse IgG and sheep anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) were coupled with rhodamine or fluorescein (Brandtzaeg, 1973) and used as second antibodies. Immunofluorescence microscopy was performed using a Zeiss photomicroscope III fitted with a planapo 63 \times , 1.4 oil immersion objective.

Immunoblotting and Immunoprecipitation

Reduced proteins were separated by SDS PAGE (Laemmli, 1970) on 4-12% or 4-15% linear acrylamide gradients, and then transferred at 30V overnight onto nitrocellulose filters (Schleicher & Schüll Co., Dassel, FRG) as described by Burnette (1981). The filters were incubated in PBS containing 10% newborn calf serum, 0.2% Triton X-100, and M3A5 ascites diluted up to 1:8,000 for 60 min at room temperature. The filters were then incubated with peroxidase-conjugated anti-mouse IgG (Sigma Chemical Co.) or alkaline phosphatase-conjugated anti-mouse IgG (Jackson Immunoresearch Laboratories Inc., Avondale, PA) for 60 min in the same buffer. The labeled bands were visualized using diaminobenzidine as substrate for the peroxidase reaction or a mixture of nitroblue tetrazolium and 5-bromo, 4-chloro, 3-indolyl phosphate (Sigma Chemical Co.) for the alkaline phosphatase reaction.

Confluent monolayers of Vero cells were labeled with 50-100 μ Ci/ml [³⁵S]methionine (Amersham-Buchler GmbH & Co. KG, Braunschweig, FRG) for 20 h in MEM lacking methionine, supplemented with 5% undialyzed FCS. 6×10^7 cells were washed with ice-cold PBS and then scraped in 1 ml ice-cold PBS. The cells were pelleted at 800 g_{av} for 30 s and resuspended in 1.5 ml 50 mM Tris, 100 mM NaCl, 0.4% (wt/vol) SDS, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.5, and sonicated until the DNA was fragmented (15-60 s). The samples were adjusted to 2% (vol/vol) Triton X-100 and 1 mg/ml leupeptin, aprotinin, antipain, and pepstatin (Sigma Chemical Co.), and then preabsorbed with non-immune mouse IgG plus 200 μ l of 1:1 slurry of protein A-sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) in washing buffer (50 mM Tris, 100 mM NaCl, 0.1% (wt/vol) SDS, 0.5% (vol/vol) Triton X-100, pH 7.5) for 30 min at room temperature. The beads were pelleted at 4,300 g_{av} for 15 min at room temperature and the supernatants were incubated with rocking at 4°C overnight with either 20 μ l M3A5 ascites or 10 μ l anti-tubulin (YL1/2) or 66 μ l mouse non-immune IgG. 100 μ l protein A-sepharose in a 1:1 slurry with washing buffer was added per 10 μ l antibody, and incubated for 60 min with rocking at 4°C. The beads were pelleted by centrifugation at 4,300 g_{av} for 30 s, allowed to stand for 60 min at 4°C in washing buffer, then rinsed three times with washing buffer at 4°C, and finally washed with 50 mM Tris, pH 7.5. The beads were boiled in 100 μ l gel sample buffer to release bound antigen and analyzed by SDS PAGE on 4-15% linear acrylamide gradients. Fluorography with sodium salicylate as fluor was performed as described (Chamberlain, 1979) using preflashed Kodak X-Omat AR5 film (Laskey and Mills, 1975).

Membrane Extraction and Washing Procedures

A rat liver fraction enriched in Golgi stacks (prepared according to Leelavathi et al., 1970) and Vero cells were extracted with Triton X-114 as described by Bordier (1981). The detergent fractions and samples of the initial extract were taken up directly in gel sample buffer; aqueous fractions were first concentrated by precipitation with 10% (wt/vol) TCA. A sample of the Golgi fraction was diluted 30-fold with 100 mM potassium phosphate buffer, pH 6.7, to lower the sucrose concentration and then centrifuged in a Ti45

rotor fitted with adaptors for 1.5-ml microfuge tubes for 2 h at 40,000 rpm, 4°C. The pellet and the TCA-precipitated supernatant were dissolved in gel sample buffer. The Golgi fraction was also washed with 100 mM sodium carbonate, pH 11.0, as described by Fujiki et al. (1982). After centrifugation at 100,000 g_{av} in a Beckman Airfuge at 4°C for 1.5 h, the carbonate-insoluble material was dissolved directly in gel sample buffer while carbonate-soluble material was dissolved after TCA-precipitation. All samples were analyzed by immunoblotting.

Protease Treatment of Golgi Fractions

Golgi membranes were treated with proteinase K (E. Merck, Darmstadt, FRG) using a modification of the method described by Fleischer (1981). Golgi membranes (230 μ g protein in 21 μ l of 0.6 M sucrose, 5 mM MgCl₂, 100 mM KH₂PO₄/K₂HPO₄, pH 6.7) were adjusted to a final concentration of 25 mM Hepes, 25 mM KCl, 2.5 mM Mg(CH₃COO)₂, pH 7.9 (final volume of 50 μ l) and were incubated for 30 min at 37°C in the presence of 0, 1, 5, or 100 μ g/ml proteinase K either with or without 0.4% (vol/vol) Triton X-100. Digestion was halted by placing on ice and addition of PMSF to a final concentration of 4 mM. After digestion, two 7.5- μ l aliquots were assayed for galactosyltransferase activity according to the method of Bretz et al. (1980), with ovalbumin as acceptor. The remaining digested samples were resuspended in gel sample buffer and analyzed on a 4–15% linear acrylamide gradient by SDS PAGE followed by immunoblotting, using M3A5 ascites fluid.

Binding of the 110K Protein to Taxol-stabilized Microtubules

A Triton X-114 extract was prepared from five confluent 245 × 245-mm dishes of CEFs according to the method of Bordier (1981), except that the detergent extraction buffer contained microtubule-polymerizing buffer (PB; 100 mM K-Pipes, pH 6.8, 1 mM MgCl₂, 2 mM EGTA, 0.1 mM GTP). The aqueous fraction (13 ml) was removed and clarified an additional two times by warming to 30°C followed by centrifugation at 300 g. The aqueous supernatant was then centrifuged at 105,000 g_{av} in a Ti75 rotor for 30 min at 4°C to remove aggregated material.

Endogenous microtubules were polymerized from 10 ml of the aqueous fraction in the presence of 1 mM GTP and 10 μ M taxol (supplied by Dr. M. Suffness, Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) by incubation at 37°C for 30 min.

Exogenous taxol-stabilized microtubules were prepared from 2.3 mg phosphocellulose chromatography-purified calf brain tubulin (Fellous et al., 1977) as described by Schiff et al. (1979). The calf brain microtubules were pelleted at 20 psi in a Beckman airfuge for 20 min at 25°C, and then rinsed once with PB. The microtubule pellet was resuspended in PB and incubated with 3 ml of the CEF Triton X-114 aqueous fraction for 20 min at 25°C. Both aqueous fractions, containing endogenously or exogenously polymerized microtubules, were layered over a 4-M glycerol cushion (in PB) at 25°C and then centrifuged in a Ti70 rotor at 100,000 g_{av} for 20 min at 25°C. The microtubule-containing pellets were resuspended directly in gel sample buffer. Both supernatants were concentrated by precipitation with 10% (wt/vol) TCA. This concentration of TCA also precipitated the K-Pipes, giving rise to very large pellets. These were resuspended in sample buffer, and in order to dissolve the pellets the bromophenol blue indicator was titrated from yellow to blue with 2 M NaOH. The two supernatant gel samples contained ~220 mM K-Pipes, so the pellet samples were also brought to 220 mM K-Pipes before SDS PAGE. The supernatants and pellets were then analyzed by immunoblotting.

Results

Immunoblotting and Immunoprecipitation of Cell Extracts with M3A5

Microtubule protein prepared from goose brain (the source of the original immunogen) and bovine brain were analyzed by immunoblotting with M3A5. M3A5 detected a high molecular weight doublet in both microtubule protein preparations (Fig. 1, lanes *a* and *e*). The same doublet was stained by a polyclonal anti-MAP-2 antibody (a generous gift from G. Scapigliati, Sclavo Institute, Siena, Italy; data not shown).

Various non-neuronal cells (Vero, MDCK II, and CEF) were extracted directly into hot sample buffer and then analyzed by immunoblotting. In these extracts M3A5 recognized only a single band of ~110,000 M_r (Fig. 1, lanes *b–d*). Neither M3A5 nor the polyclonal anti-MAP-2 antibodies detected proteins in the 270,000–300,000 M_r MAP-2 region of the gel, suggesting that MAP-2 was either present at concen-

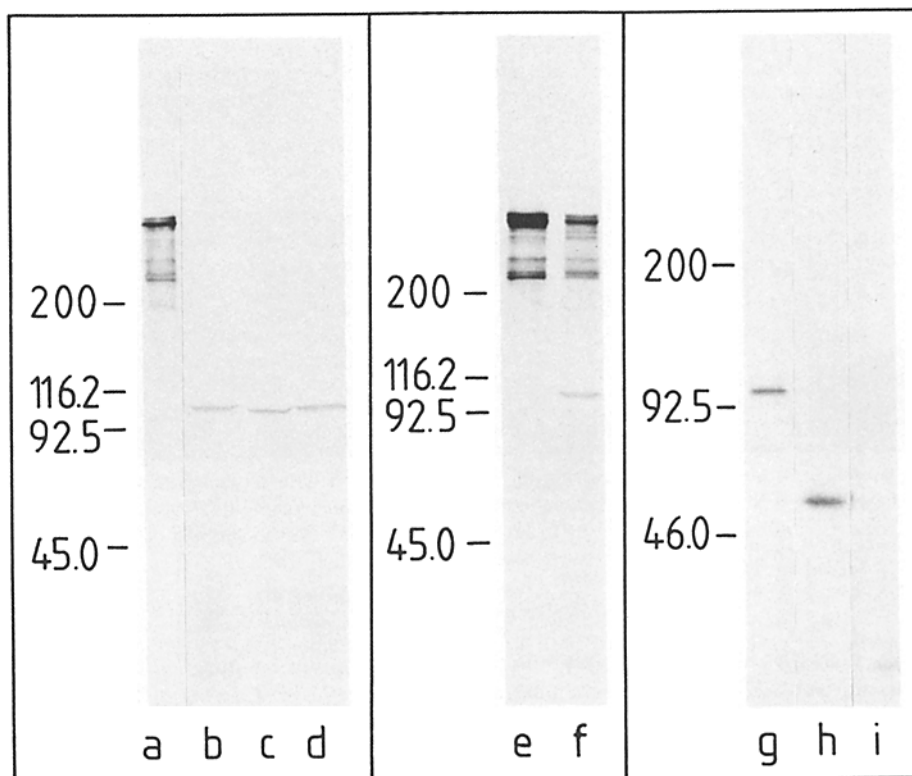


Figure 1. Identification of the M3A5 antigens by immunoblotting and immunoprecipitation. Bovine brain (lane *a*) and goose brain (lane *e*) microtubule proteins were separated by SDS PAGE and then immunoblotted along with Vero (lane *b*), MDCK II (lane *c*), and CEF cells (lane *d*) extracted directly into hot sample buffer. Vero cells were also extracted in the presence of goose brain microtubule protein (lane *f*). The bands below the MAP-2 doublet in lanes *a*, *e*, and *f* are MAP-2 degradation products. Linear acrylamide gradients of 4–12% (lanes *a–d*) and 4–15% (lanes *e* and *f*) were used. Lanes *g–i* show immunoprecipitation of [³⁵S]methionine-labeled Vero cells with M3A5 (lane *g*), anti-tubulin (lane *h*), and preimmune mouse IgG (lane *i*). 10,000 cpm were loaded per lane on a 4–12% gel.

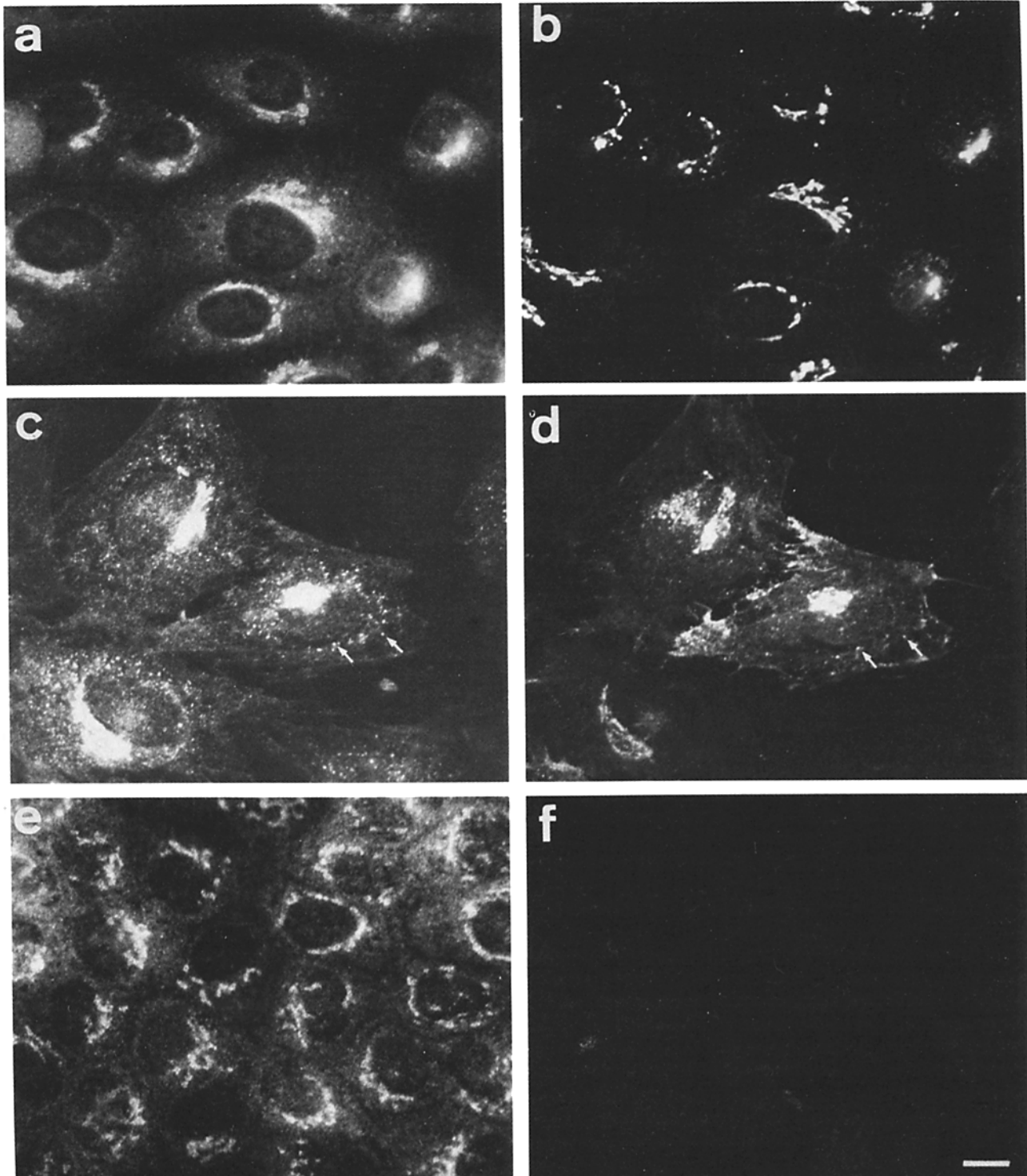


Figure 2. M3A5 stains the Golgi apparatus in a variety of tissue culture cell types. Vero cells were double stained with M3A5 (*a*) and anti-galactosyl transferase (*b*). CEF cells infected with ts045 VSV and shifted to permissive temperature for 30 min were double stained with M3A5 (*c*) and anti-VSV-G (*d*). MDCK cells (*e*) and Vero cells (*f*) were stained with M3A5 alone. M3A5 staining of the Golgi was abolished after preabsorption of M3A5 with 7.5 μ g of boiled MAPs per μ l of ascites fluid (*f*). Bar, 10 μ m.

trations too low to detect in these cell types, or that M3A5 does not recognize the non-neuronal form of MAP-2. To try to ensure that the 110K protein observed in non-neuronal cell extracts was not a degradation product of MAP-2, formed despite the rapid extraction procedure, 235 μ g of goose brain

microtubule protein was combined with 4×10^6 Vero cells and then extracted as before. MAP-2 was not degraded during the extraction (Fig. 1, lane *f*) and so it seems likely that the 110K protein is not an artefactual MAP-2 degradation product. It remains possible, however, that endogenous Vero

MAP-2, if present, may have a different sensitivity to proteolytic enzymes compared to goose brain MAP-2. M3A5 did not bind to the 110K protein on immunoblots of Vero cell extract after preincubation of the antibody with a boiled bovine microtubule protein fraction which was enriched with MAP-2 (Fellous et al., 1977; data not shown). We conclude, therefore, that MAP-2 and the 110K protein share an antigenic determinant.

M3A5 immunoprecipitated a protein of $\sim 110,000 M_r$ (Fig. 1, lane g) from Vero cells labeled with [35 S]methionine for 20 h. As a positive control tubulin was immunoprecipitated with YL1/2 (Fig. 1, lane h), a rat monoclonal anti-tubulin antibody (Kilmartin et al., 1982). M3A5 immunoprecipitated the 110K protein from non-neuronal cell extracts and MAP-2 from a bovine brain microtubule protein mixture in either the presence or absence of SDS (data not shown).

Distribution of the 110K Protein in Tissue Culture Cells

M3A5 stained a reticular structure in the perinuclear area of the following non-neuronal cells of various species and tissue origins: Vero (Fig. 2 a), CEF (Fig. 2 c), MDCK II (Fig. 2 e), as well as in PtK₂, baby hamster kidney, and HeLa cells (not shown). Double-labeling with an antibody to galactosyltransferase (Roth and Berger, 1982), a marker for the *trans* Golgi cisternae (Fig. 2 b), revealed that M3A5 stained the Golgi apparatus and a population of vesicles scattered throughout the cytoplasm. In CEF cells infected with ts045 VSV, M3A5, and polyclonal anti-VSV-G gave similar immunofluorescence staining patterns (Fig. 2, c and d), again localizing the M3A5 antigen in the Golgi apparatus. Both M3A5 and anti-VSV-G also stained a number of vesicles in the cytoplasm (arrows, Fig. 2, c and d) which may correspond to transport vesicles carrying VSV-G within the cell, perhaps from the Golgi apparatus to the plasma membrane. The Golgi apparatus staining pattern of M3A5 was abolished after preabsorption of M3A5 (Fig. 2 f) with a boiled microtubule protein fraction. This fraction contained predominantly MAP-2 with some of the Tau proteins, prepared from bovine brain as described by Fellous et al. (1977).

In cultured neuronal cells prepared from chicken brain, M3A5 stained cell processes as well as the cell body. It was not possible, however, to distinguish which structures were labeled within the small, rounded cell bodies. M3A5 and rat anti-tubulin gave similar staining patterns on these cells (Fig. 3, a and b), except that M3A5 labeling was more discontinuous than anti-tubulin labeling. Some filamentous structures were stained with both antibodies; these filaments are probably microtubules or microtubule bundles. The similarity between the M3A5 and anti-tubulin staining patterns is in sharp contrast to the situation in the non-neuronal cells tested, and reflects the fact that the chick neuronal microtubules contain significant amounts of MAP-2.

The co-localization of the M3A5 antigen and galactosyltransferase was confirmed in cells treated with either 10 μ M monensin or 10 μ M nocodazole. Monensin caused vacuolation of the Golgi, giving the dark circular patches seen in Fig. 4, a and b. M3A5 and anti-galactosyltransferase staining patterns were only partially superimposable after monensin treatment, as in some cases M3A5 stained crescents adjacent to vacuoles, whereas the anti-galactosyltransferase staining appeared to extend around the perimeter of the vacuoles (arrows, Fig. 4, a and b). Neither the microtu-

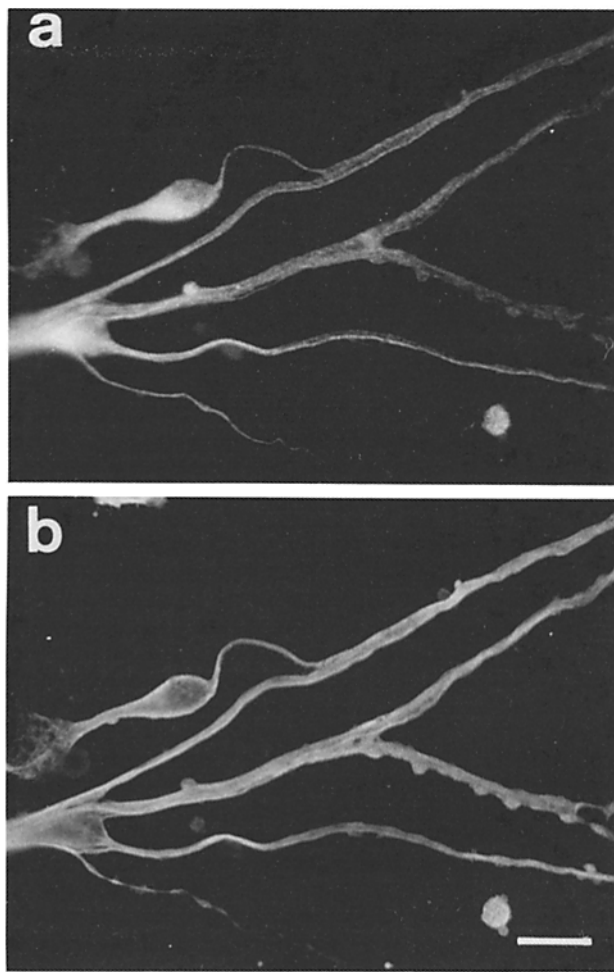


Figure 3. M3A5 labels cell processes in chicken brain neuronal cells. Chick brain neuronal cells prepared according to Pettmann et al. (1979) were grown on poly-L-lysine-coated coverslips and double-labeled with M3A5 and YL1/2 (a rat anti-tubulin antibody). Both M3A5 and YL1/2 stain filamentous structures within the cell processes. Bar, 10 μ m.

bule network (Fig. 4 c) nor the overall perinuclear location of the Golgi apparatus (Fig. 4, a and b) were affected by 10 μ M monensin. Nocodazole, in contrast, caused complete depolymerization of microtubules (Fig. 4 f) and scattering of the Golgi apparatus throughout the cytoplasm (Fig. 4, d and e). M3A5 and anti-galactosyltransferase staining patterns remained virtually coincident after nocodazole treatment. The 110K protein did not redistribute into the nucleus when Vero cells were heat shocked (not shown), and so M3A5 seems not to be specific for the 100/110K protein recognized by the JLJ5a monoclonal antibody (Lin and Queally, 1982; Welch et al., 1983).

Immunofluorescence staining with M3A5 was successful only with methanol/acetone, paraformaldehyde, or ethylene glycol *bis* (succinimidylsuccinate) (Mitchinson and Kirschner, 1985) fixation, followed for the latter two methods by permeabilization with 0.1% Triton X-100 and 0.05% SDS. No M3A5 staining was observed when SDS was not present during the permeabilization step. One explanation for this could be that the M3A5 antigenic site is "cryptic" because the 110K protein is involved in a protein-protein interaction which

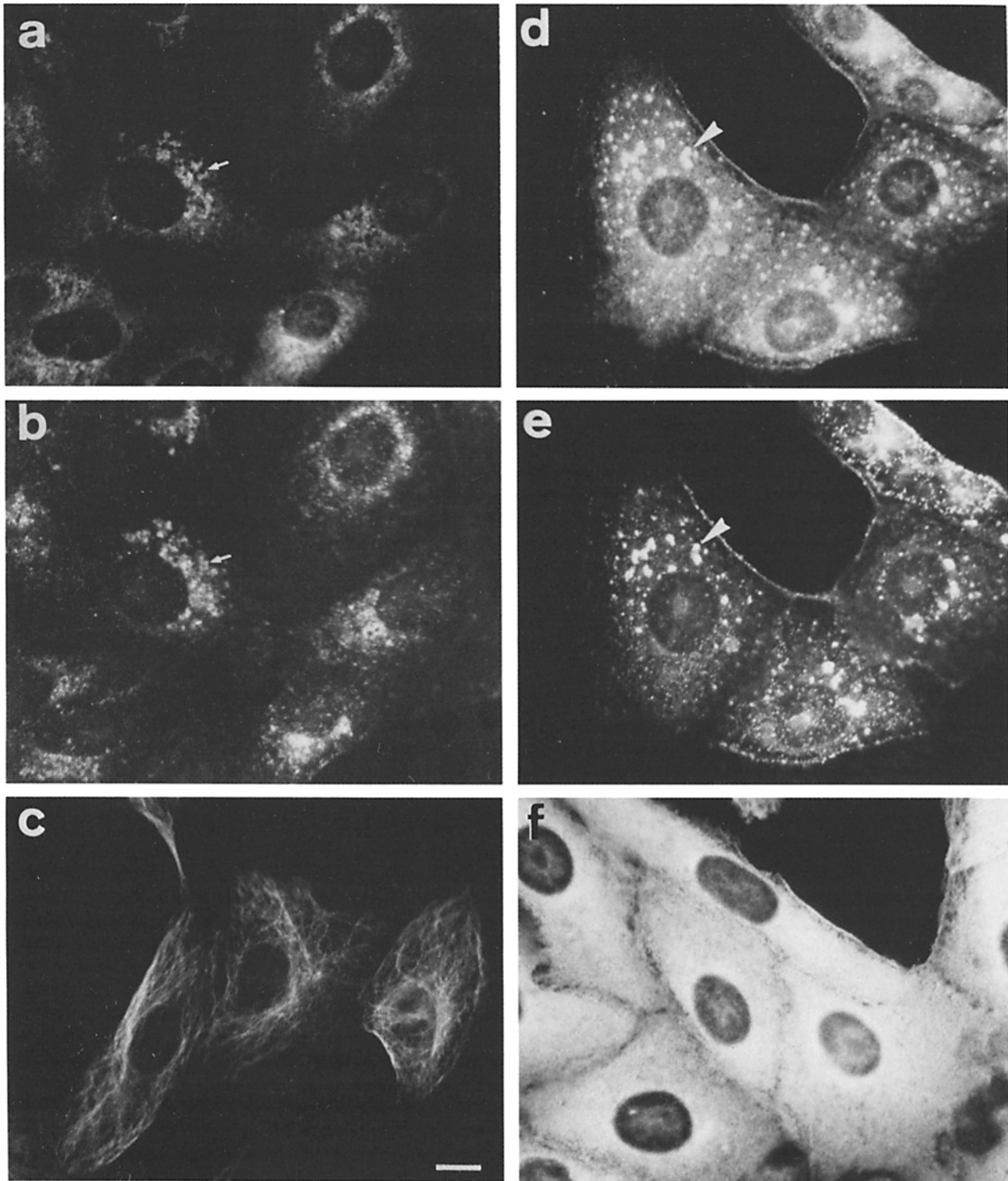


Figure 4. Effect of monensin and nocodazole on the distribution of the 110K protein. Vero cells grown on coverslips and treated with 10 μ M monensin for 15 min (*a-c*) or 10 μ M nocodazole for 45 min (*d-f*) were fixed and then the coverslips were divided. Half of each coverslip was double-stained with M3A5 (*a* and *d*) and anti-galactosyltransferase (*b* and *e*) and each other half was single stained with YL1/2 (*c* and *f*), an anti-tubulin antibody. Arrows (*a* and *b*) point to a monensin-induced vacuole with associated M3A5 and anti-galactosyltransferase staining. M3A5 and anti-galactosyltransferase both stain the same Golgi elements after nocodazole treatment (*arrowheads* in *d* and *e*). Monensin had no effect on the microtubule network, whereas nocodazole induced complete depolymerization. Bar, 10 μ m.

needs to be broken by SDS, for example, before the antigenic site is available. It has therefore so far proved difficult to obtain conclusive results using M3A5 for immunoelectron microscopy on frozen sections. To determine the precise loca-

tion of the 110K protein within the Golgi apparatus, a polyclonal antibody which can recognize more accessible regions of the protein may be required.

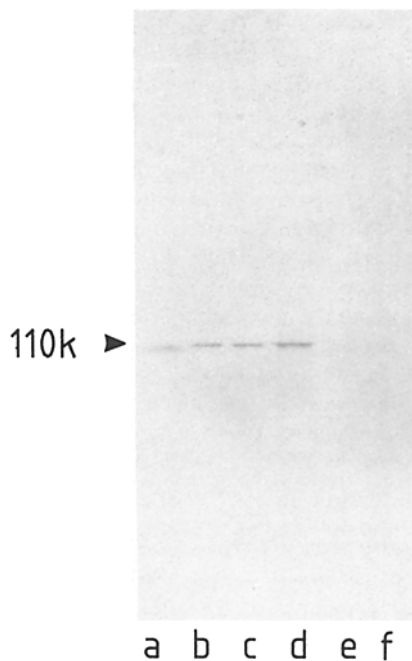


Figure 5. The distribution of the 110K protein in subcellular fractions prepared from rat liver. Rat liver fractionation was performed according to Leelavathi et al. (1970), and fractions were immunoblotted with M3A5. (Lane *a*) Total homogenate; (lane *b*) S₁ Golgi stack fraction (from 0.25/0.50 M sucrose interface); (lane *c*) S₂ Golgi stack fraction (0.50/0.86 M sucrose); (lane *d*) 0.86 M sucrose fraction; (lane *e*) 0.86/1.30 M sucrose interface; (lane *f*) pellet. 75 μg of protein was loaded per lane.

The 110K Protein Is Enriched in Rat Liver Golgi Fractions

In detergent extracts of all non-neuronal cell types tested by immunoblotting, M3A5 labeled one protein with an M_r of ~110,000. Immunofluorescence patterns observed using M3A5 in the same cell types suggested that the M3A5 antigen was associated with the Golgi apparatus. Therefore, a Golgi stack fraction prepared from rat liver according to the method of Leelavathi et al. (1970) was analyzed by immunoblotting, along with the total homogenate and other gradient fractions from the same preparation (Fig. 5). A 110K band was detectable in the total homogenate (Fig. 5, lane *a*). The 110K protein was enriched, however, in the two Golgi stack fractions, S₁ (Fig. 5, lane *b*) and S₂ (Fig. 5, lane *c*), which were collected from the 0.25/0.5 M and 0.50/0.86 M sucrose interfaces, respectively. Electron microscopy showed that the S₁ and S₂ fractions contained a variety of membrane vesicles and recognizable stacked Golgi cisternae (data not shown). The 110K protein was also present in the 0.86-M fraction (Fig. 5, lane *d*), but not at the 0.86/1.30 M sucrose interface (Fig. 5, lane *e*) or in the pellet (Fig. 5, lane *f*). The 110K protein could not be detected in a purified coated vesicle preparation (data not shown).

The 110K Protein Is a Peripheral Membrane Protein

When the Golgi fraction was centrifuged at 100,000 g_{av} for 120 min, all of the 110K protein was found in the pellet (Fig. 6, lane *a*) suggesting that it was associated with membranes. The same results were obtained with Vero cell homogenates

(not shown). The membrane location of the 110K protein was investigated by Triton X-114 extraction (Fig. 6, lanes *c-e*) of the Golgi fraction (460 μg protein). Samples of the total extract, detergent phase, and TCA-precipitated aqueous phase were analyzed by immunoblotting. The total extract (Fig. 6, lane *c*) contained the 110K protein, as did the aqueous phase (lane *e*). The Triton X-114 pellet (detergent phase) did not contain detectable 110K protein (lane *d*). This distribution was also observed when Vero cells were extracted with Triton X-114 (data not shown). In addition, the Golgi fraction (230 μg protein) was washed with 200 mM NaHCO₃, pH 11.0, and the soluble and insoluble material was analyzed by immunoblotting (Fig. 6, lanes *f* and *g*). The 110K protein was found only in the carbonate-soluble fraction (lane *g*) and not in the insoluble pellet (lane *f*). Both these approaches suggest that the 110K protein is either a peripheral membrane protein or a secretory protein, but not an integral membrane protein.

The 110K Protein Is Located on the Cytoplasmic Face of the Golgi Membrane

It was clearly of interest to discover with which face of the Golgi membrane the 110K protein interacted. This was determined by digestion of Golgi vesicles with proteinase K (Fig. 7) or TPCCK trypsin (data not shown) in both the presence and absence of Triton X-100. The extent of 110K protein degradation was established by immunoblotting with M3A5.

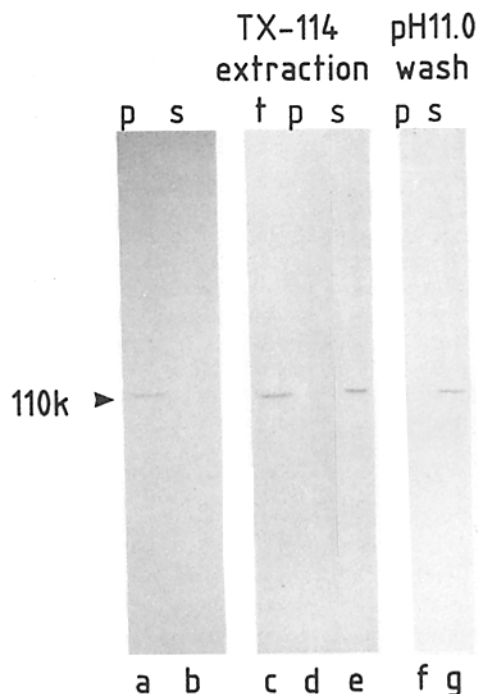


Figure 6. The 110K protein recognized by M3A5 is a peripheral membrane protein. A fraction enriched with rat liver Golgi stack membranes (S₂, collected from the 0.50/0.86 M sucrose interface), was resuspended in 100 mM potassium phosphate buffer, pH 6.7, and then centrifuged at 100,000 g_{av} for 2 h. The membrane pellet (lane *a*) and the supernatant (*b*) were analyzed by immunoblotting. The same Golgi stack fraction was extracted with Triton X-114 (lanes *c-e*) or washed with 100 mM sodium carbonate, pH 11.0 (lanes *f* and *g*) and the resulting pellets (*p*), supernatants (*s*) were analyzed by immunoblotting. *t*, Total Triton X-114 extract (lane *c*).

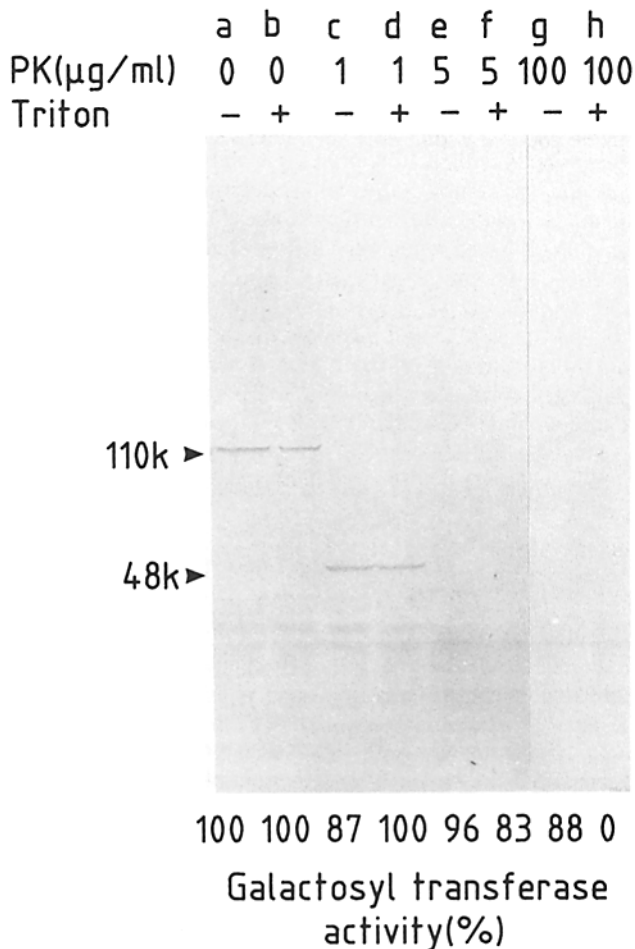


Figure 7. Digestion of the 110K protein with proteinase K. Aliquots of rat liver Golgi membranes (230 μg protein) were incubated for 30 min with 0 (a and b), 1 (c and d); 5 (e and f), or 100 (g and h) $\mu\text{g/ml}$ proteinase K either in the absence (a, c, e, and g) or presence (b, d, f, and h) of 0.4% Triton X-100. After incubation and addition of PMSF to block protease activity, duplicate aliquots were assayed for galactosyltransferase activity. The remaining samples were reduced and analyzed by immunoblotting. The 110K protein is accessible to proteinase K in both intact and detergent-disrupted vesicles. It is digested to a 48-kD fragment (48K) which is recognized by M3A5 after treatment with 1 $\mu\text{g/ml}$ of proteinase K (c and d), or completely digested at higher protease concentrations (e-h).

The integrity of the Golgi vesicles was monitored in parallel by assaying galactosyltransferase activity. Galactosyltransferase is a Golgi enzyme which is found exclusively in the lumen of the Golgi cisternae and so should not be accessible for digestion by proteinase K or trypsin unless Triton X-100 is also present in the digestion mixture (Fleischer, 1981).

The 110K protein was proteolyzed to give a fragment of ~ 48 kD by 1 $\mu\text{g/ml}$ proteinase K both in the absence (lane c) and presence (lane d) of Triton X-100. When 5 $\mu\text{g/ml}$ or 100 $\mu\text{g/ml}$ proteinase K was used (lanes e-h), the fragment of the 110K protein carrying the M3A5 epitope was completely degraded. Thus, the ~ 48 -kD fragment observed after treatment with 1 $\mu\text{g/ml}$ proteinase K is an intermediate form, and is not resistant to digestion. A similar intermediate fragment (~ 51 kD), observed after treatment with 10 $\mu\text{g/ml}$ TPCK-trypsin, disappeared after digestion with 100 $\mu\text{g/ml}$

both with and without Triton X-100 (data not shown). The vesicles were shown to be intact, as digestion with 100 $\mu\text{g/ml}$ proteinase K in the absence of Triton resulted in a decrease in galactosyltransferase activity of only 12%, compared to total loss of activity after incubation with 100 $\mu\text{g/ml}$ proteinase K in the presence of Triton X-100 (Fig. 7, lanes g and h). With 100 $\mu\text{g/ml}$ TPCK-trypsin, galactosyltransferase activity was reduced by 17 or 100%, without and with Triton X-100, respectively (data not shown). Both the ~ 48 -kD and ~ 51 -kD fragments produced by proteinase K and TPCK-trypsin, respectively, remained associated with the membrane, as judged by ultracentrifugation (data not shown). These results show that the 110K protein is accessible for protease digestion when Golgi vesicles are intact, and so must be associated with the cytoplasmic surface of Golgi membranes and not with the luminal face of the vesicle membrane.

The 110K Protein Interacts with Taxol-stabilized Microtubules In Vitro

To find out whether the 110K protein and MAP-2 share any functional properties in addition to being immunologically related, we determined whether the 110K protein could interact with microtubules in vitro. As a source of soluble non-membrane-bound 110K protein, we used the Triton X-114 aqueous fraction from cultured primary CEFs after removal of insoluble material by centrifugation at 105,000 g_{av} for 30 min. We then either polymerized microtubules at 37°C from the endogenous tubulin in the extract, in the presence of 10 μM taxol and 1.0 mM GTP, or, we added exogenously taxol-polymerized calf brain tubulin microtubules to the aqueous fraction. The microtubules were then collected by centrifugation through a 4-M glycerol cushion at 100,000 g_{av} for 20 min. Immunoblotting was used to analyze the dis-

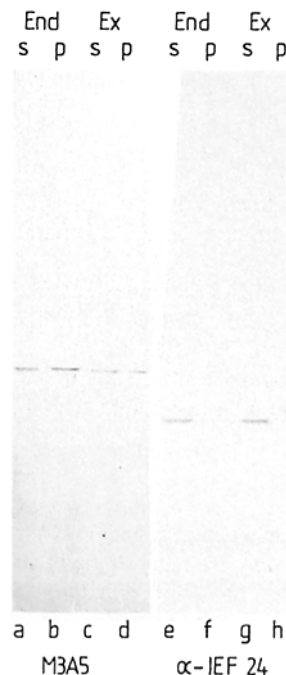


Figure 8. Interaction of the 110K protein with microtubules in vitro. The high speed supernatant of a Triton X-114 aqueous fraction of CEFs was used as the source of the 110K protein. Endogenous microtubules were polymerized from this extract with 10 μM taxol and 1 mM GTP, and then centrifuged at 100,000 g_{av} through a 4-M glycerol cushion to give a supernatant (lanes a and e) and a microtubule pellet (lanes d and h). Samples were analyzed by immunoblotting using M3A5 (lanes a-d) or rabbit anti-IEF 24, an antibody to a mitochondrial protein (lanes e-h). The relative gel loadings were as follows: 1.25% of the supernatant was loaded in lanes a and e; 3.0% of the microtubule pellet (lanes b and f); 4.6% of the supernatant (lanes c and g); and 15% of the microtubule pellet (lanes d and h).

tribution of the 110K protein and a number of other proteins within the microtubule pellets and the supernatants (Fig. 8).

The 110K protein was present in both endogenous and exogenous microtubule pellets (Fig. 8, lanes *b* and *d*) as well as in the supernatant fractions (lanes *a* and *c*), and so appeared to interact with microtubules. As a control to test whether soluble proteins were becoming nonspecifically pelleted with microtubules, we studied the distribution of IEF 24, a mitochondrial protein (Mose-Larsen et al., 1982) which is located in the lumen of mitochondria (Griffiths, G., and R. Bravo, unpublished results) and so is not normally accessible to microtubules in the cytoplasm. A polyclonal antiserum to IEF 24 (a generous gift from R. Bravo, EMBL, Heidelberg, FRG) revealed that the protein was present in both supernatants (Fig. 8, lanes *e* and *g*) but was absent from both microtubule pellets (lanes *f* and *h*). The faint band observed above IEF 24 is present in lanes *e-h*, irrespective of the distribution of IEF 24, and is therefore most likely to be unspecific.

A monoclonal antibody to β -actin (6B1) revealed that actin was present in low amounts in both microtubule pellets (data not shown). Actin was considerably enriched, however, in the supernatant fractions. The 110K protein was clearly enriched in both microtubule pellets compared to actin. Thus it seems unlikely that the presence of the 110K protein in the pellets can be ascribed to its forming a complex with actin. As an additional control, the distribution of vimentin was studied, using another monoclonal antibody (7A3). Vimentin was absent from all pellets and supernatants (data not shown), as would be expected considering that an early step in the Triton X-114 extraction procedure involves the removal of nuclei and insoluble material by centrifugation.

The results described above show that CEF 110K protein can interact specifically with both endogenous CEF microtubules and with calf brain microtubules *in vitro*. The 110K protein is not present, however, in taxol-polymerized microtubules prepared from CEF according to the method of Vallee (1982) (data not shown). This is to be expected, since the microtubules were polymerized from a membrane-free supernatant prepared from homogenized CEF, which should not contain the 110K protein (cf. Fig. 6, lanes *a* and *b*).

We have shown that the 110K protein interacts with taxol-stabilized microtubules. One possibility was that this ability was due to the 110K protein and MAP-2 possessing similar, immunologically related tubulin-binding sites. To test this, we subjected bovine brain MAP-2 to chymotryptic digestion followed by polymerization with tubulin, so as to generate the 35-kD microtubule-binding domain of MAP-2 as described previously (Vallee, 1980). M3A5 recognized as fragment of 30–35-kD and also some higher molecular weight fragments, but none co-polymerized with tubulin (data not shown). M3A5 must therefore recognize a site on the projecting arm of MAP-2, and not on the tubulin-binding domain.

Discussion

Some of the protein components involved in translocation of vesicles along microtubules in axons have recently been discovered. Kinesin is a translocator molecule which is involved in anterograde movement of vesicles (Vale et al., 1985a), whereas a protein which is immunologically and pharmacologically distinct from kinesin seems to be in-

involved in generating retrograde transport (Vale et al., 1985b). No proteins mediating interactions between microtubules and organelles or vesicles have yet been characterized in non-neuronal cells. MAPs or MAP-related proteins may be good candidates for such interactions. So far several MAPs have been shown to be associated with microtubules in tissues and cultured cells of non-neuronal origin (for reviews see Vallee, 1984; Wiche, 1985). Our aim was to raise monoclonal antibodies against neuronal MAPs and to select for those antibodies which also recognized components of organelles in non-neuronal cells.

With this strategy we obtained M3A5, a monoclonal antibody specific for neuronal MAP-2. In all non-neuronal cells tested, this antibody labeled the Golgi complex but not the microtubule network. Also Wiche et al. (1986) have reported that a polyclonal MAP-2 antibody labeled the perinuclear region of mouse 3T3 cells. In extracts of non-neuronal culture cells and in rat liver Golgi fractions, M3A5 recognized a protein of 110K. This protein was shown to behave like a peripheral membrane protein by two criteria: (*a*) partitioning into the aqueous phase on extraction with Triton X-114; and (*b*) removal from the membrane by washing with sodium carbonate at pH 11.0. Furthermore, since it is susceptible to digestion by proteinase K in the absence of detergent, the 110K protein must be located on the cytoplasmic face of vesicles derived from a Golgi fraction. So far, only one other monoclonal antibody to cytoplasmic domains of Golgi-specific proteins has been described; the antibody recognizes two peripheral membrane proteins of *M*_r 54,000 and 86,000 (Chicheportiche et al., 1984). Smith et al. (1984) have described two monoclonal antibodies which recognize an *M*_r 103,000–108,000 protein in rat pancreatic acinar cells, but as this protein is an integral membrane protein located on the luminal side of the Golgi membrane, it appears to be distinct from the 110K protein recognized by M3A5. The 110K protein, therefore, is a peripheral, cytoplasmically oriented Golgi protein, which has not been previously described.

In addition to staining the Golgi complex, M3A5 also stained a population of vesicles devoid of galactosyltransferase, which were scattered throughout the cytoplasm. It is not clear to which class of vesicles they belong. The colocalization of VSV-G and the 110K protein (Fig. 2, *c* and *d*) may suggest that they are exocytic vesicles transporting protein from the Golgi apparatus to the cell surface. We cannot exclude, however, that the 110K protein is present in another organelle type, such as endosomes, for example.

MAP-2 and the 110K Golgi protein obviously share an antigenic determinant. Using polyclonal antibodies, several such immunological relationships between MAP-2 and other cytoskeletal proteins, such as the α subunit of erythrocyte spectrin (Davis and Bennett, 1982) and a variety of astrocyte MAPs (Couchie et al., 1985) have been reported. In addition, a relationship between MAP-2 and an extracellular glycoprotein has recently been reported (Briones and Wiche, 1985). Rodionov et al. (1985) have described a monoclonal antibody, RN17, that recognizes MAP-1 and -2 and also a 100-kD protein in various non-neuronal cells, and which stained microtubules and coated vesicles in the same cells. RN17 and M3A5 both appear to recognize distinct proteins, as RN17 did not label the Golgi complex, and M3A5 neither stained microtubules in non-neuronal cells nor detected any 110K protein in a coated vesicle fraction. Whether any of

these observations imply a truly functional, rather than antigenic relationship between MAP-2 and the other proteins, is not clear. When monoclonal antibodies such as M3A5 or RN17 (Rodionov et al., 1985) are used instead of polyclonal antibodies, the likelihood of picking up purely antigenic relationships between two otherwise unrelated proteins must be increased. We have shown, however, that the 110K protein can interact with taxol-stabilized microtubules in a membrane-free system in vitro, so it appears that the 110K protein and MAP-2 are not only immunologically related, but also share the ability to bind to tubulin. This interaction between the 110K protein and microtubules clearly requires further investigation, such as analyzing for co-polymerization with tubulin using a multiple polymerization/depolymerization protocol (without taxol) such as that described by Weatherbee et al. (1980).

Unlike MAP-2, the 110K protein is not located along microtubules in vivo but rather in the Golgi apparatus. The 110K protein therefore should not be termed a MAP. Instead it might act as a microtubule-binding protein which is located on the Golgi membrane and which can also interact with microtubules. In this way, the 110K protein may play a part in linking the Golgi apparatus, and possibly also Golgi-derived transport vesicles, to the microtubule network. A structural link between the Golgi apparatus and microtubules has long been implied, as reviewed above. The 110K protein, being located on the cytoplasmic face of the Golgi apparatus membranes, is clearly in the right position to perform this function. It is also possible that the 110K protein could be involved in linking transmembrane proteins within the Golgi apparatus and transport vesicles, to the cytoskeleton, as has been suggested in a number of other systems (e.g. Ranscht et al., 1984; Bourgignon et al., 1985).

M3A5 recognizes an epitope on the projecting portion of MAP-2 rather than the microtubule-binding domain (data not shown). The projecting arm of MAP-2 has been shown to interact with cyclic AMP-dependent protein kinase (Vallee et al., 1981) and in particular, with its regulatory subunit (RII) (Theurkauf and Vallee, 1982; Lohmann et al., 1984). RII has also been found in association with the Golgi complex and MTOC in epithelial and fibroblastic culture cells (Nigg et al., 1985) and also in a variety of cell types in the nervous system (De Camilli et al., 1986). The 110K protein could be an RII receptor by virtue of its immunological relationship with MAP-2 and its location in the Golgi apparatus. It could not, however, account for the localization of RII at the MTOC, as we have never observed staining of the MTOC with M3A5. Proposing a role for the 110K protein in linking the Golgi apparatus to the microtubular network need not necessarily exclude an interaction with RII, as the presence of RII and the catalytic subunit might provide a mechanism for controlling via phosphorylation the microtubule-Golgi apparatus interaction. Some sort of control system must exist which allows the Golgi apparatus first to scatter and then recluster at the onset and end of mitosis, respectively. As yet we do not know whether the 110K protein is a substrate for cAMP-dependent protein kinase.

If the 110K protein binds to microtubules directly, then there are at least two ways in which it might mediate the redistribution of the Golgi complex to its correct perinuclear location; after mitosis, for example. The 110K protein may interact only at the minus ends of microtubules, and this

would involve a passive redistribution of the Golgi complex. Alternatively, the 110K protein could form part of a retrograde translocation complex which would cause movement from the plus to the minus ends of microtubules. The Golgi complex would then be actively transported to the minus ends of microtubules, resulting in a build-up of elements of the Golgi complex around the MTOC. A concentration of actively transported vesicles at the ends of microtubules has indeed been observed in dissociated squid axoplasm (Allen et al., 1985).

Clearly, further experiments are required to enable us to understand the precise function of the 110K protein in the interaction between the Golgi apparatus and the microtubule network, and also in the positioning of the Golgi apparatus within the perinuclear region of the cell.

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