# Epithelial and fibroblastoid cells contain numerous cell-type specific putative microtubule-regulating proteins, among which are ezrin and fodrin

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**Upon cell junction formation, the microtubules of polarizing epithelial cells become reorganized by unknown signaling mechanisms and regulating proteins. Microtubule-associated (MAPs) and other types of proteins are likely to be involved in this process, but most of these are unknown. Such proteins are called here collectively microtubule-regulating proteins (MRPs). As a first step towards their characterization, we used co-sedimentation of cytosolic proteins of MDCK cells and A72, a dog fibroblastoid line, with an excess of taxol-stabilized MTs, to obtain a cell fraction enriched in putative MRPs ("MRPs"). Additional tests have led to the inventory of around 40 "MRPs" among the 80 proteins present in the microtubule pellet. We also found that "MRPs" are recovered in higher amounts from MDCK cytosol, and that half of these are cell-type specific. These results corroborate data from yeast cells and insect eggs, and show that in mammalian somatic cells too, a large number of proteins seems to be involved in microtubule regulation, and that different cell types use a specific set of MRPs. "MRPs" found in both cell types are the intermediate chain of cytoplasmic dynein, Arp1, the major subunit of the dynactin complex, and CLIP-170. Two MDCK-specific "MRPs" were identified as the actin-binding proteins ezrin and α-fodrin. These results are discussed with regard to a possible involvement of ezrin and fodrin in morphogenetic interactions of microtubules with the membrane cytoskeleton in polarizing epithelia upon junction formation.**

*Abbreviations:* MAPs Microtubule-associated proteins. – MDCK Madin Darby Canine Kidney. – MRPs Microtubule-regulating proteins. – MT Microtubule. – PBS Phosphate-buffered saline.

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## Introduction

The microtubule (MT) network of vertebrate somatic cells is usually organized in a dynamically unstable, radial pattern, converging at the centrosome region [30, 32], but in some cases, it can become transiently or stably polarized [47] and even no longer converge at the centrosome region, like in polarized epithelia, striated muscle fibers and neurons [33]. This polarization of the network is essential in development and cell differentiation, and for proper cell functioning [11, 14, 33] since it contributes directly or indirectly to the establishment and maintenance of cellular polarity [7, 17]. The mechanisms regulating these important aspects of MT network control remain poorly understood. A frequently cited possibility is that MTs extending into the cell periphery become involved in morphogenetic interactions with peripheral cues [32]. A role for motor complexes, tethered to special cortical domains, in organizing interphase and mitotic MT networks, has been proposed [17]. The contributions of non-motor MAPs and other regulating proteins to these processes in non-neuronal cells remain very poorly understood [14, 22, 35].

Polarizing epithelial cell lines form an attractive model system for identifying the proteins involved in the morphogenetic signaling between the cell cortex and the MT network. Indeed, they reorganize their MT-network in response to cell-cell contact formation [3, 16, 38, 43], and the reorganization of the cortical cytoskeleton induced by it [37, 46]. Morphological findings in highly specialized epithelial cells suggest that in epithelial cell lines, cell surface sites may be involved in the control of their MT nucleation and positioning [20, 39, 54]. Recently, PAR-1, an element of a signal transduction pathway that communicates cell polarity to the cytoskeleton has been shown to be associated with the cellular cortex in the lateral plasma membrane domain of MDCK cells [6, 19]. On the cytoskeletal side, it has been proposed that these cells contain a set of MT-regulating proteins which are different from those of other cell types, like fibroblasts, which are motile and do not reorganize their MTs upon reaching confluency [3, 16, 38,

43]. Consistent with this view, a 115 kDa MAP (E-MAP115) is preferentially expressed in epithelial cells [36], but its role is unknown. Studies identifying minor MAPs (defined by their retention on and salt elution from a microtubule affinity matrix and their intracellular localization with specific antibodies) from early Drosophila embryos [29] and the budding yeast Sacharomyces cerevisiae [4] have shown that these model systems contain numerous MAPs. In contrast, in vertebrate somatic cell lines, only few MAPs have been described until now, and it is likely that many remain to be discovered. So far, no MDCK-specific proteins involved in MT regulation have been reported. Identifying and characterizing these will contribute to understand their particular way of MT regulation.

MTs used as affinity matrix provide the most commonly used technique for probing for MAPs [52]. The studies in yeast [4] and Drosophila [29] have lent new credibility to this approach. They established that only a minority of proteins recovered on a MT affinity matrix bind fortuitously. Furthermore, these studies revealed two important points: first, a large number of the proteins identified in this way were not localized along intracellular MTs, but instead, displayed various distributions (MT organizing centers, kinetochores, spindle fibers only) illustrating the complexity of the MT cytoskeleton, and second, some of these may not be MAPs, but instead, interacting with or being part of MAP-containing protein complexes. So, even those proteins recovered on MTs which do not represent genuine MAPs, may help identifying protein complexes functioning in regulating MT organization. Inventorizing such proteins is therefore useful as a first step towards the characterization of the proteins involved in MT regulation. In the following, we reserve the term MAPs for proteins interacting with MTs via a specific MT-binding domain, and playing a proven role in MT functioning and/or organization. We propose the term microtubule-regulating proteins (MRPs) to designate a more general class of proteins which contribute to microtubule organization. They comprise some of the MAPs and other types of proteins. We will use the term putative MT-regulating proteins ("MRPs") for those proteins found in MT pellets which, according to a number of criteria, defined in this study, show a specific co-sedimentation with endogenous and exogenous MTs in vitro. In the light of the data from Drosophila and yeast, these "MRPs" are good candidates to be involved in regulating MT organization.

The few available studies using non-neuronal vertebrate cell lines or even tissues [9, 36, 45, 52, 56] as source for probing for "MRPs" have identified variable numbers of proteins which co-sedimented with MTs. The methods used were not designed for recovering minor proteins, and accordingly, only 5 to 10 proteins were found, mostly resolved by 1D gels. Moreover, very quickly, the efforts were centered on one or two major proteins to show that they were genuine MAPs. The very few non-motor MAPs thus identified in vertebrate cells are relatively abundant proteins, which, with the exception of CLIP-170 [45], bind along the whole length of the MTs. Almost no efforts were made to define the nature of the remaining proteins, also because no rigorous criteria were used to at least define whether they co-sedimented specifically or fortuitously. In order to detect minor, but potentially important "MRPs" these methods are not suitable.

As a first step towards the characterization of genuine MRPs of mammalian epithelial cells, we have probed for "MRPs" in a cytosolic extract of MDCK cells, a well characterized polarizing epithelial cell line from canine kidney origin, and have compared these with the "MRPs" of A72 cells, a canine fibroblastoid cell line. We report here: *a)* that both cell types contain at least 100 proteins cosedimenting with MTs, only 40 of which can be termed "MRPs", *b)* that the latter are more abundant in MDCK cells, and *c)* that half of these are cell-type specific. These findings substantiate beliefs that in mammalian somatic cells numerous proteins may be involved in MT-regulation. Two common "MRPs" were identified as the intermediate chain of cytoplasmic dynein (a protein complex known to interact with MTs) and as CLIP-170. Interestingly, ezrin and the α-chain of non-erythroid spectrin (fodrin), both known as actin-binding proteins, were identified as "MRPs", but only in MDCK cells. This latter finding is discussed with regard to a possible involvement of ezrin and fodrin in morphogenetic interactions of MTs with the membrane cytoskeleton of polarizing epithelia upon junction formation.

## Materials and methods

### Preparation of large amounts of cytosolic extracts of MDCK and A72 cells

MDCK II cells were propagated [55] successively in 85 $cm^2$ T-flasks, double-deck (1200 $cm^2$) and 10 deck (6320 $cm^2$/factory) Cell Factories (Nunc, Life technologies, Cergy-Pontoise, France). Confluent cells from 4 factories (ca. $5 \times 10^9$ cells) were harvested as follows. The factories were washed twice with PBS, trypsinized, and washed out with cold washing buffer (0.5 mM $MgCl_2$, 1 mM EGTA, 5.5 mM glucose in PBS). The cell suspension (about 3 l) was centrifuged for 20 min at 700*g*. The packed cells were resuspended in 200 ml cold washing buffer supplemented with trypsin inhibitor (Sigma, Saint Quentin Fallavier, France) (20 mg/100 ml) and pelleted for 5 min at 400*g*. They were resuspended in 200 ml washing buffer and pelleted for 5 min at 400*g*. Then they were resuspended in 200 ml cold homogenization buffer (HB) (100 mM MES, pH 7.0, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.52 M sucrose, 1 mM DTT [28]) and pelleted for 5 min at 700*g*. The final pellets (around 10 ml of packed cells) were resuspended in 1.5 volume of "homogenization buffer complete" (HBC) (100 mM K-MES, pH 7.0, 0.52 M sucrose, 0.5 mM MgAcetate, 1 mM EGTA, 1 mM DTT, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 20 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml pepstatin A) and sonified with the Microtip of a sonifier (Branson, model 250), at power 7. Sonification was repeated 3–4 times during 6 seconds each. The suspension was cooled on ice between cycles of disruption. The breakage of the cells was controled by phase-contrast light microscopy. The cell homogenate was centrifuged for 10 min at 10000*g*. The supernatant was centrifuged for 1 h at 100000*g*, and the recovered supernatant was centrifuged once more for 1 h at 100000*g*. The final high speed supernatant was drop-frozen in liquid nitrogen and stored at –80 °C. Protein concentration was measured using the Bradford assay [8] (Biorad, Ivry-sur-Seine, France) and BSA (Sigma) as standard.

A cytosolic extract of the dog fibroblastoid cell line A72 [55] was obtained in the same way from cells grown in 10 Nunc 576 $cm^2$ squared dishes.

### Tentative identification of putative MRPs ("MRPs") by co-sedimentation with and salt elution from MTs taxol-polymerized from the endogenous tubulin pool

Aliquots (600 μl) of cytosol (from MDCK or A72 cells), adjusted to 5 mg protein/ml with HBC (see above), were centrifuged at 150000*g* for 20 min at 4 °C (Beckman TL-100 Ultracentrifuge, TLA 100.1 rotor, 0.5 ml tubes) (Gagny, France). Taxol (20 μM) and 2 mM GTP were added to the supernatant. The mixture was incubated for 30 min at 23 °C, loaded onto a sucrose cushion (500 μl of 30 % sucrose in HBC

supplemented with 20 μM taxol), and centrifuged at 30000*g* for 30 min at 22 °C, acceleration 9, deceleration 6 (Beckman TL-100 Ultracentrifuge, TLS 55 rotor, 2 ml tubes). Salt sensitive proteins were eluted by resuspending the MT pellet in 120 μl of HBC containing 0.8 M KCl and 20 μM taxol. The eluate was cleared by centrifugation at 30000 *g* for 30 min at 22 °C (Beckman TL-100 Ultracentrifuge, TLA 100.2 rotor, 2 ml tubes). The proteins in the supernatant were named crude "MRPs" and prepared for analytical 1D and 2D gel electrophoresis (see below).

## Testing for the recovery of crude "MRPs" upon the addition of taxol-stabilized MTs made of brain tubulin, of inhibiting MT assembly in the cytosol, and of washing the crude "MRPs" containing MT pellet

Tubulin was purified from porcine brain [57]. Taxol-stabilized MTs [52] were pelleted at 108000*g* for 20 min at 25 °C, just before their use as affinity matrix. They were resuspended at 2 mg MTs/ml into the cleared cytosol, supplemented with 20 μM taxol (Molecular Probes, Interchim, Montluçon, France), and crude "MRPs" were obtained as described above.

As a control for the dependency of protein sedimentation on the presence of MTs, cleared MDCK cytosol was supplemented with 10 μM nocodazole (Sigma), centrifuged as above, and the sedimenting proteins were salt eluted from the small pellet under identical conditions.

In the washing experiment, taxol-stabilized MTs were added to the cleared cytosol, which was split in two parts. One part was used for the preparation of crude "MRPs" as before. The MT pellet, obtained from the other part was washed by resuspension in HBC (twice the initial cytosol volume) and repelleting (108000*g* for 20 min at 23 °C). The washed crude "MRPs" were salt eluted in ½ of the initial cytosol volume.

## Testing for the ability of dialyzed crude "MRPs" to rebind to newly added MTs

Crude MRPs, salt eluted from pellets of largely brain MTs were microdialyzed [41] against HBC (two changes of the buffer). They were diluted 1:2 with HBC and incubated with resuspended taxol-stabilized MTs as described above. The MTs were sedimented at 30000*g* for 30 min at 22 °C (Beckman TL-100 Ultracentrifuge, TLA 100.2 rotor, 2 ml tubes) and the supernatant containing the non-rebound fraction was recovered quantitatively. The MT pellet was resuspended in 120 μl of HBC supplemented with 0.8 M KCl and 20 μM taxol and repelleted by centrifugation at 30000*g* for 30 min at 22 °C. The supernatant containing the rebound fraction was recovered quantitatively. For analytical 2D gel electrophoresis, two volumes of 2D sample buffer (see below) were added to the "rebound" and "non-rebound" fractions and the samples were concentrated at 30 °C (to avoid urea precipitation) in Ultrafree-MC Filter Units (Millipore, Saint-Quentin-en-Yvelines, France) to the same volume (40 μl).

## Testing for crude MRPs' heat stability

Crude MRPs, prepared with largely brain MTs, were dialyzed and supplemented with dithiothreitol (DTT, 1 mM). They were boiled for 5 min, cooled on ice and centrifuged at 30000*g* for 30 min at 4 °C to remove denatured protein. The resultant pellet and supernatant containing respectively heat labile and stable proteins were processed for 1D and 2D gel electrophoresis (see below).

## Analytical 2D gel electrophoresis and protein pattern comparison

The salt eluted protein fractions were microdialyzed against homogenization buffer (HB, see above), and two volumes of 2D sample buffer (9.8 M urea, 2.0 % Triton X-100, 2.0 % Servalyte 7–9, 100 mM DTT) (Serva, Biowittaker, Fontenay-sous-Bois, France) were added to them. Per 100 μl of sample, 1 μl carbonic anhydrase (Carbamalyte pI-calibration kit, Pharmacia, Saclay, France) was added, and the mixture was incubated for 15 min at 25 °C. Samples were aliquoted and stored at –20 °C.

The following conditions for the first dimension isoelectric focusing step were developed to improve the reproducibilty of the mini-2D gels (BioRad mini-Protean II, 2D capillary gel system) used in this study. The general conditions of use were those indicated by the manufacturer. The first dimension capillary gels were composed of 9.2 M urea, 4 % polyacrylamide, 2 % Triton X-100, 0.6 % Pharmalyte 3–10, 1.0 % Pharmalyte 4–6.5, 1.4 % Pharmalyte 5–8. Note the use of 3 % total ampholytes. Pre-electrophoresis was carried out as follows: 200 V for 10 min; 300 V for 15 min; 400 V for 15 min. 20 μl of sample was loaded per gel tube and run at: 400 V for 15 min; 750 V for 15 min and 1000 V for 2.5 h. The extruded capillary tube gels were incubated with 100 μl of SDS sample buffer for 10 min and loaded onto slab SDS-polyacrylamide gels (BioRad mini-Protean II slab gel system, 1 mm thick, 8 % polyacrylamide). Molecular weight markers were applied aside the transferred tube gel as 0.5 cm long agarose capillary tube gels containing 1 % agarose and high molecular weight (HMW)-markers (BioRad) diluted 1:100 in SDS sample buffer. The slab gel was run at constant 180 V until the bromophenol blue front had reached the bottom of the gel. The gels were silver stained [21], scanned in Adobe Photoshop, and the file transferred into Canvas for cropping and labeling. The plates were printed with a Kodak Colorease printer. Comparison of the protein pattern in different gels was done manually making use of the size and pI markers present in each gel.

## Analytical 1D gel electrophoresis of high molecular weight proteins

To an aliquot of sample prepared for 2D gel electrophoresis, two volumes of SDS sample buffer were added. The sample was not heated. It was loaded on slab gels (BioRad mini-Protean II slab gel system, 1 mm thick, 4 % polyacrylamide, no SDS in the separation gel, but instead, 2 M urea) [5]. The slab gel was run at constant 180 V and silver stained [21].

## Preparation of large amounts of MDCK crude MRPs

Fractions (10 ml) of cytosol, adjusted to 5 mg protein/ml with HBC, were centrifuged at 150000*g* for 20 min at 4 °C. The supernatants were warmed to 22 °C, and 20 μM taxol and pelleted porcine brain MTs (prepared as described above, 2 mg MTs/ml of cytosol) were resuspended in them, and the mixture was incubated for 30 min at 22 °C. The preparation was divided into two parts and each fraction was sedimented through a 5 ml cushion (30 % sucrose in HBC with 20 μM taxol) at 30000*g* for 30 min at 22 °C. The crude MRPs were eluted from the pelleted MTs by resuspending the latter in 4 ml of HBC supplemented with 0.8 M KCl and 20 μM taxol followed by centrifugation at 30000*g*, for 30 min at 22 °C. The crude MRPs were pooled, drop-frozen in liquid nitrogen and stored at –80 °C.

## Preparative 2D gel electrophoresis

For preparative 2D gel electrophoresis, crude MRPs were dialyzed against HBC and the heat labile fraction was obtained by heat precipitation in the presence of DTT as described above. The precipitated proteins of 4 ml of dialyzed crude MRPs were pelleted and directly resuspended in 400 μl 2D sample buffer. Per 2D gel, 20 μl of sample was loaded. The gels were stained with Coomassie Brilliant Blue. Ten spots corresponding to "MRPs" could be identified using the information contained in Fig. 3 and were numbered (see Fig. 7). In order to obtain enough material for microsequencing of these "MRPs", spots were cut out and pooled from each of 80 gels and stored at –20 °C until microsequencing.

## Protein characterization

Coomassie-stained spots were combined and equilibrated in sample buffer. Proteins were eluted and concentrated from the primary gel pieces into a secondary gel matrix and in-gel digested with trypsin [15]. Proteins were identified by MOWSE peptide mass-fingerprinting as described [42] or by internal microsequencing and homology searches using sequences stored in protein or DNA sequence databases.

### Immunofluorescence of MDCK ezrin cosedimenting with brain MTs

Sixty µl of a micture of brain MTs and MDCK cytosol were diluted in 2 ml of 0.25 % glutaraldehyde in 80 mM K-Pipes, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA (RG). Fractions (0.5 ml) were loaded on 5 ml of 25 % glycerol in a glass Corex tube, containing a round coverslip placed on top of a plexiglas insert. The tubes were centrifuged at 12000 rpm, 20 °C for 30 min in a HB4 rotor (Sorvall), and the coverslips were placed in six-well plates for double immunofluorescence with anti-tubulin (FITC) and anti-ezrin (Cy-3), or for single immunofluorescence with a monoclonal anti-ezrin (gift of Dr. P. Mangeat). The coverslips were mounted on slides with Mowiol, containing 10 % DABCO as antibleach [34].

### Density gradient centrifugation analysis of crude MDCK "MRPs"

Dialysed crude "MRPs" (500 µl) prepared with excess brain MTs as described above were loaded on a 4.5 ml 5–25 % sucrose gradient in 20 mM Tris-HCl, pH 7.6, 50 mM KCl, 5 mM EDTA, 5 µg/ml leupeptin, 5 µg/ml pepstatin, and centrifuged in a Beckman SW50.1 rotor at 26500 rpm for 18 h at 4 °C. Thirteen fractions were collected. The proteins were TCA-precipitated, ethanol-washed, and solubilized in equal volumes of hot SDS sample buffer, and separated on 8 or 6 % PAGE slab gels. These were either silver stained, or transferred to nitrocellulose for alkaline phosphatase Western blotting with antibodies to Arp1 (gift from D. Meyer, UCLA, Los Angeles, CA), "74.1", monoclonal anti-cytoplasmic dynein intermediate chain (gift from Dr. K. Pfister, University of Virginia, Charlottesville, VA), CLIP-170 (gift from J. E. Rickard and T. E. Kreis, Univ. Geneva, Switzerland), ezrin (gift from P. Mangeat), fodrin (gift from J. Nelson) and ankyrin (gift from E. Kordeli, Institut Jacques Monod, Paris, France).

## Results

The aims of this study have been *1)* to detect putative MRPs ("MRPs"), including minor ones, in cytosolic extracts of epithelial (MDCK) and fibroblastoid (A72) dog cell lines; *2)* to find out whether there exist cell-type specific "MRPs"; and *3)* (to prepare amounts of a number of MDCK "MRPs" sufficient for microsequencing as a first step towards their molecular characterization.

In order to render the last aim feasable, we have developed a method yielding large amounts of adherent MDCK cells grown to confluency. A total of 12 ten deck "Cell Factories" (Nunc) yielded 40 g of packed MDCK cells (approximately $1.5 \times 10^{10}$ cells). After cell disruption and clearing, 50 ml of

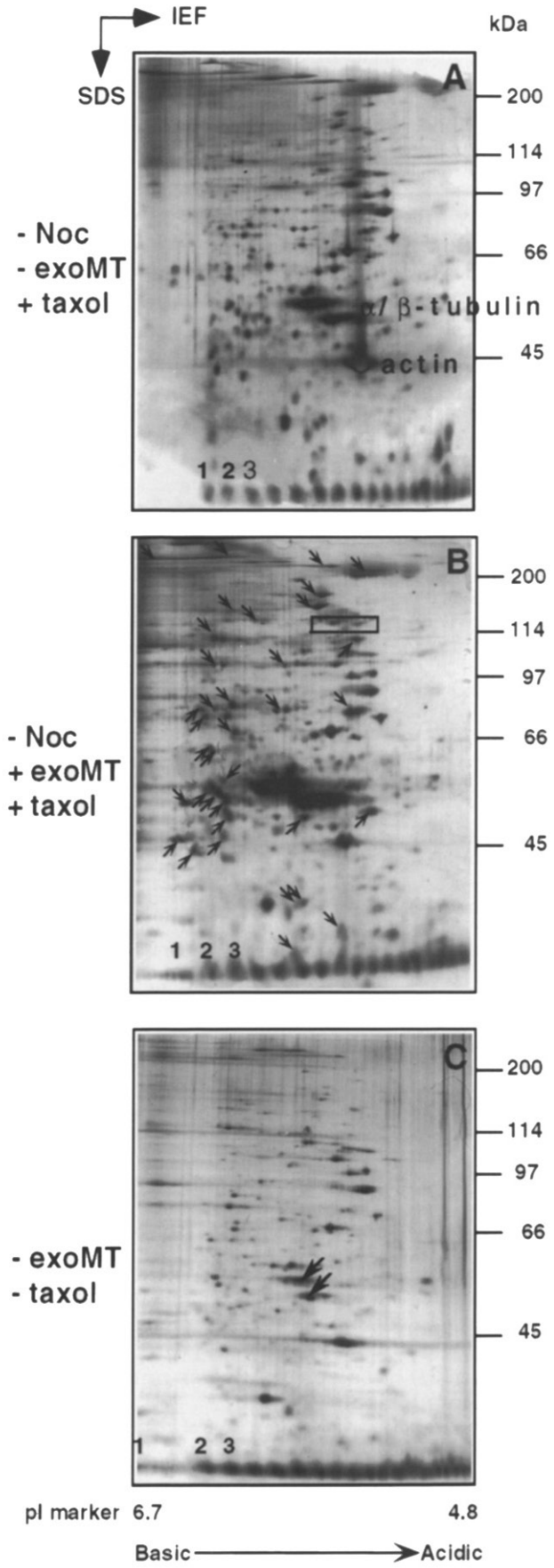



**Fig. 1.** Comparative 2D-electrophoretic analysis of crude MRPs obtained from MDCK cytosol under different conditions. Each gel shows silver stained proteins which sedimented through a sucrose cushion out of 600 µl of cleared MDCK cytosol (5 mg protein/ml) and were eluted from the pellet with 0.8 M KCl in ⅓ of the starting volume. In (**A**) the endogenous tubulin was polymerized by the addition of taxol. In (**B**) exogenous taxol-stabilized MTs and taxol were added to the cytosol. In (**C**) the polymerization of the endogenous tubulin was inhibited by nocodazole. Because of some instability of the pH-gradient at the basic end of the gel, the positions of the first three pI marker spots are numbered. In (**B**), the *arrows* indicate proteins which sediment in a largely MT-dependent fashion, and whose amount increases upon the addition of exogenous MTs. The amounts of unmarked proteins in the three gels were only slightly increased by the presence of MTs. Their amount was variable between different experiments (compare with Figs. 2A, 3 and 4).

cytosol at a protein concentration of around 15 mg/ml was obtained. For the binding assays, the cytosol was further diluted to 5 mg protein/ml, cleared again and used at once. For the probing for "MRPs", we first tried the well established method of adding taxol to the cytosol to induce the polymerization of the endogenous tubulin, pelleting the MTs and co-sedimenting proteins through a sucrose cushion, and eluting the "MRPs" with salt [52]. In order to obtain a more concentrated eluate, we used the salt solution at 1:5 of the starting cytosol volume. The protein mixtures were analyzed by mini-2D gel electrophoresis. Figure 1 A shows a silver stained MT eluate from MDCK cells. About 80 spots are visible. They were termed crude "MRPs".

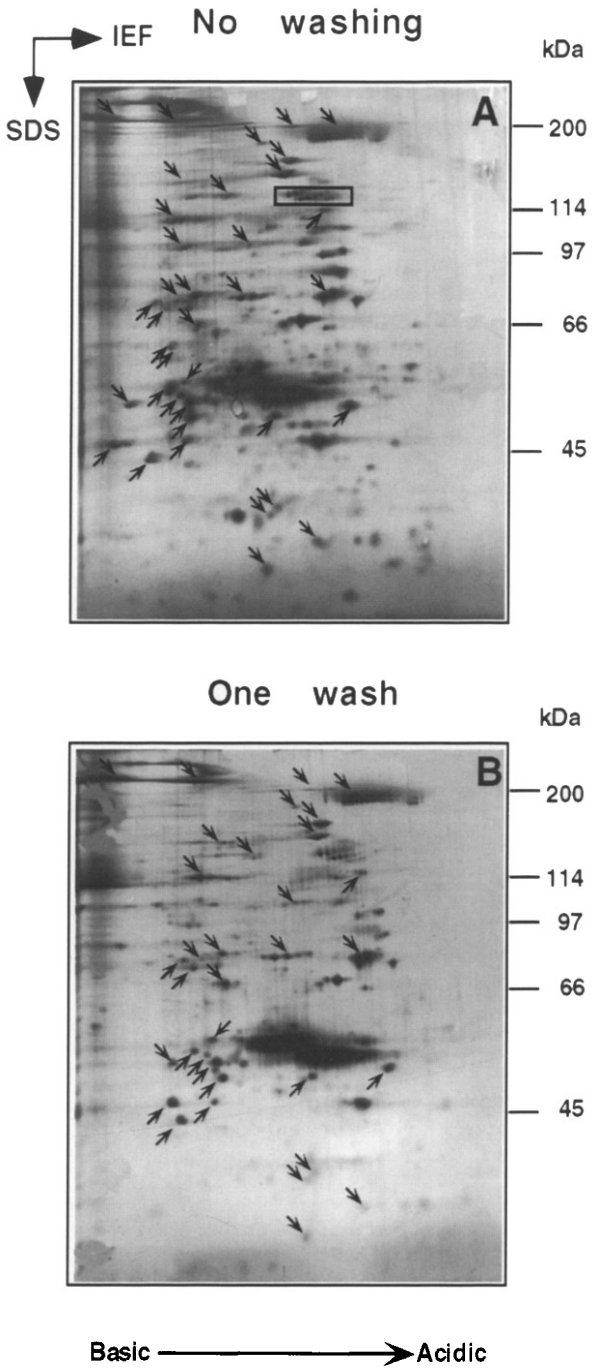



**Fig. 2.** Effect of washing of the crude MDCK MRP-MT pellet on the recovery of crude MRPs. (**A**): crude MRPs eluted from an unwashed pellet. (**B**): crude MRPs eluted from the same, washed pellet. The assay was performed as in Fig. 1B. The MT pellet was washed once by resuspending it in twice the initial cytosol volume of HBC and repelleting before salt elution of the crude MRPs in ½ of the initial cytosol volume. Comparison of the gels shows that many proteins are lost or diminished upon washing, whereas others are hardly affected. The latter, marked in Fig. 2B, largely correspond to the proteins identified in Fig. 1B which are also marked in Fig. 2A.

In order to investigate which of the crude "MRPs" were co-sedimenting because they were directly or indirectly attached to the MTs, and which proteins were recovered as non-specific background we have used several tests.

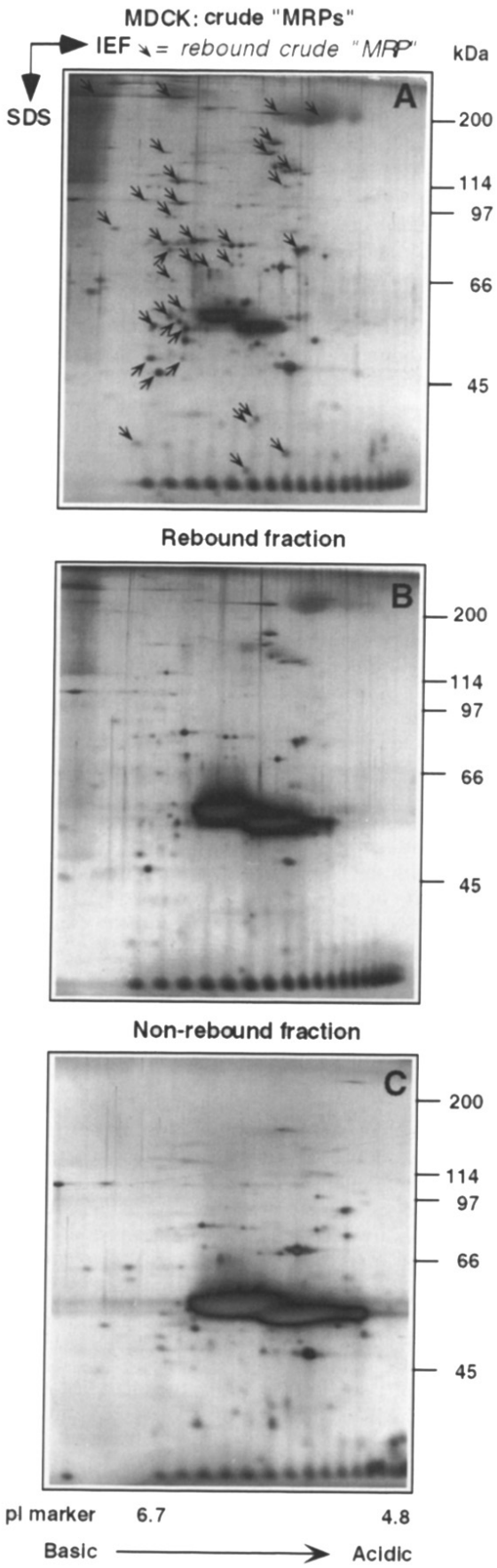
We have analyzed the effect of the addition of a large amount of exogenous MTs to the cytosol on the protein pattern of the crude "MRPs" (Fig. 1B). This addition did not significantly change the qualitative protein pattern of the crude MRPs. Only a series of closely spaced spots with a molecular mass of around 120 kDa (surrounded by a rectangle) newly appeared. Interestingly, a remarkable enrichment of several of the proteins seen in Fig. 1A was observed, while many others were not or much less enriched. The former were marked by arrows.

In order to see which of the crude "MRPs" would sediment in the absence of MTs, we added nocodazole to the cytosol to inhibit tubulin polymerization. In Fig. 1C, proteins salt eluted from the small pellet formed after sedimenting through the sucrose cushion are shown. Their number was clearly lower than in the presence of MTs (Figs. 1A and B). The presence of a small amount of tubulin (thick arrows) indicates that some MT assembly took place. Comparison of the protein pattern of Figs. 1B and C shows that most of the marked proteins do not sediment in the absence of MTs. A few are present in small, but significant amounts in Fig. 1C. They could have been associated with the small amount of tubulin recovered in the eluate, in spite of the presence of nocodazole.

We also tested the effect of washing of the crude "MRPs" shown in Fig. 1B, prior to elution with salt (see Material and methods). Figs. 2A and B show the crude "MRPs" recovered from the same binding experiment without (A) and after washing (B). In Fig. 2A, the same proteins as in Fig. 1B are marked. Numerous proteins were almost quantitatively retained in the MT pellet upon washing. Comparison of the spots marked by arrows in Figs. 2A and 1A, B with those in Fig. 2B, which correspond to proteins not or only moderately eluted by washing, shows an excellent correspondence between the marked proteins. As a more stringent test of MT-dependent co-sedimentation of crude "MRPs" with MTs, we tested their ability to co-sediment with newly added MTs upon dialysis (Fig. 3). Proteins which were recovered in the rebound fraction for more than 50 % were labeled by an arrow and named rebound "MRPs". Comparison of these proteins with those identified in Figs. 1 and 2 also shows an excellent correspondence.

Collectively, these tests allow for the identification of putative MRPs ("MRPs"). Those of MDCK cells are shown in Fig. 4A.

A similar rebinding test has been used to assign which of the A72 crude "MRPs", eluted from MT pellets largely made of brain tubulin, were "MRPs". In Fig. 4B, the proteins identified as rebound "MRPs" (experimental data not shown) among crude A72 MRPs, obtained as in Fig. 1B, were marked by arrows and designated "MRPs".




## Comparison of "MRPs" of MDCK and A72 cells

The comparison of "MRPs" of MDCK and A72 cells (Figs. 4A, B) revealed that the number of "MRPs" in MDCK and A72 cells is similar, but that globally, they are more abundantly recovered from MDCK cytosol. Interestingly, around 20 "MRPs" were identified as being MDCK-specific (thick arrows in Fig. 4A), 20 as A72-specific (thick arrows in Fig. 4B) and 20 being shared by both cell types (thin arrows).

## MDCK and A72 cells contain a few high molecular weight "MRPs"

By analyzing the crude "MRPs" using high resolution 1D electrophoresis [5], a few additional proteins were further identified as "MRPs" (Fig. 5). Three and two HMW rebound "MRPs" are found in MDCK and A72 cells. One of these HMW-"MRPs" of MDCK cells displays heat stability. The ability to elute the smallest HMW-"MRP" with Mg-ATP (not shown) suggests that it may be the cytoplasmic dynein heavy chain. This was confirmed by its co-immunoprecipitation with an antibody to the dynein intermediate chain (not shown), and its sedimentation as a 19S particle in density gradients (see below). None of these HMW-"MRPs" reacted with monoclonal antibodies to brain MAP 1A or B (not shown), but this may be due to a lack of cross-reaction.

## Purification and microsequencing of MDCK "MRPs" identifies the actin-binding proteins ezrin and α-fodrin as MDCK-specific "MRPs"

With the exception of two proteins with molecular masses around 200 and 120 kDa and one HMW protein (Fig. 5) all other crude "MRPs" of MDCK cells are heat labile (Fig. 6). These heat stable "MRPs" are also found in A72 cells (see Figs. 4 and 5). We have exploited the fact that almost all the crude "MRPs" are heat labile for preparing large amounts of concentrated MDCK crude "MRPs". The position of heat labile crude "MRPs" in the gel after 2D gel electrophoresis and Coomassie blue staining did not change (Fig. 7A and B). The numbered arrows indicate "MRPs" that are well visible as Coomassie blue-stained spots. Six MDCK specific and four common "MRPs" were collected as Coomassie blue-stained spots from 80 gels for microsequencing, and three of these have been microsequenced (Fig. 8). Two of these show 100% homology with ezrin, a microfilament-associated protein (No. 6), and the α-chain of non-erythroid spectrin (fodrin) (No. 1), a protein of the cortical membrane-associated cytoskeleton. Interestingly, ezrin and fodrin were only found as

**Fig. 3.** Test for the capacity of MDCK crude MRPs to rebind to MTs upon dialysis. (**A**) Crude MRPs. (**B**) Rebound fraction. (**C**) Non-rebound fraction. Crude "MRPs" were microdialyzed against HBC, diluted 1:2 with HBC and incubated with pelleted taxol stabilized MTs as described in Fig. 1B. The suspension was pelleted, and the supernatant was recovered quantitatively. Proteins were salt eluted from the pellet, cleared and the supernatant was recovered quantitatively. For analytical 2D gel electrophoresis, two volumes of 2D sample buffer were added to each of the supernatant fractions, the samples were concentrated to the same volume, and analyzed by 2D electrophoresis. Proteins were considered rebound if at least 50% of them rebound to MTs. They are called rebound MRPs. Comparison with the proteins marked in Figs. 1B and 2A shows very good correspondence.

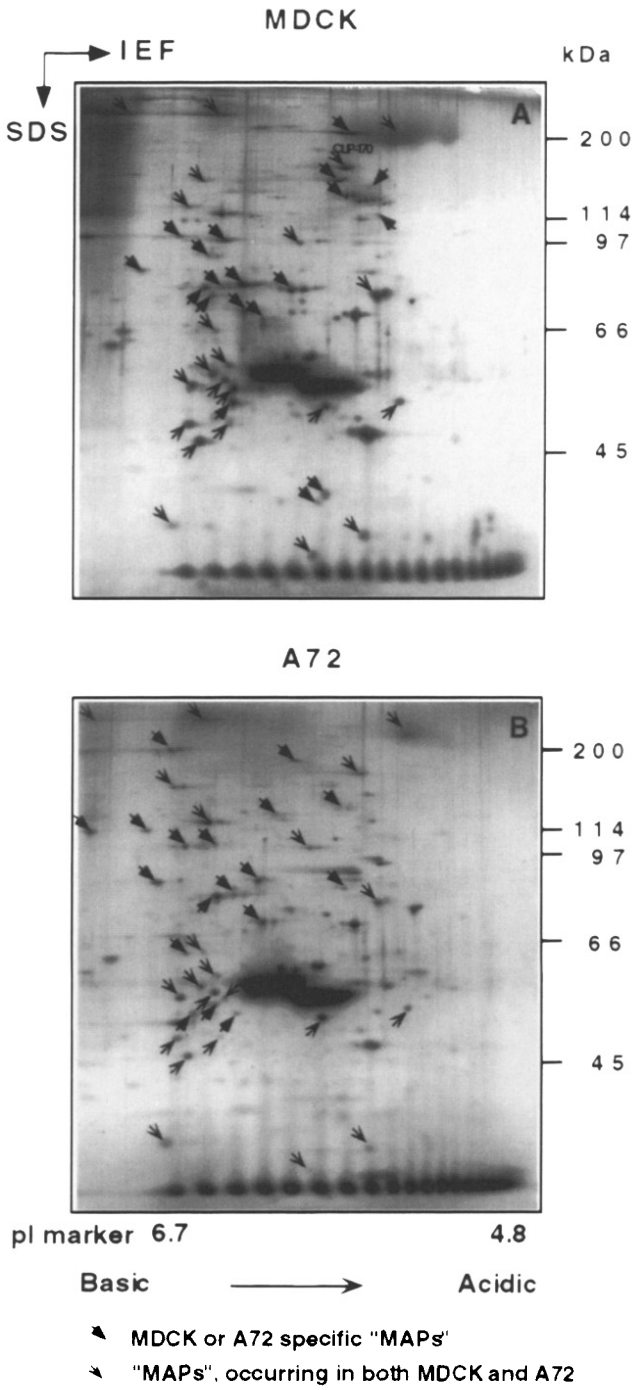



**Fig. 4.** 2D-electrophoretic comparison between MDCK "MRPs" and "MRPs" of the fibroblastoid cell line A72. (**A**) MDCK crude MRPs. (**B**) A72 crude MRPs. Crude MRPs were prepared as in Fig. 1B. The proteins marked by *arrows* are those marked in Figs. 1B, 2A, and 3A, and called "MRPs" to indicate that they have a good chance of being putative MRPs, i.e., proteins binding directly or indirectly to MTs in our in vitro tests. Comparison of the position of protein spots in the gels was done manually, making use of the size and pI markers present in each gel. *Thin arrows* indicate "MRPs" which are shared by both cell types. *Thick arrows* indicate "MRPs" which are unique to each cell type. Around 20 of each type of "MRPs" were found in both cell types.

"MRPs" in MDCK cells. The third protein (No. 8) shows high homology with the intermediate chain of rat brain cytoplasmic dynein. The latter is, as expected, found in both MDCK and A72 cells. Using specific antibodies, spot No. 2 (Fig. 7) has been identified as CLIP-170 [44], and the presence of Arp1, the major subunit of the dynactin complex [48], has been found by 1D Western blotting (see below).

## Immunofluorescence of co-sedimented MDCK ezrin along brain MTs

Ezrin (Fig. 9B) was visible as fine patches, distributed along the MTs (Fig. 9C). Patches were also found in the background (Figs. 9B, C). The linear arrangement of several of these suggests that they may have been associated with MTs lost during the preparation. Similar arrays of patches were stained by monoclonal anti-ezrin, but the background staining was insignifant (Fig. 9D). In this experiment, the MTs were less dense and shorter (not shown). These results show that ezrin sedimented by being associated with the MTs.

## Sucrose density gradient centrifugation of crude "MRPs"

In order to assess whether larger protein complexes were present in the crude "MRPs" fraction, and to study the sedimentation behavior of ezrin and fodrin, we separated the crude "MRPs" on a sucrose gradient (Fig. 10). The bulk of the pro-

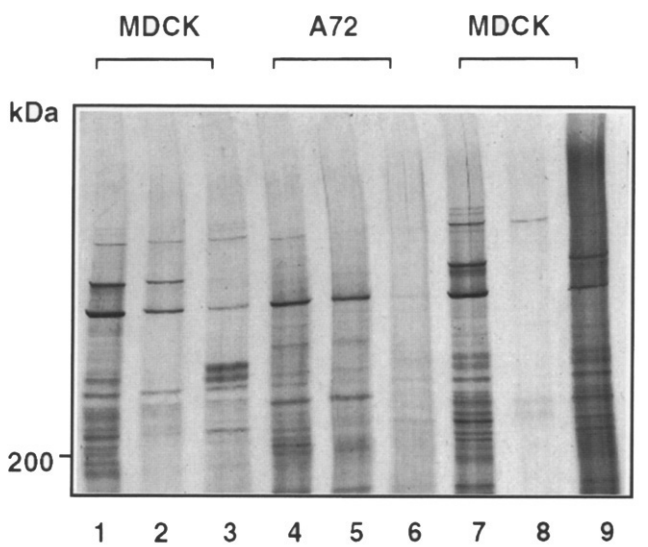



**Fig. 5.** 1D electrophoresis of high molecular weight proteins obtained as in Figs. 3 and 6. (*lanes 1–3, 7–9*): MDCK proteins; (*lanes 4–6*): A72 proteins. (*lanes 1 and 4*): crude MRPs; (*lanes 2 and 5*): rebound fraction; (*lanes 3 and 6*): non-rebound fraction; (*lane 7*): crude MRPs; (*lane 8*): heat stable proteins. (*lane 9*): heat labile proteins. Electrophoresis was carried out on 4% urea slab gels as described in [5]. Respectively three and two high molecular weight rebound "MRPs" are found in MDCK and A72 cells. The largest "MRP" of MDCK displays heat stability. The smallest "MRP" is eluted for >50% with Mg-ATP (not shown) and is probably cytoplasmic dynein heavy chain.

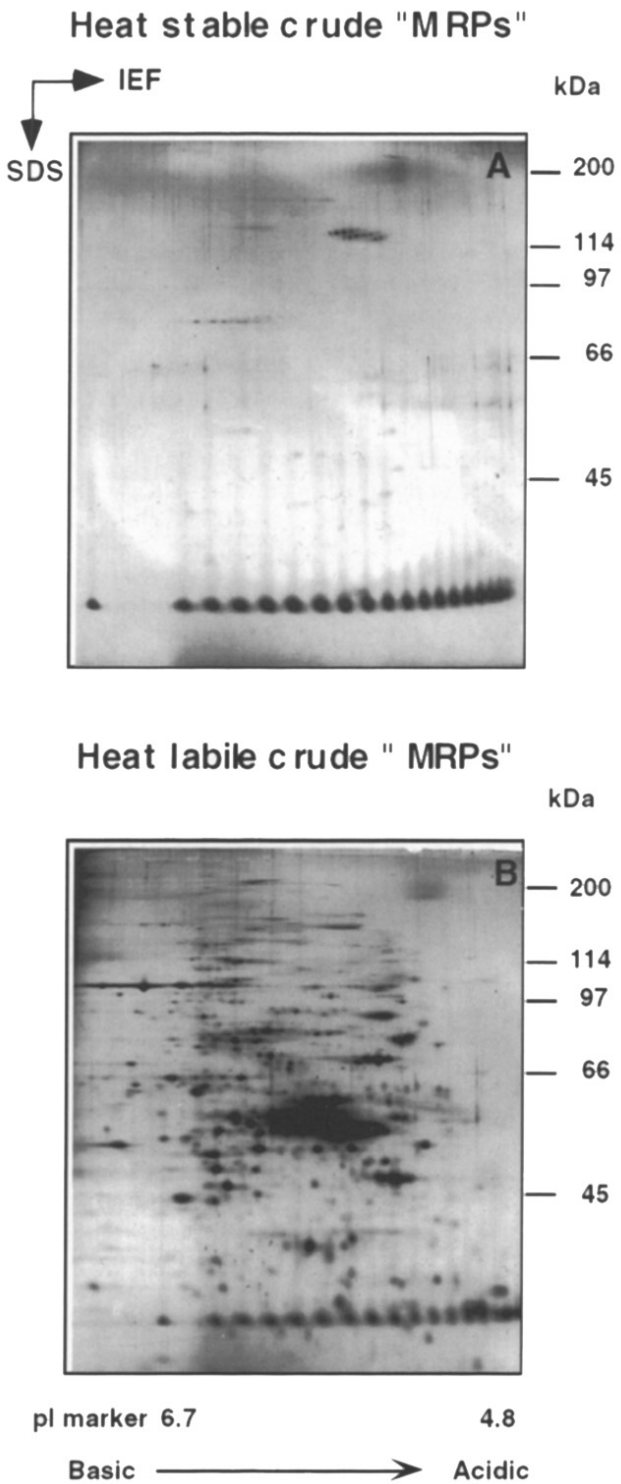



**Fig. 6.** Testing for heat stability of MDCK "MRPs". (**A**): Heat stable MDCK crude "MRPs". (**B**): Heat labile MDCK crude "MRPs". Crude "MRPs" were dialyzed and supplemented with 1 mM dithiothreitol. They were boiled for 5 min, cooled on ice and centrifuged at 30000*g* for 30 min at 4 °C to remove denatured protein. The resultant pellet and supernatant containing respectively the heat labile and stable fractions were processed for 2D electrophoresis. Only two proteins show heat stability.

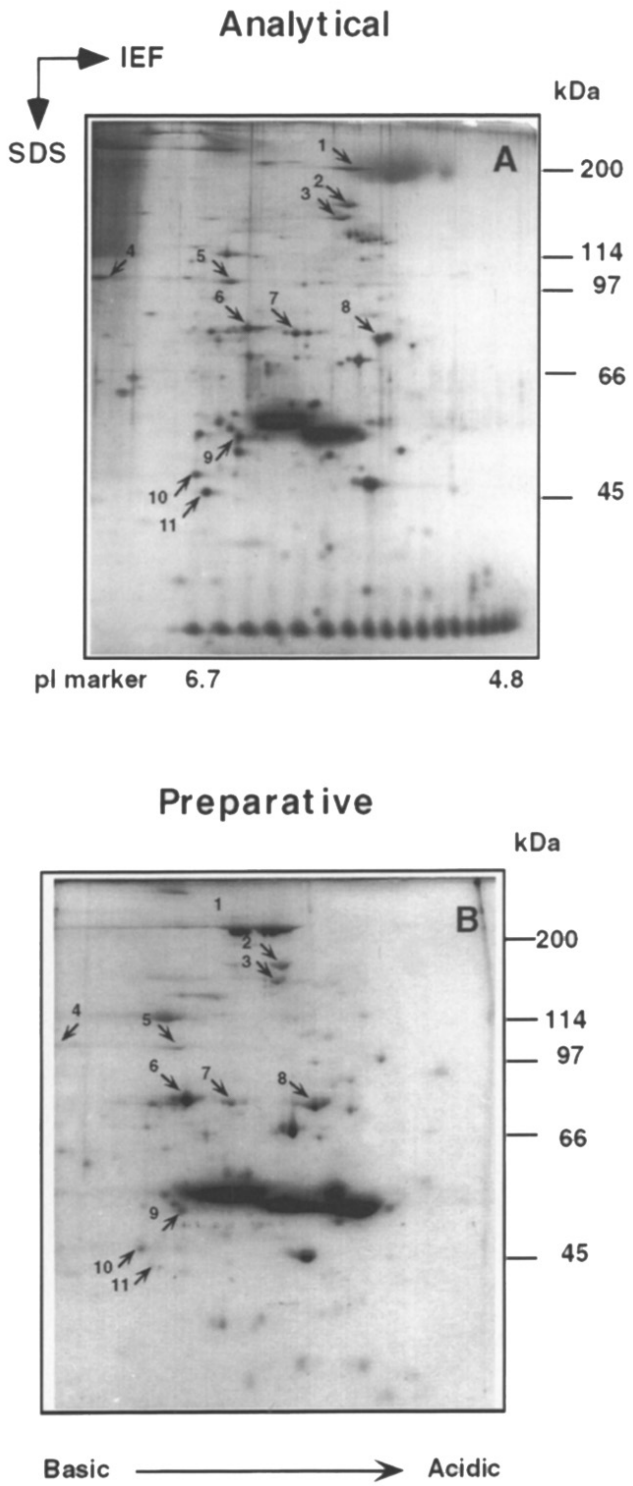



**Fig. 7.** Comparison of MDCK crude "MRPs" prepared by the analytical assay or by the preparative method. (**A**): Silver stained analytical crude "MRPs". (**B**): Coomassie blue-stained preparative crude "MRPs". Coomassie blue-stained spots corresponding to "MRPs" (see Fig. 4A), were identified and numbered.

Nr 1. **FODRIN**

G-N-A-M-V-E-E-G-H-F-A-A-E-D-V-K (100%)

Nr. 6. **EZRIN**

R-K-E-D-E-V-E-e-W-Q-H-R (100%)

Nr. 8. **CYTOPLASMIC DYNEIN INTERMEDIATE CHAIN (71 kDa)**

a) L-W-T-T-K (100%)

b) S-V-S-T-P-S-A-G-S-Q-D-D (100%)

c) E-A-V-A-P-V-Q-E-E-S-D-L-E-K (78%)

d) Q-R-L-A-Q-I-R-E-E-K (100%)

e) A-P-P-H-E-L-T-E-E-E-K (91%)

**Fig. 8.** Characterization of MDCK "MRPs" No. 1, 6 and 8 by microsequencing. Peptides from each digest, selected by mass spectrometry, were subjected to automated Edman degradation and their sequences compared with the information stored in protein sequence or DNA sequence data bases. The sequences are shown in the one-letter notation. Comparison is each time made with the human counterparts. For No. 1 and No. 6, where only limited sequence information was obtained, complete identity was noticed. For No. 8, 100% identity is observed for three peptides, while two other peptides show a high degree of identity (given in brackets).

teins present in the crude "MRPs" fraction were found in the light fractions (<7S). This was also the case for most of the ezrin and CLIP-170. As expected, cytoplasmic dynein was found in the heaviest fractions. Dynactin (as identified by anti-Arp1) peaked in the same heavy fractions. The behavior of Arp1, however, was abnormal, since part of it smeared over the entire gradient compatible with a dissociation of the complex during centrifugation. Fodrin sedimented at 11.5S, whereas ankyrin, also present in the crude "MRP" fraction, was found in the 6-7S fractions.

## Discussion

Our results identify around 80 proteins in cytosolic preparations of MDCK cells which co-sediment with MTs. Forty of these *a)* sediment in a MT-dependent fashion, *b)* are enriched by the addition of excess exogenous MTs, *c)* are largely retained on MTs upon washing, and *d)* rebind for at least 50% to new MTs after dialysis. Based on these criteria, we call these proteins putative MRPs ("MRPs"). A similar number of "MRPs" was identified in extracts of A72 cells. The co-sedimentation could be the result of a direct binding to MTs as expected for a potential MAP, or of an indirect association as a subunit of a protein complex interacting with MTs. The intermediate chain of cytoplasmic dynein identified here is an example of an indirect co-sedimentation through its association with the heavy chain of cytoplasmic dynein, a multiprotein complex shown to be present in the crude "MRPs" by sucrose density centrifugation. The same holds for Arp1, which is a subunit of the dynactin complex, which binds to MTs via its $p150^{Glued}$ subunit. The results also show that like for early Drosophila embryos and the yeast S. cerevisiae, MTs made of

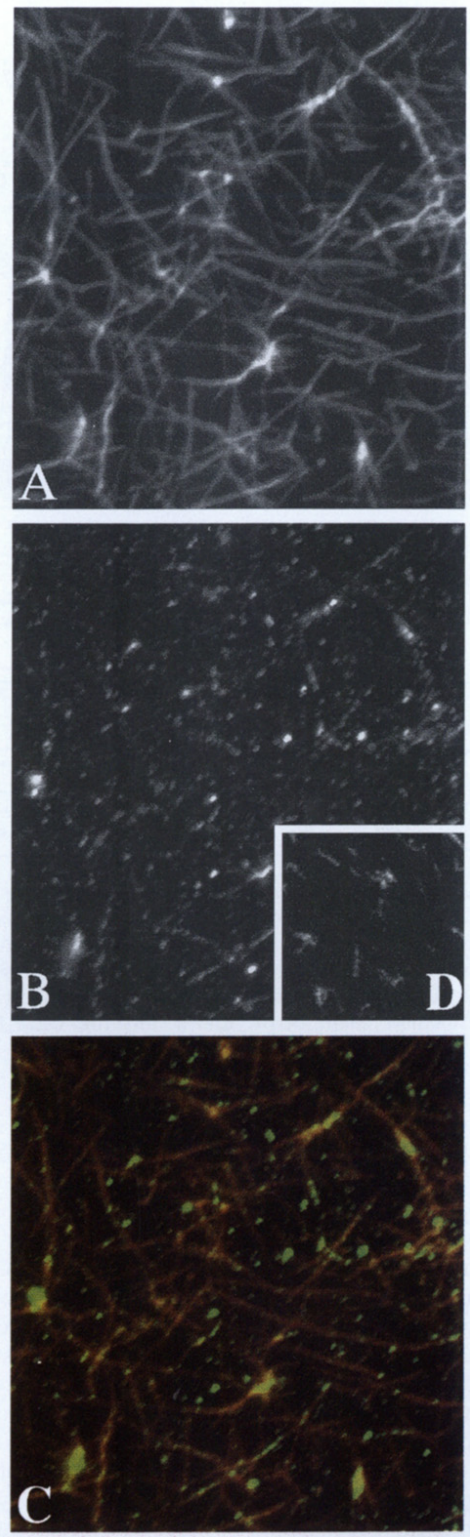



**Fig. 9.** Localization of MDCK ezrin along taxol-stabilized microtubules. Upon incubation of brain MTs and MDCK cytosol, the mixture was diluted 10 times in 0.25% glutaraldehyde and the microtubules were centrifuged on coverslips. These were labeled for tubulin (FITC, **A**) and ezrin (Cy-3, **B**) or for ezrin alone (CY-3, **D**) and mounted. Panel **C** shows the combination of the signals in (**A**) and (**B**) after pseudocoloring, whereby the contrast of the MTs was kept dim to better show ezrin associated along MTs. Images were taken using a conventional fluorescence microscope equipped with a cooled CCD camera.

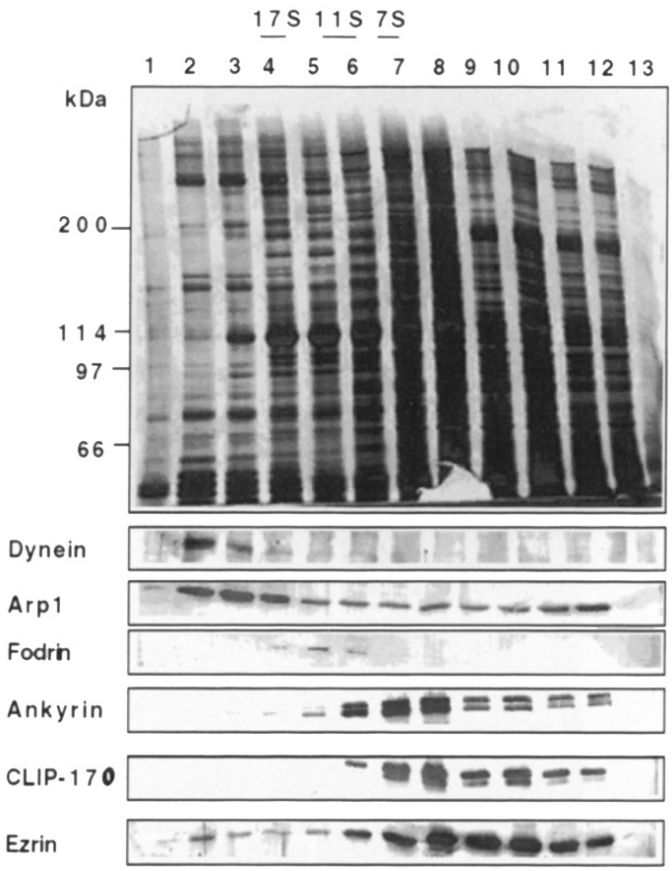



**Fig. 10.** Sucrose density gradient centrifugation of crude MDCK "MRPs". A crude MDCK "MRPs" fraction was separated on a sucrose gradient and proteins of 13 fractions separated on 8 or 6 % PAGE slab gels. These were either silver stained (Top, 8 % gel), or transferred to nitrocellulose for Western blotting with antibodies to cytoplasmic dynein intermediate chain, Arp1, fodrin, ankyrin, CLIP-170, and ezrin.

brain tubulin can substitute for the endogenous tubulin [4, 29]. They further show the interest of using an excess of such MTs for optimizing the recovery of "MRPs" in view of their purification.

The number of "MRPs" reported here is considerably higher than that of previous studies of mammalian cell lines and is very similar to the number found in early Drosophila embryos [29]. This illustrates the progress made possible by our method. We are aware that proteins which co-sediment specifically and saturably with MTs in vitro are not necessarily of physiological significance for MT functioning in living cells. On the other hand, genuine MAPs or MRPs do not always need to be associated with MTs in living cells or may not be present along the entire length of MTs, but instead near their ends or localized at peripheral cues. For example, the cytoplasmic linker protein CLIP-170 shows an accumulation at peripheral MT segments, where it may link endocytic compartments to MTs [32, 44, 45], and we have found that dynein and dynactin co-distribute in cortical sites which capture aster MTs during mitosis (D. Dujardin et al., submitted). Some MAPs, like CP190 and $p150^{Glued}$ are subunits of multiprotein complexes [31, 48] which are most of the time not associated with MTs. Many of the proteins described in the Drosophila [29] and yeast study [4] have been shown to display an association with a variety of structures which play a role in MT organization (centrosome, kinetochore, spindle pole, midbody) merely indicating a role in MT functioning. In view of these precedents, several of the "MRPs" in our study can be expected to be of significance for MT functioning.

The existence of numerous epithelia- and fibroblast-specific "MRPs" supports the idea that MT systems of a wide variety of cell-types are regulated by a set of specific MRPs [4, 29]. It also indicates that different cell-types control their MT network at the level of gene expression, by regulating both qualitatively and quantitatively the expression of MRPs.

The new "MRPs" we have identified are known proteins, and their intracellular localization has been documented. Microsequencing of the remaining "MRPs" and characterizing possible MT-related functions for ezrin and fodrin will provide us with several opportunities on the way to a better characterization of the MT system of epithelial cells. Establishing the possible role of any new "MRP" in MT functioning often requires extensive functional analysis. Work is in progress to assess whether ezrin and fodrin play such a role. We further plan to clone those "MRPs" that will turn out to be new proteins and to characterize the in vivo function at the cellular and molecular level. It will be of particular interest to determine their intracellular localization and to establish whether they interact either directly with MTs or indirectly through a multiprotein complex. In the latter case, the identification of other subunits could be of great use to identify the MAP responsible for the MT co-sedimentation of the protein complex.

The identification of ezrin (a protein of the ERM (ezrin, radixin, moesin) protein family) [1, 50, 51] as a putative MRP is unexpected but seems very interesting, since this is only the case in MDCK cells. In simple epithelia, ezrin is associated with the microvilli at the apical pole [2]. Our data clearly establish the capacity of ezrin to specifically interact with MTs: *1)* we show here that a fraction of the cytoplasmic ezrin pool co-sediments specifically with MTs; *2)* the co-sedimenting ezrin is detectable along these by immunofluorescence. The result from the density gradient analysis indicates that the ezrin recovered in the crude "MRPs" fraction is much heavier than monomeric ezrin (3.5S). It could be a subunit of a multiprotein complex, or be oligomeric. Our data do not allow to distinguish between these possibilities and thus, the interaction of ezrin with MTs may be direct or indirect. Previous reports have suggested that ERM proteins play a role in certain aspects of MT functioning. Ezrin and radixin are present in a MT-based structure: the marginal band of chicken erythrocytes [58]. A 78 kDa chicken erythrocyte plasma membrane protein binds tubulin in a Western blot overlay assay, colocalizes with the microtubular marginal band [49], and reacts with monospecific antibodies to ezrin (R. Cassoly, personal communication), indicating a direct interaction between ezrin and tubulin. Thus, at least in this cell-type, ezrin could function as a genuine MRP. In the growth cone of hippocampal neurons too, a protein of the ERM family depends upon MTs [18] for its localization in the cell cortex. In polarized MDCK cells, we have observed that non-centrosomal MTs, displaying a high level of acetylated tubulin, form a dense network just beneath the apical plasma membrane (P. Guillaud and J. De Mey, unpublished data). The MTs of the marginal band of chicken erythrocytes are also acetylated. We speculate that a particular form of ezrin, localized in the plasma membrane domain, may be involved in maintaining the apical localization of these

MTs, much like it may function in the marginal band of erythrocytes.

The identification of the α-chain of non-erythroid spectrin (fodrin) [53] as an MDCK-specific "MRP" seems also very interesting. Recently, it has been shown that it is essential for the maintenance of epithelial cell morphology [24]. Fodrin can bind to cytoplasmic membranes by directly interacting with inositol lipids through its pleckstrin homology domain [25], and by binding to the integral membrane protein CD45 [26]. Our data establish that the fodrin recovered as a "MRP" in MDCK extracts interacts specifically with the MTs used as an affinity matrix and sediments as a 11.5S particle, larger than the spectrin tetramer. It may therefore be a subunit of a multiprotein complex. It has been shown that brain spectrin may associate with dynactin via the Arp1 filament of the complex [23]. The fodrin-containing protein complex studied here, however, clearly does not co-sediment with the dynein/dynactin complex, as was shown in a brain extract [23]. At the plasma membrane of MDCK cells, fodrin forms complexes with ankyrin and the transmembrane proteins, $Na^+/K^+$-ATPase or E-cadherin [40]. We show here that ankyrin, present in the crude "MRPs" fraction, is not part of the fodrin-containing complex. We tentatively propose that the fodrin form studied here co-sediments with MTs in an indirect way, possibly through its association with a MAP. A Tau-like MAP may play this role. Indeed, in vitro, erythroid spectrin binds to the brain MAP, Tau protein [10]. The fodrin-containing fractions of the sucrose density gradient will be further analyzed to test this possibility. Additional reports have also raised the possibility that fodrin plays a role in MT functioning. It can bind to MTs in vitro and bundle them [27]. In addition, it co-purifies with brain MTs obtained by direct isolation into a stabilizing buffer [12, 13].

Future work is aimed at testing whether these results have identified elements of the signaling between the cell surface and the MTs.

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