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A microtubule-interacting protein involved in coalignment of vimentin intermediate filaments with microtubules

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SUMMARY

A protein of M_r 210 000 was identified in 3T3 cells by immunoblotting and by immunoprecipitation with a monoclonal antibody MA-01. The protein was thermolabile and was located on 3T3 microtubules prepared by taxol-driven polymerization in vitro. On fixed cells the MA-01 antigen was located on interphase and mitotic microtubular structures, vinblastine paracrystals, taxol bundles and colcemid-resistant microtubules. Microinjection experiments with purified MA-01 antibody followed by double immunofluorescence have shown that the injection of antibody led to disruption of vimentin filaments, whereas the distribution of cytoplasmic microtubules was unchanged. The collapse of vimentin filaments started 30 minutes after injecting the antibody at immunoglobulin concentrations 2 mg ml^{-1} or higher and reached its maximum 3-6 hours after the injection. Within 20 hours after the injection vimentin filaments became reconstituted. Microinjection of the antibody into cells pre-treated with vinblastine resulted in localization of the MA-01 antigen on vinblastine paracrystals as well as on coiled vimentin filaments. The data presented suggest that the MA-01 antigen is a new microtubule-interacting protein that mediates, directly or indirectly, an interaction between microtubules and vimentin intermediate filaments.

Key words: microtubules, vimentin filaments, microtubule-interacting protein

INTRODUCTION

The cytoskeleton of mammalian cells is a complex filamentous assemblage of actin microfilaments, microtubules and intermediate filaments in association with accessory proteins. Intermediate filaments of type III (i.e. vimentin, desmin or glial fibrillary acidic protein) form, in cultured cell lines, extended networks that are partially coaligned with microtubules (Geiger and Singer, 1980; Ball and Singer, 1981; Traub, 1985). Several lines of evidence indicate that these two cytoskeletal systems, although composed of distinct polymers, are physically interconnected and that the morphology of intermediate filaments depends on microtubules. Thus, disruption and redistribution of microtubules induced by microtubule-acting drugs, such as colchicine, nocodazole, vinblastine and taxol, resulted in a coil of intermediate filaments (Osborn et al., 1980; Geuens et al., 1983; Forry-Schaudies et al., 1986); microinjection of antibodies against tubulin had a similar effect (Wehland and Willingham, 1983; Blose et al., 1984). On the other hand microinjection of antibodies against proteins of type III intermediate filaments (Klymkowsky, 1981; Gawlitta et al., 1981; Tölle et al., 1986) or intermediate filament-associated proteins (Lin and Feramisco, 1981) had no effect on distribution of microtubules, yet the intermediate filaments coiled up in the perinuclear region.

Little is known about the molecules involved in interactions between microtubules and intermediate filaments. Specific binding proteins on microtubules or intermediate filaments might be responsible for such interactions. Microtubule-associated proteins have been identified as components that stimulate tubulin polymerization and co-polymerize with microtubules during repeated cycles of assembly and disassembly in vitro (reviewed by Olmsted, 1986; Wiche, 1989). Among them the tau, MAP2 and MAP4 classes are characterized by the stability of corresponding fibrillar structural proteins at elevated temperatures. The largest number of proteins described have been attributed to the MAP4 class, particular members having molecular masses ranging from 180 to 240 kDa (reviewed by Olmsted, 1991). Other proteins display more transient interactions with microtubules, and include the microtubule-based motor proteins kinesin, cytoplasmic dynein and dynamin (reviewed by Vallee and Shpetner, 1990; Bloom, 1992), as well as proteins potentially involved in microtubule-membrane interactions (Allan and Kreis, 1986; Bloom and Brashear, 1989; Mithieux and Rousset, 1989; Rickard and Kreis, 1990; Scheel and Kreis, 1991). Alternatively, intermediate filament-associated proteins may be capable of interlinking intermediate filaments and microtubules (reviewed by Steinert and Roop, 1988; Foisner and Wiche, 1991). It has been shown, for example, that plectin,

one of the best-characterized intermediate filament-associated proteins, binds to high molecular mass microtubule-associated proteins MAP2 and MAP1 (Herrmann and Wiche, 1987). Although there are many potential protein candidates that may play a role in connecting microtubules and vimentin filaments in cultured cells, data on the involvement of the corresponding proteins in these interactions in vivo are limited. More recent data showed that kinesin was involved not only in plus-end-directed movement of membraneous organelles along microtubules, but also in interaction between intermediate filaments and microtubules. Microinjection of anti-kinesin antibody into fibroblasts was found to cause a retraction of vimentin filaments from the peripheral cytoplasm, and the formation of dense, perinuclear aggregates (Gyoeva and Gelfand, 1991).

In the present study using monoclonal antibody MA-01 we have identified a thermolabile 210 kDa protein in 3T3 cells that was located on cellular microtubular structures as well as on microtubules prepared in vitro. Microinjection of the antibody caused reversible aggregation of all vimentin filaments within the cell in the perinuclear region without any apparent alteration of microtubules. In injected cells pre-treated with vinblastine the protein was found on paracrystals as well as on coiled vimentin filaments. The microinjection experiments indicate a role for the MA-01 antigen in mediating the interaction of microtubules with intermediate filaments.

MATERIALS AND METHODS

Materials

Taxol was a generous gift from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Vinblastine was purchased from Gedeon Richter (Budapest, Hungary) and colcemid from CIBA (Basel, Switzerland). Molecular mass markers and polybrene were from Sigma Chemie (Deisenhofen, Germany), Mowiol 4-88 from Calbiochem AG (Lucerne, Switzerland). Chemical reagents used were of analytical grade.

Antibodies

Preparation of mouse monoclonal antibodies MA-01, TU-04 and VI-01 was described elsewhere (Dráberová et al., 1986a,b; Dráber et al., 1989). The MA-01 antibody (IgG_1) was raised against porcine brain microtubule-associated protein MAP-2; it cross-reacts with 210 kDa protein in different non-neuronal cells of various species (Dráberová et al., 1986b; Hašek et al., 1992). The TU-04 antibody (IgM) is directed against the antigenic determinant on -tubulin (Dráber et al., 1989) and the VI-01 antibody (IgM) reacts with vimentin (Dráberová et al., 1986a; Lukáš et al., 1989). The monoclonal antibody HTF-14 (IgG_1) against human transferrin (Viklický et al., 1983) was used as a negative control. Antibodies of the IgG class were purified from ascitic fluid by DEAE-chromatography following ammonium sulphate precipitation (Harlow and Lane, 1988); IgM antibodies were precipitated by polyethylene glycol and purified by hydroxylapatite chromatography (Stanker et al., 1985). The antibody purity attained was higher than 80% as assessed by SDS-PAGE. The antibodies TU-04 and VI-01 were labelled with rhodamine B sulphonyl chloride according to Brandtzaeg (1973). For microinjection experiments the antibodies were extensively dialysed against microinjection buffer containing 114 mM KCl, 20 mM NaCl, 3 mM $MgCl_2$, 3 mM NaH_2PO_4, pH 7.0 (Klymkowsky, 1981). The rabbit antibody against MAP4 proteins of primates was a generous gift from Dr J. C. Bulinski, Columbia University, New York (Bulinski and Borisy, 1980). Swine anti-mouse IgG antibody was kindly donated by Dr F. Franék (Institute of Molecular Genetics, Prague). Goat anti-mouse Ig antibody conjugated with alkaline phosphatase was purchased from Promega Biotec (Madison, WI), FITC-conjugated anti-mouse Ig antibody was from Sevac (Prague, Czech Republic).

Tissue culture

Mouse embryonal 3T3 cells or human embryonal LEP cells were grown at 37°C in air with 5% CO_2, in RPMI-1640 medium supplemented with 3 mM L-glutamine, 1 mM sodium pyruvate, penicillin (100 i.u. ml^{-1}), streptomycin (100 μg ml^{-1}), gentamycin (20 μg ml^{-1}) and 10% (v/v) heat-inactivated bovine serum. To some of the cultures vinblastine or taxol at 10 mM were added for 18 hours and colcemid at 10 μM for 1 hour.

Preparation of microtubule protein

3T3 cells were harvested by a rubber policeman into RPMI-1640 medium, centrifuged at 500 ***g*** for 10 minutes, washed twice in PBS at 4°C and once at 4°C in PEM buffer (100 mM K-Pipes, 2 mM EGTA, 1 mM $MgSO_4$, pH 6.8). The washed cell pellet was left to swell in 5 vols of ice-cold 2 mM EGTA, 1 mM $MgSO_4$, pH 6.8, for 4 minutes and then pelleted at 500 ***g*** for 10 minutes at 4°C. The supernatant was aspirated and cells were resuspended in PEM buffer (2 vols of the original cell pellet) containing proteinase inhibitors (1 μg ml^{-1} each of aprotinin, leupeptin, antipain, pepstatin, 100 μg ml^{-1} of soybean trypsin inhibitor and 1 mM PMSF). Cells were then homogenized with a Teflon-on-glass homogenizer to achieve more than 70% cell breakage, as evaluated by the trypan blue exclusion test. The resulting homogenate was centrifuged at 40,000 ***g*** for 10 minutes (4°C) and the supernatant was further centrifuged at 200,000 ***g*** for 90 minutes (4°C). The high-speed extract was polymerized in the presence of 20 μM taxol and 1 mM GTP for 15 minutes at 37°C and 15 minutes at 4°C. In some preparations GTP was not included in the polymerization mixture. Microtubules were spun down at 30,000 ***g*** for 30 minutes (4°C) through a 10% (w/v) sucrose cushion in PEM containing 20 μM taxol and 1 mM GTP (Vallee, 1986). The microtubules were washed twice by resuspending them in cold PEM with 2 μM taxol and 1 mM GTP (0.2 vol. of the high-speed supernatant), and spinning them down at 30,000 ***g*** for 30 minutes (4°C). To prepare heat-stable fraction of 3T3 or LEP cells, NaCl and 2-mercaptoethanol were added to the high-speed extract at final concentrations of 0.8 M and 1% (v/v), respectively, and the extract was boiled at 100°C for 3 minutes. After chilling in an ice-water bath, the heat-treated extract was centrifuged at 12,000 ***g*** for 30 minutes at 4°C to remove denatured proteins. The supernatant was dialysed against water and lyophilized.

Immunoprecipitation and immunoblotting

Swine anti-mouse antibody was covalently coupled to CNBr-activated Sepharose 4B at a ratio of 5 mg protein ml^{-1} gel according to the manufacturer's directions and the gel was equilibrated in TBST (10 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween-20). Then 150 μl of sedimented gel with immobilized anti-mouse antibody was incubated with rocking, at 4°C for 2 hours with 1 ml of MA-01 antibody at a concentration of 1 mg ml^{-1} in TBST or with HTF-14 antibody at the same concentration. The beads were pelleted by centrifugation at 4,000 ***g*** for 30 seconds and washed four times (5 minutes each) in cold TBST. The beads were further incubated with rocking for 2 hours at 4°C with 1 ml of 3T3 high-speed extract, prepared as for the isolation of microtubules

and diluted 1:1 with TBST. The beads were pelleted and washed four times, for 5 minutes each, in cold TBST before boiling for 3 minutes in 100 μl of SDS-sample buffer to release bound proteins.

SDS-electrophoresis was carried out according to the method of Laemmli (1970) and the separated proteins were transferred onto nitrocellulose sheets by electroblotting (Towbin et al., 1979). Details of the immunostaining procedure using secondary antibody labeled with alkaline phosphatase are described elsewhere (Dráber et al., 1988). The monoclonal antibody MA-01 was used as ascitic fluid diluted $1:10^5$, the rabbit anti-MAP4 antibody was diluted $1:10^4$, the secondary antibody was diluted 1: 7,500.

Microinjection

Microinjection of antibodies into the cells was carried out according to Graessman and Graessman (1976) using an inverted Opton Axiovert 35 microscope and Opton micromanipulator MR. Glass capillaries were about 1 μm diameter and pressure was applied by means of a 20 ml syringe. Approximatelly one tenth of a cell volume was injected with purified antibodies at concentrations ranging from 0.5 to 14 mg ml^{-1}. Typically, 50 cells were injected on each coverslip within 5 minutes. Injected cells were incubated at 37°C for various time periods prior to extraction and fixation. Cytoskeleton preparations were processed for double-label immunofluorescence. In some cases the antibody was injected into cells pre-treated with vinblastine. During microinjection cells were kept in a medium containing vinblastine. The cells were thereafter incubated for 1 hour in the same medium and subsequently fixed.

Immunofluorescence

Extraction and fixation steps were carried out in a microtubule stabilizing buffer (MSB) consisting of 0.1 M K-Mes, 2 mM EGTA, 2 mM $MgCl_2$, 4% polyethylene glycol 6000, pH 6.9. Fixed cytoskeletons were prepared as described (Dráber et al., 1989). Briefly, cells grown on coverslips were routinely extracted for 1 minute with 0.2% Triton X-100 at 37°C and fixed for 30 minutes in 3% formaldehyde at the same temperature. Alternatively, cells were first fixed for 30 minutes in 3% formaldehyde before extraction for 4 minutes with 0.5% Triton X-100 at 37°C; or cells were fixed at 37°C for 3 minutes in 0.25% glutaraldehyde/0.5% Triton X-100 mixture, followed by 20 minutes of incubation at the same temperature in 1% glutaraldehyde and then treated three times (5 minutes each) in 0.5 mg ml^{-1} $NaBH_4$. Cells were also fixed for 10 minutes in methanol followed by 6 minutes in acetone at −20°C. Taxol-stabilized cytoskeleton was prepared by extracting the cells for 4 minutes at 37°C with 0.2% Triton X-100 in MSB containing 10 μM taxol; further incubation for 3 hours in MSB at 37°C preceded the formaldehyde fixation. The buffer was changed three times during the incubation period.

For staining of isolated 3T3 microtubules, the coverlips were treated with 0.02% polybrene and inverted over a drop of microtubules resuspended in MSB, at a protein concentration of 2 mg ml^{-1}. The slides were incubated at 37°C for 10 minutes and gently washed with MSB. The preparations were then fixed by 3% formaldehyde in MSB for 10 minutes, washed in MSB and used for immunostaining.

All antibody dilutions were made in 2% BSA in PBS, all subsequent incubations were at room temperature. Fixed cytoskeletons or isolated microtubules were incubated for 30 minutes with MA-01 antibody at a concentration of 5 μg ml^{-1}, washed (three times, 5 minutes each) in PBS, incubated for 30 minutes with FITC-conjugated anti-mouse Ig antibody (dilution 1:30) and washed again. For double-label immunofluorescence the remaining binding sites on the FITC-conjugated antibody were blocked by 30 minutes of incubation with normal mouse serum diluted 1:10, prior to 30 minutes of incubation with rhodamine-conjugated anti-tubulin or anti-vimentin antibodies diluted, respectively, 1:100 or 1:50.

The slides were mounted in Mowiol 4-88 and examined with a Leitz Orthoplan microscope equipped with a 63/1.30 Fluoreszenz oil-immersion objective, epi-illumination using the filter combination cubes N_2 and I_2, and an Orthomat 35 mm camera. Photographs were taken using Kodak Tri-Xpan film and printed on hard paper. Neither the control antibody HTF-14 nor the FITC conjugates alone gave any detectable staining.

RESULTS

Immunofluorescence colocalization of MA-01 antigen with microtubules

The mouse monoclonal antibody MA-01, originally raised against porcine brain MAP2 proteins, reacts with MAP2 proteins in brains of various species (Dráberová et al., 1986b; Kuznetsov et al., 1986). When used for double-label immunofluorescence staining of fixed 3T3 cells, this antibody stained cytoplasmic microtubules in interphase cells (Fig. 1A,B). The antigen was found, within the limit of detection of immunofluorescence microscopy, in all cytoplasmic microtubules with uniform distribution lengthwise. In mitotic cells, MA-01 antibody clearly labeled mitotic spindles and midbodies (Fig. 1C). Because in double-label indirect/direct immunofluorescence we applied two mouse monoclonal antibodies (one of them was conjugated with rhodamine), normal mouse serum was used to block the remaining binding sites on FITC-conjugated secondary anti-mouse antibody. As shown in Fig. 1C,D, the serum effectively blocked the remaining binding sites, and mitotic spindles with midbodies were not visualized with rhodamine-conjugated anti-vimentin antibody used in double-label staining. The strongest immunofluorescence with MA-01 antibody was seen in 3T3 cells extracted with 0.2% Triton X-100 and subsequently fixed with 3% formaldehyde. The microtubule staining pattern was however not changed when cells were first fixed with formaldehyde and then extracted with Triton X-100, or when glutaraldehyde/Triton X-100 fixation was used. The background staining was however higher. Since detergent pretreatment led to a clearer image of the microtubules, it is possible that a significant soluble cytoplasmic pool of the antigen is present in the cells. In methanol/acetone-fixed cells only microtubule organizing centres were intensely labeled but there was, in addition, homogeneous diffuse staining. This indicates that the availability of the corresponding antigenic determinants on microtubular structures depends on fixation conditions. To exclude the possibility that microtubular staining with MA-01 antibody results from binding of the soluble cytoplasmic proteins to cytoskeletal structures during the fixation process, cells were first extracted with 0.2% Triton X-100 in MSB containing 10 μM taxol and the resulting cytoskeleton was incubated for 3 hours in MSB at 37°C before fixation. During this incubation MSB was changed three times. Staining of cytoskeleton prepared in this way with MA-01 antibody revealed a typical microtubular staining pattern (Fig. 1E,F); moreover, the staining

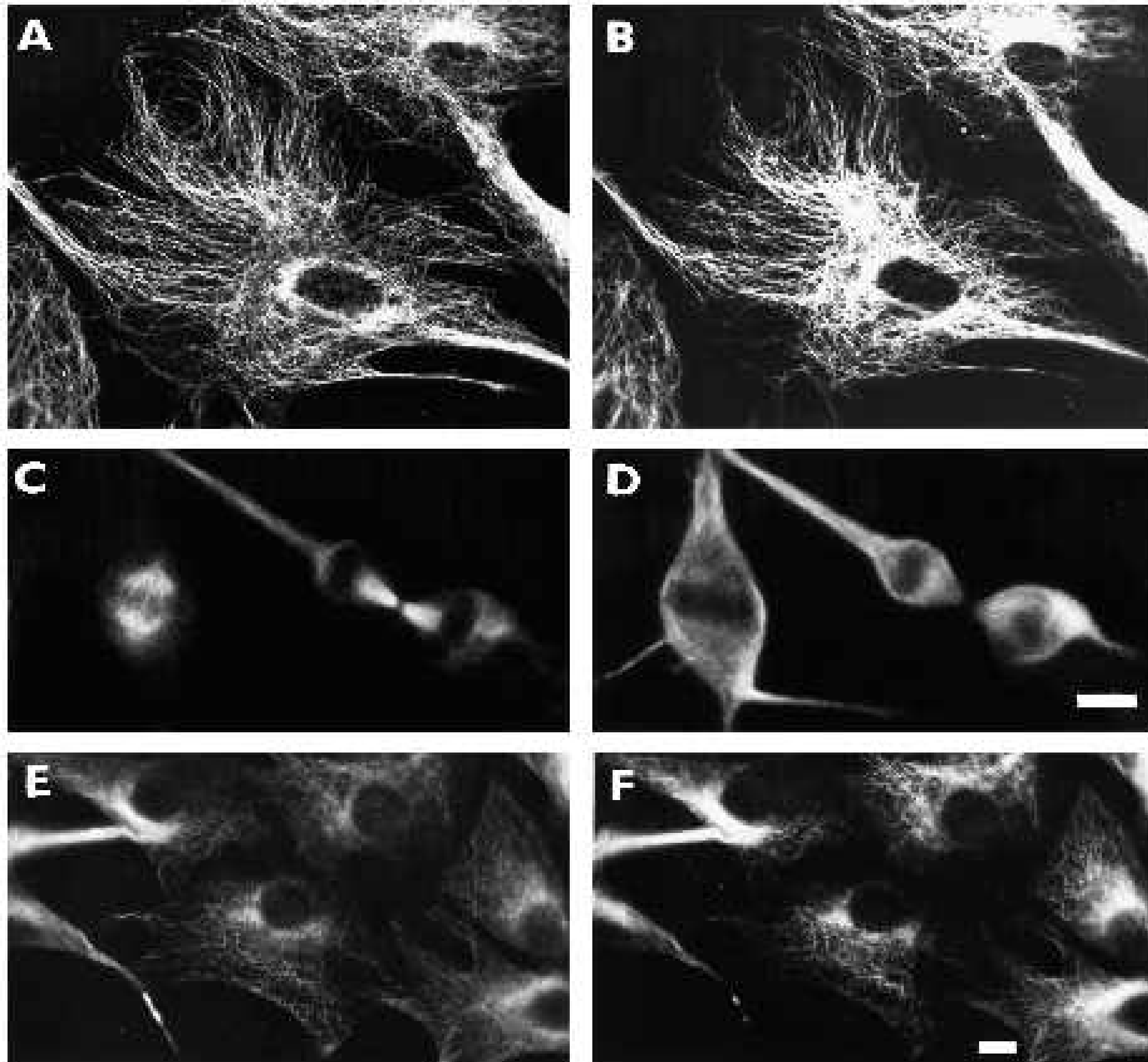

Fig. 1. Immunofluorescence double-label staining of 3T3 cells and taxol-stabilized cytoskeleton of 3T3 cells with MA-01 antibody (A,C,E), anti-tubulin (B,F) and anti-vimentin (D) antibodies. Taxol-stabilized cytoskeleton was incubated for 3 hours at 37°C in MSB, fixed and processed for immunostaining with MA-01 antibody (E) and anti-tubulin antibody (F). Each pair (A-B, C-D and E-F) represents the same cells. Bar, 10 μm.

intensity remained the same. Thus, the antigen is bound to the microtubules and is not easily detached.

The pattern of MA-01 antigen localization in interphase and mitotic cells suggested an in vivo association of the antigen with microtubules. Experiments were therefore designed to test the effect of microtubule-acting drugs. In vinblastine-treated cells the antibody was found to stain paracrystals (Dráberová et al., 1986b), whereas in taxol-treated cells it stained microtubule bundles (Fig. 2A,B). In colcemid-treated cells the antigen became diffusely distributed in the majority of cells, even though some cells displayed colcemid-resistant filaments that were also stained by anti-tubulin antibody (Fig. 2C,D). The MA-01 antibody did not stain coiled vimentin filaments in drug-treated cells. The effects of microtubule-acting drugs on the organization of the MA-01 antigen further indicated an association of this antigen with microtubules.

MA-01 antigen is a 210 kDa thermolabile protein that is associated with microtubules in vitro

To identify the protein recognized by MA-01 antibody, total whole cell lysate, high-speed extract and microtubule protein were prepared from 3T3 cells and examined by immunoblot analysis. In whole cell lysates or high-speed extracts the antibody reacted strongly with a protein of relative electrophoretic mobility corresponding to 210 kDa (Fig. 3, lanes 4 and 5). Secondary antibody alone gave no staining. In some preparations faint staining of 170 and 100 kDa proteins was occasionally observed, which could reflect proteolytic degradation during the process of sample

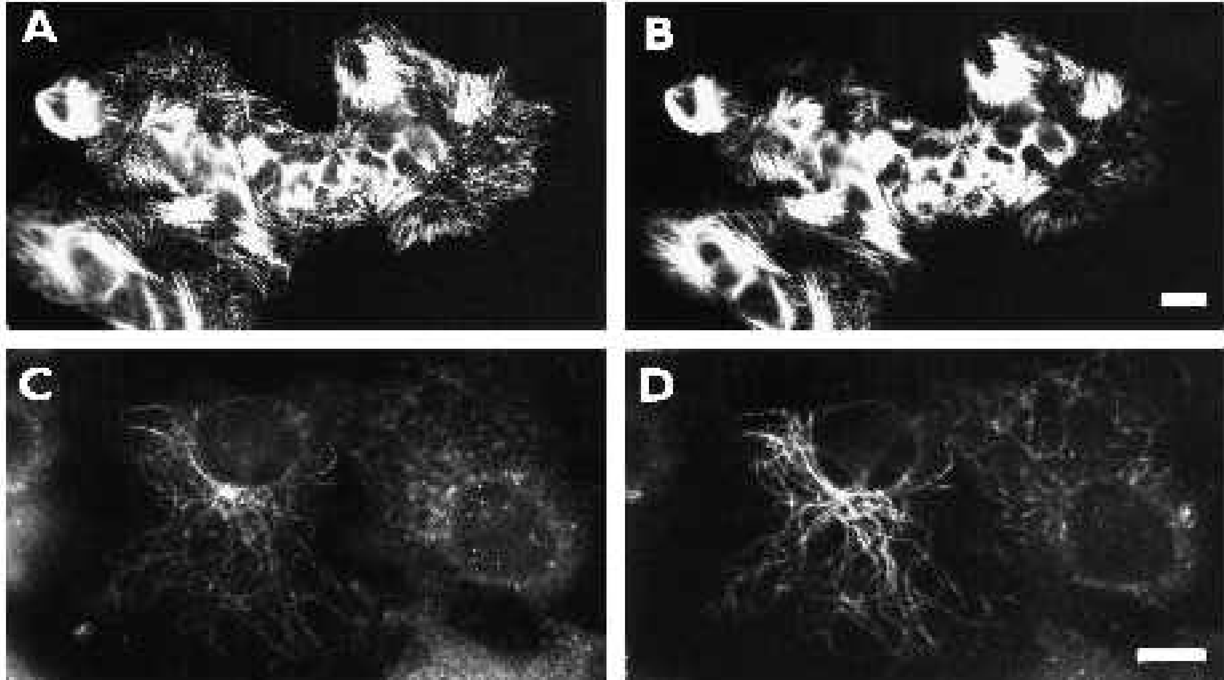

Fig. 2. Effect of taxol and colcemid on the distribution of MA-01 antigen. 3T3 cells were treated for 18 hours with 10 μM of taxol (A,B) or for 1 hour with 10 μM colcemid (C,D), fixed and stained with MA-01 antibody (A,C) or anti-tubulin antibody (B,D). Each pair (A-B and C-D) represents the same cells. Bar, 10 μm.

preparation. No reactivity was detected at the positions corresponding to brain high molecular mass MAP1 and MAP2 proteins, kinesin, tubulin, tau proteins or vimentin. The 210 kDa protein was also detectable in other specimens: in a cytoskeleton fraction prepared according to the method for immunofluorescence staining, in cells treated with colcemid, vinblastine or taxol (not shown) as well as in twice-washed microtubules isolated in vitro by polymerization in the presence of taxol and GTP (Fig. 3, lane 6) or in the presence of taxol only. The antigen, however, was not substantially enriched in microtubule preparations and was also present in microtubule-depleted supernatant. The 210 kDa protein was detected in the heat-labile fraction of high-speed extract from 3T3 cells, but no staining was observed in the concentrated heat-stable fraction (Fig. 4A, lanes 3 and 4). As the MA-01 antibody had been shown to react with 210 kDa protein also in human lung fibroblast LEP (Dráberová et al., 1986b), a concentrated heat-stable fraction of these cells was prepared and used for immunoblotting with the MA-01 antibody and with antibody specific for MAP4 proteins of primates. While the MA-01 antibody stained 210 kDa protein only in high-speed extracts, not in the thermostable fraction (Fig. 4B, lanes 3 and 4), the reaction of anti-MAP4 antibody with proteins in positions around 210 kDa was detectable both in high-speed extracts and in the thermostable fraction (Fig. 4B, lanes 5 and 6). Staining of proteins below 210 kDa in the thermostable fraction probably results from degradation of MAP4 proteins during the concentration of this preparation. A protein of 210 kDa isolated from 3T3 cells by immunoprecipitation with MA-01 antibody reacted in subsequent immunoblotting with MA-01 antibody (Fig. 4C, lane 1). A control antibody HTF-14 did not precipitate any protein that

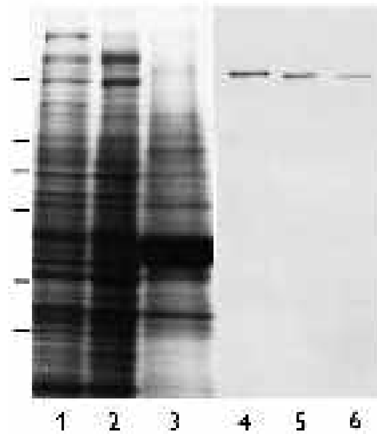

Fig. 3. Immunoblot of 3T3 microtubule protein with MA-01 antibody. Lanes 1 and 4, total cell lysate; lanes 2 and 5, high-speed supernatant; lanes 3 and 6, washed microtubules prepared by taxol-driven polymerization. Lanes 1-3, Coomassie Blue staining; lanes 4-6, immunostaining with MA-01 antibody; 5% to 10% linear polyacrylamide gel. Bars on left margins indicate positions, from top to bottom, of specific molecular mass markers (205 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa, 29 kDa).

would show reactivity with MA-01 antibody in immunoblotting (Fig. 4C, lane 3). Prominent labeling of bands of around 55 kDa (Fig. 4) is caused by staining of the heavy chains of mouse IgG (originating from antibodies immobilized on Sepharose beads and used for immunoprecipitation) with a conjugated secondary anti-mouse antibody (Fig. 4C, lane 2). MA-01 antibody immunoprecipitated the MAP2 from a porcine microtubule protein (not shown). As expected, the antibody also stained 3T3 micro-

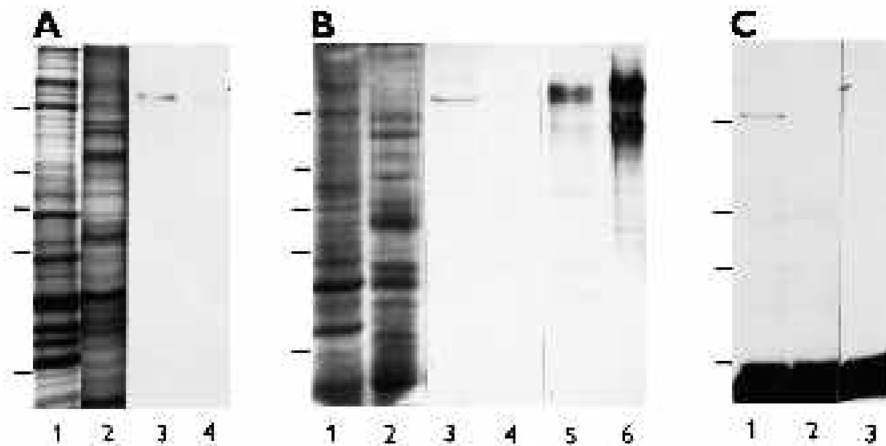

Fig. 4. Characterization of MA-01 antigen by immunoblotting of thermostable proteins and immunoprecipitation. (A) Immunoblot of 3T3 thermostable proteins. Lanes 1 and 3, thermolabile proteins prepared from high-speed extract; lanes 2 and 4, the corresponding concentrated thermostable proteins. Lanes 1 and 2, Coomassie Blue staining; lanes 3 and 4, immunostaining with MA-01 antibody; 7.5% polyacrylamide gel. (B) Immunoblot of thermostable proteins prepared from the human cell line LEP. Lanes 1,3 and 5, high-speed supernatant; lanes 2,4 and 6, concentrated thermostable proteins prepared from high-speed extract. Lanes 1 and 2, Coomassie Blue staining; lanes 3 and 4, immunostaining with MA-01 antibody; lanes 5 and 6, immunostaining with polyclonal antibody against human MAP-4 proteins; 7.5% polyacrylamide gel. (C) Immunoblot of 3T3 cells immunoprecipitated by MA-01 antibody. Lane 1, immunoprecipitation of 3T3 cells with MA-01 antibody bound to immobilized anti-mouse antibody; lane 2, MA-01 antibody; bound to immobilized anti-mouse antibody; lane 3, immunoprecipitation of 3T3 cells with control antibody HTF-14 bound to immobilized anti-mouse antibody. Lanes 1-3, immunostaining with MA-01 antibody; 6% polyacrylamide gel. Prominent bands around 55 kDa are heavy chains of mouse IgG stained with secondary anti-mouse antibody. Bars on left margins indicate positions, from top to bottom, of specific molecular mass markers (205 kDa, 116 kDa, 97.4 kDa, 66 kDa, 45 kDa, 29 kDa).

tubules that had been washed twice and fixed before examination by immunofluorescence microscopy (Fig. 5). Combined data indicate that the 210 kDa protein recognized by MA-01 antibody interacts with microtubules in vitro; it can however be distinguished from structural thermostable proteins of the MAP4 family.

Involvement of MA-01 antigen in interaction of microtubules and vimentin-type intermediate filaments

In order to investigate whether the 210 kDa protein takes part in interactions between cytoskeletal filaments, experiments involving microinjection of 3T3 cells with purified MA-01 antibody were undertaken. After microinjection, extraction and fixation the antibody was found distributed diffusely in the cytoplasm and no cytoskeletal structures were stained. However, with prolonged incubation the MA-01 immunofluorescence was accumulated around the nucleus; after 3 hours the pattern was characterized by a perinuclear cap and faint diffuse staining (Fig. 6A,C,E). Double immunofluorescence with anti-tubulin antibody showed that the microtubular network remained unchanged (Fig. 6B,D,F). If however anti-vimentin antibody was used in the double immunofluorescence assay, it became obvious that injection of MA-01 antibody (Fig. 7A,C,E) induced a gradual change in the network of the intermediate filaments; after incubation for 6 hours the vimentin filaments were completely disrupted (Fig. 7B,D,F). Six hours after injection, vimentin and MA-01 antigen colocalized in the perinuclear cap (Fig. 7E,F). Cells injected with control antibody HTF-14 at concentrations ranging from 1 to 15 mg ml^{-1} and incubated for 1-10 hours did not show collapsed vimentin filaments (not shown), thus eliminating the possibility that the collapse was a response to microinjection. When MA-01 antibody-injected cells were stained with rhodamine-conjugated phalloidin, stress fibres of microfilaments resembled those observed in cells injected with the control antibody (not shown).

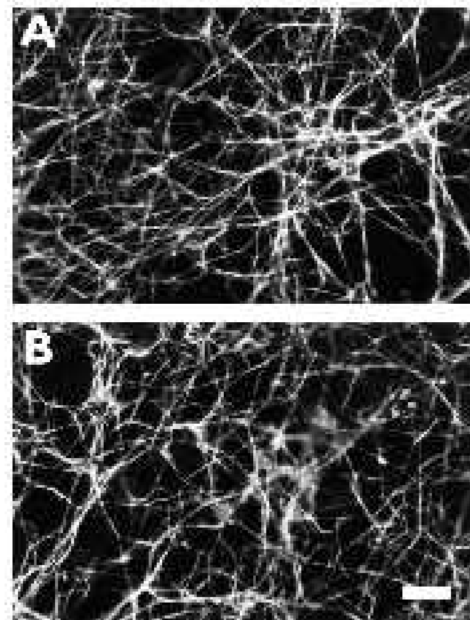

Fig. 5. Immunofluorescence staining of 3T3 microtubules with anti-tubulin antibody (A) and MA-01 antibody (B). Microtubules were adsorbed onto polybrene-treated glass coverslips, fixed and stained with antibodies. Bar, 10 μm.

To determine the time and concentration necessary to initiate the described collapse of filaments, cells were injected with various concentrations of MA-01 antibody (0.5, 1.0, 2.0, 5.0 and 14.0 mg ml^{-1}) and incubated for different time periods (5, 15, 30 minutes and 1, 3,10, 20 hours). The collapse of vimentin filaments started 30 minutes after an injection of the antibody at concentrations of immunoglobulin of 2 mg ml^{-1} or higher. Lower antibody concentrations had no effect on the distribution of intermediate filaments. Collapse was discernible in about 85% of cells after injec-

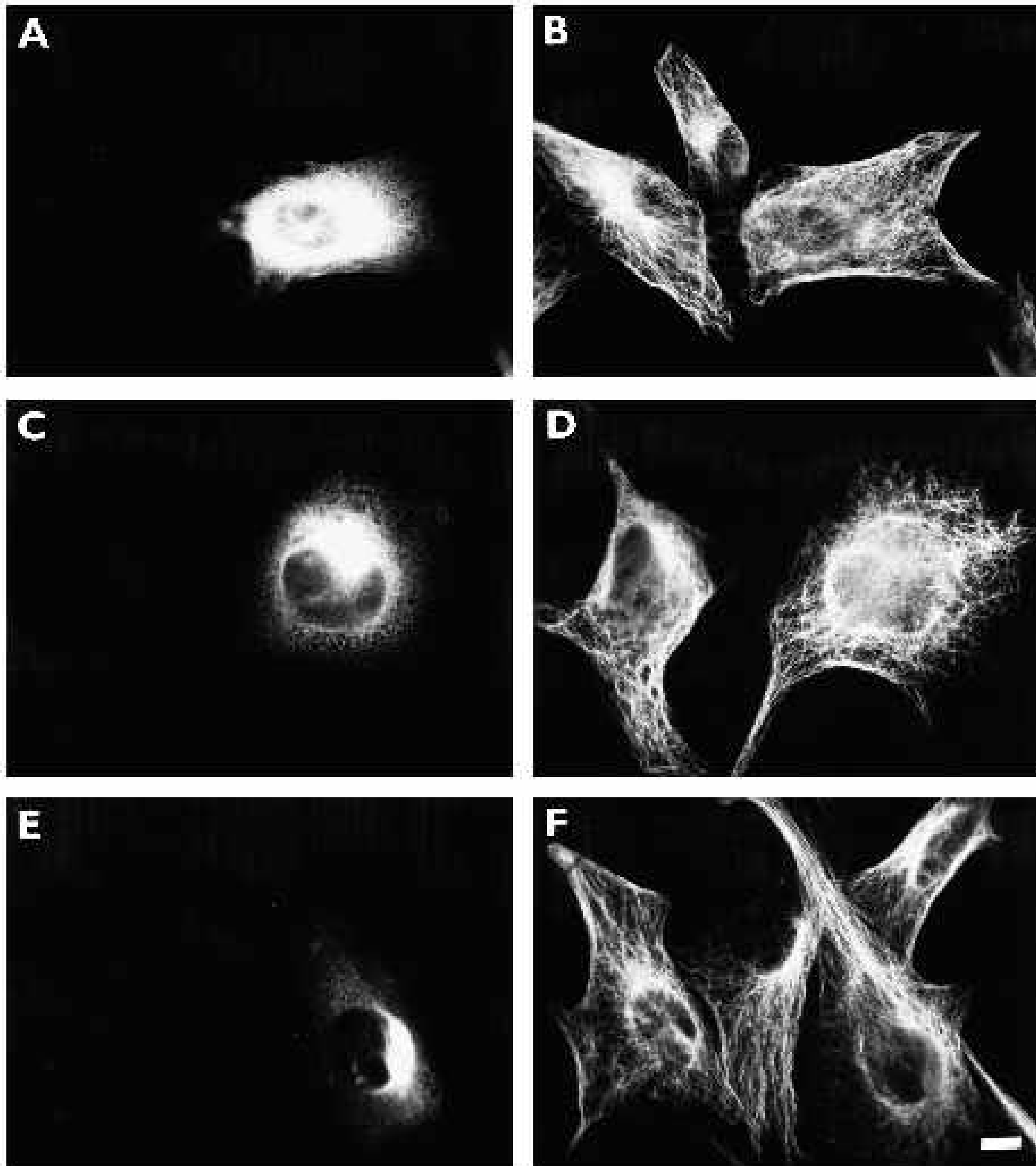

Fig. 6. Effect of MA-01 antibody injection on the distribution of microtubules. 3T3 cells were injected with MA-01 antibody at a concentration of 5 mg ml^{-1}, fixed after 5 minutes (A,B), 1 hour (C,D) or 3 hours (E,F) and processed for immunostaining. Distribution of injected MA-01 antibody (A,C,E) and microtubules (B,D,F) is shown by double-label fluorescence. Each pair (A-B, C-D and E-F) represents the same cells. Bar, 10 μm.

tion with antibody at a concentration of 5 mg ml^{-1} and incubation for 3 hours. The remaining cells were not recovered, probably due to injection trauma. Time-course experiments demonstrated that the collapse was reversible and most marked 3-6 hours after injection. With time the extent of filament collapse in individual cells decreased and extendend vimentin filaments reappeared after 20 hours. MA-01 staining was, at that time, restricted to spots that could reflect either autophagy of the injected antibody or aggregation of the antigen with antibody.

Effect of microtubule-acting drugs on distribution of MA-01 antigen

The microinjection experiments indicated that MA-01 anti-

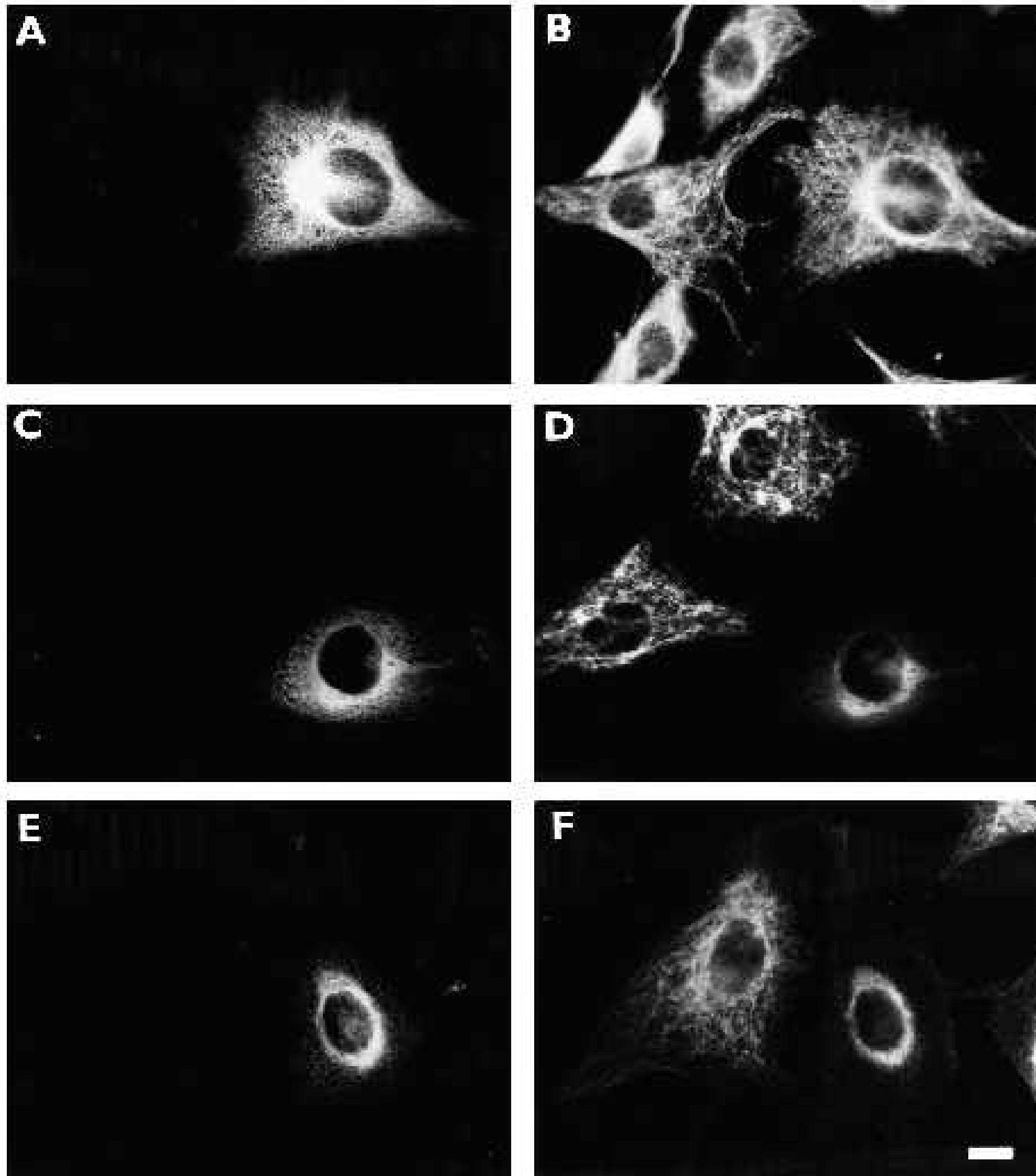

Fig. 7. Effect of MA-01 antibody injection on the distribution of intermediate filaments of vimentin-type. 3T3 cells were injected with MA-01 antibody at a concentration of 5 mg ml^{-1}, fixed after 1 hour (A,B), 3 hours (C,D) or 6 hours (E,F) and processed for immunostaining. Distribution of injected MA-01 antibody (A,C,E) and vimentin filaments (B,D,F) is shown by double-label fluorescence. Each pair (A-B, C-D and E-F) represents the same cells. Bar, 10 μm.

gen could be involved in interactions between microtubules and intermediate filaments of the vimentin type. It is known that treatment of cells with vinblastine results in a rearrangement of microtubular and vimentin fibres into, respectively, paracrystals and perinuclear coils (Traub, 1985). The location of MA-01 antigen was therefore tested by microinjection of the antibody into cells pre-treated with vinblastine. After injection the MA-01 antibody brightly stained vinblastine paracrystals as well as coiled fibrillar structures (Fig. 8A,C). Double immunofluorescence with anti-tubulin antibody (Fig. 8B) revealed co-localization of MA-01 antigen and tubulin in paracrystals. Double immunofluorescence with anti-vimentin antibody (Fig. 8D) confirmed the co-localization of MA-01 antigen and vimentin in coiled filaments as well. The same redistribution of the MA-01 antigen was observed when MA-01 anti-

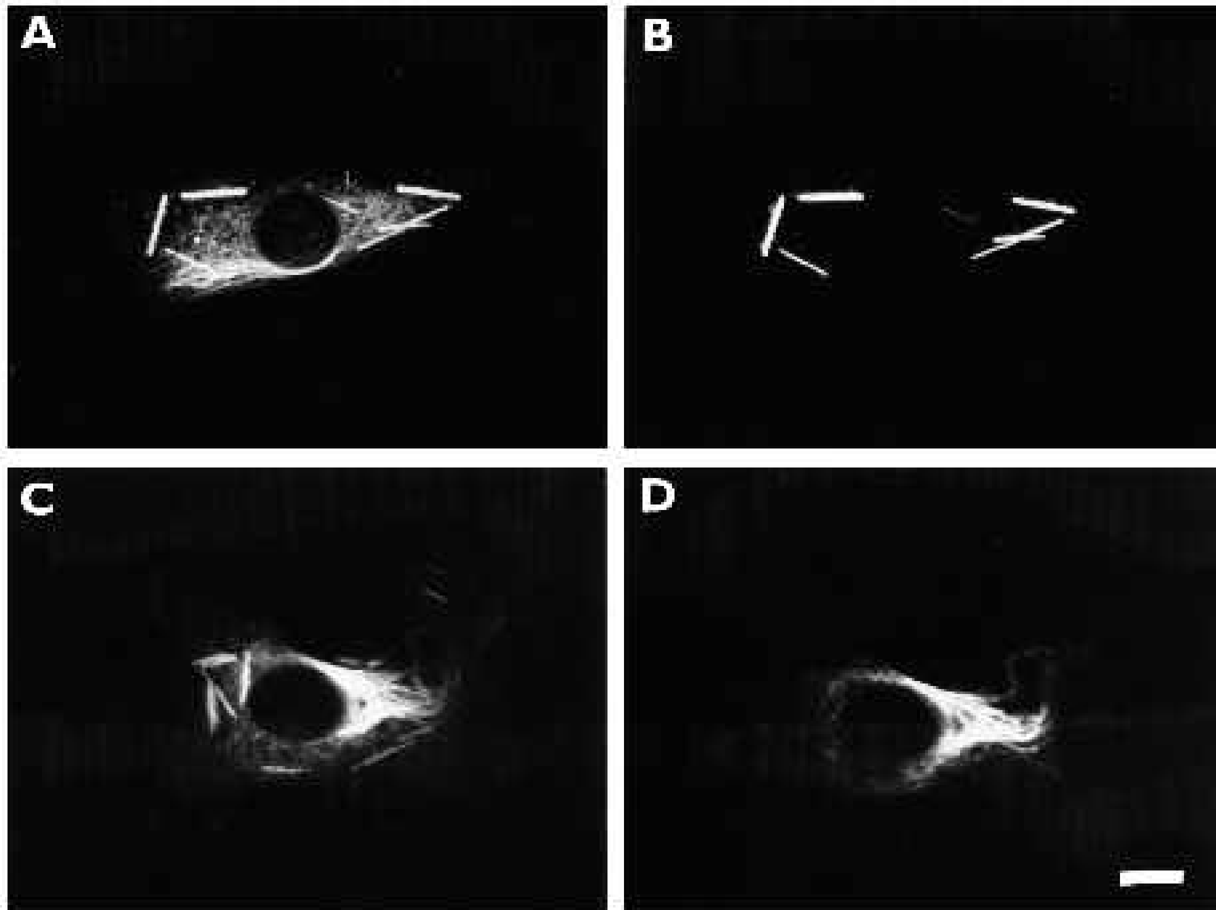

Fig. 8. Effect of vinblastine treatment on the distribution of MA-01 antigen in microinjected cells. 3T3 cells were incubated for 17 hours with 10 μM of vinblastine before being injected with MA-01 antibody at a concentration of 8 mg ml^{-1}. After 1 hour the cells were fixed and processed for immunostaining. Distribution of injected antibody (A,C), tubulin (B) and vimentin (D) is shown by double-label immunofluorescence. Each pair (A-B and C-D) represents the same cells. Bar, 10 μm.

body was injected into vinblastine-pre-treated cells or when cells were first microinjected with antibody and incubated thereafter in a medium containing vinblastine. Similarly, microinjection of the antibody into cells treated with taxol showed that MA-01 antigen was located on partially coiled vimentin filaments as well as on microtubule bundles; in microinjected colcemid pre-treated cells the MA-01 antigen was detected merely on coiled vimentin filaments, since the microtubules were disrupted (not shown).

DISCUSSION

Previously it has been shown that MA-01 antibody recognizes an epitope on MAP2 proteins (Dráberová et al., 1986b; Kuznetsov et al., 1986). On immunoblots of various types of non-neuronal cells this antibody, however, reacted with a 210 kDa protein (Dráberová et al., 1986b; Hašek et al., 1992). A protein of the same relative mobility was also detected by immunoblotting in lysates of 3T3 cells and by immunoprecipitation of high-speed extracts of 3T3 cells (Figs 3,4). Not the MA-01 antibody nor other monoclonal antibodies prepared in our laboratory against porcine MAP2, nor the polyclonal affinity-purified antibody against bovine MAP2 (kindly donated by Dr V. I. Gelfand) reacted with proteins in the 280-300 kDa region. Furthermore, except for the MA-01 antibody, none of these antibodies stained microtubules in 3T3 cells (E. Dráberová, unpublished data). Thus, our data suggest that MAP2 and the 210 kDa protein of 3T3 cells share an epitope recognized by MA-01 antibody. Sharing of epitopes between MAP2 and other proteins has already been described. An immunological relationship with MAP2 protein was reported for erythrocyte spectrin (Davis and Bennett, 1982), extracellular glycoprotein (Briones and Wiche, 1985), a 100 kDa protein located on coated vesicles and microtubules (Rodionov et al., 1985), and a 110 kDa protein associated with membranes of the Golgi apparatus (Allan and Kreis, 1986). We have observed microtubule staining with MA-01 antibody not only in 3T3 cells, but also in the yeast *Saccharomyces cerevisiae* (Hašek et al., 1992), melanophores of the teleost black tetra *Gymnocorymbus ternetzi*, the mouse embryonal carcinoma cell line P19, the canine kidney cell line MDCK and the human lung cell line LEP, suggesting that the antibody recognizes microtubule protein(s) conserved in various tissues and in various species ranging from yeast to man. MA-01 antigen, however, is not only immunologically related to MAP2, but it co-localizes

with microtubules, its distribution is sensitive to microtubule-acting drugs and it is among the proteins that co-assemble in vitro with tubulin from 3T3 lysates. Unlike MAP2 or 210 kDa MAP4 proteins (Bulinski and Borisy, 1979; Weatherbee et al., 1980, 1982) it is thermolabile. The association of MA-01 antigen with microtubules requires further investigation. Our preliminary data, however, imply that the MA-01 antigen was extracted from microtubules by 0.4 M NaCl but not by 10 mM ATP, as were some microtubule-associated motor proteins (Sloboda, 1992).

Localization of MA-01 antigen on interphase microtubules, mitotic spindles and midbodies as well as on vinblastine paracrystals, taxol bundles and colcemid-resistant microtubules demonstrated its association with microtubules in fixed 3T3 cells. The finding that microinjected MA-01 antibody did not decorate microtubules or any cytoskeletal structures indicates that the antigenic determinant is not available for binding with antibody on unfixed microtubules. The higher intensity of diffuse immunofluorescence could reflect the binding of the antibody to a soluble pool of MA-01 antigen. Fixation therefore either changes conformation of this protein or releases other proteins that effectivelly mask the MA-01 antigen. A similar effect was observed with a panel of monoclonal antibodies against the N-terminal structural domain of tubulin. These antibodies stained microtubules after fixation, but no staining of microtubules was observed after microinjection (Dráber et al., 1989). Incubation of injected interphase cells for different times or microinjection of the antibody into cells pre-treated with microtubule-acting drugs showed, however, that MA-01 antigen can be associated with unfixed cytoskeletal components. After prolonged incubation of injected cells, the MA-01 antigen was found on aggregated vimentin filaments in the perinuclear region; and extensive rearrangement of the cytoskeleton after vinblastine treatment led to the location of MA-01 antigen on vinblastine paracrystals as well as on coiled vimentin filaments. Our previous immunofluorescence data on well-spread Leydig's cells treated with cytocholasin B suggested that the MA-01 antigen might be involved in the interaction of microtubules with intermediate filaments or microfilaments in vivo (Dráberová et al., 1986b). Since in microinjection experiments the MA-01 antibody failed to change the distribution of microfilaments, it is likely that the MA-01 antigen is not engaged in interaction of microtubules and microfilaments in untreated interphase 3T3 cells. Substantial redistribution of vimentin filaments after microinjection of the antibody, however, indicates that the MA-01 antigen could be a multibinding protein that can directly or indirectly associate with microtubules and intermediate filaments.

Although the mechanism whereby injected antibodies cause a collapse of vimentin filaments is unclear, one can propose the hypothesis that a high intracellular concentration of antibody (up to 500 $\mu g\ ml^{-1}$ at an initial concentration of antibody of 5 $mg\ ml^{-1}$ and injection of one tenth of the cell volume) could deplete the cytoplasmic moiety of the protein. The other molecules of MA-01 antigen could thereafter be released from the coupling of vimentin filaments to microtubules. The vimentin filaments would then collapse to the perinuclear region. It has been shown that not all microtubule-associated proteins of the total pool are associated with microtubules (Nixon et al., 1990) and that microtubule-associated proteins turn over in cells more rapidly than tubulin (Olmsted et al., 1989). The collapse of intermediate filaments was not limited to 3T3 cells but was also observed in the human lung fibroblast cell line LEP containing vimentin filaments. Microinjection of the antibody into a HeLa cell had no obvious effect on the distribution of keratin filaments. The results presented further confirm that intermediate filaments can be withdrawn from the cytoskeleton with no effect on the orientation of microtubules and cell morphology (Traub, 1985).

Only a few previous reports have described the involvement of cytoskeleton-associated proteins in interactions between microtubules and vimentin filaments in vivo. Lin and Feramisco (1981) showed that microinjection of monoclonal antibody against a 95 kDa protein associated with intermediate filaments of fibroblasts induced aggregation of intermediate filaments into tight bundles around the nucleus. Bloom and Vallee (1983) have shown by immunofluorescence with polyclonal and monoclonal antibodies against MAP2 on the primary culture of rat brain cells that MAP2 is associated with microtubules and vimentin filaments. Since MAP2 is probably not universally distributed in cells (Lewis et al., 1986), MA-01 antigen could substitute for this function in various non-neuronal cells. Goyeva and Gelfand (1991) have shown that kinesin is also involved in positioning of vimentin filaments in human fibroblasts, and they suggested that interaction of vimentin filaments with microtubules resulted from association of kinesin with some vimentin-associated proteins or that it was mediated by membrane-bound organelles.

The combined data suggest therefore that the MA-01 antigen is a new microtubule-interacting protein of non-neuronal cells that mediates interactions between microtubules and intermediate filaments of vimentin type.

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