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The Involvement of the Intermediate Chain of Cytoplasmic Dynein in Binding the Motor Complex to Membranous Organelles of *Xenopus* Oocytes

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Cytoplasmic dynein is one of the major motor proteins involved in intracellular transport. It is a protein complex consisting of four subunit classes: heavy chains, intermediate chains (ICs), light intermediate chains, and light chains. In a previous study, we had generated new monoclonal antibodies to the ICs and mapped the ICs to the base of the motor. Because the ICs have been implicated in targeting the motor to cargo, we tested whether these new antibodies to the intermediate chain could block the function of cytoplasmic dynein. When cytoplasmic extracts of *Xenopus* oocytes were incubated with either one of the monoclonal antibodies (m74–1, m74–2), neither organelle movement nor network formation was observed. Network formation and membrane transport was blocked at an antibody concentration as low as 15 μg/ml. In contrast to these observations, no effect was observed on organelle movement and tubular network formation in the presence of a control antibody at concentrations as high as 0.5 mg/ml. After incubating cytoplasmic extracts or isolated membranes with the monoclonal antibodies m74–1 and m74–2, the dynein IC polypeptide was no longer detectable in the membrane fraction by SDS-PAGE immunoblot, indicating a loss of cytoplasmic dynein from the membrane. We used a panel of dynein IC truncation mutants and mapped the epitopes of both antibodies to the N-terminal coiled-coil domain, in close proximity to the p150^{Glued} binding domain. In an IC affinity column binding assay, both antibodies inhibited the IC–p150^{Glued} interaction. Thus these findings demonstrate that direct IC–p150^{Glued} interaction is required for the proper attachment of cytoplasmic dynein to membranes.

INTRODUCTION

Microtubule-based motor proteins provide the machinery for most membrane trafficking within the cytoplasm of higher eukaryotes, and the microtubule cytoskeleton provides the polarized framework on which an oriented transport can take place. Oriented transport is achieved by two classes of motor proteins with opposite directionality, kinesins and cytoplasmic dyneins. Cytoplasmic dynein is a minus-end–directed motor complex that is responsible for centripetal transport. Cytoplasmic dynein has been colocalized with components of the Golgi apparatus (Corthesy-Theulaz *et al.*, 1992; Fath *et al.*, 1994; Vaisberg *et al.*,

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1996), endocytic vesicles (Lin and Collins, 1992), lysosomes (Lin and Collins, 1992), and mitochondria (Hirokawa *et al.*, 1990). Furthermore, Aniento *et al.* (1993) have demonstrated that cytoplasmic dynein is essential for the fusion of endocytic vesicles.

The extension of tubular networks of the endoplasmic reticulum (ER) is another process that most likely will depend on microtubular motors (Lee and Chen, 1988; Lee *et al.*, 1989; Terasaki and Reese, 1994). The ER, which is linked to microtubules via cross-bridges (Franke, 1971), represents the largest membrane system within a cell. Experiments examining recovery from nocodazole treatment in cultured cells have demonstrated that the extension of the ER tubules is preceded by the polymerization of microtubules (Lee *et al.*, 1989). The extension of the ER networks is oriented toward the cell periphery along microtubular tracks (Terasaki and Reese, 1994) and it is believed to be caused by the plus-end–directed motor kinesin (Dabora and Sheetz, 1988; Vale and Hotani, 1988; Toyoshima *et al.*, 1992). ER network formation has also been reconstituted in vitro in cytoplasmic extracts of cultured cells (Dabora and Sheetz, 1988) and of *Xenopus* oocytes (Allan and Vale, 1991). Interestingly, in the case of *Xenopus* oocytes, Allan and Vale (1991, 1994) have demonstrated that cytoplasmic dynein but not kinesin is involved in the formation and maintenance of ER networks (Allan and Vale, 1994; Allan, 1995) in vitro. It is currently not understood why *Xenopus* ER should move exclusively by cytoplasmic dynein. The large size of the *Xenopus* oocyte may, however, require an egg-specific function (Allan, 1995). Cytoplasmic extracts of *Xenopus* oocytes provide, therefore, an excellent in vitro system to study the function of cytoplasmic dynein.

Cytoplasmic dynein is a large complex consisting of four subunit classes: heavy chains, intermediate chains (ICs), light intermediate chains, and light chains (Paschal *et al.*, 1987). Although cytoplasmic dynein interacts with microtubules dynamically via the catalytic heavy chains, it had been postulated that ICs link the motor complex to membranous organelles (King *et al.*, 1991; Paschal *et al.* 1992). In a previous study, we have demonstrated that the ICs are localized at the base of the motor complex (Steffen *et al.*, 1996). The position of the ICs is, therefore, in complete agreement with them being responsible for docking the motor protein to cargo.

Dynein-dependent vesicle transport in vitro depends on the presence of dynactin (Gill *et al.*, 1991). More recent work suggests that dynactin might function as the cytoplasmic dynein "receptor" (Vaughan and Vallee, 1995; Echeverri *et al.*, 1996). By using blot overlay and immunoprecipitation assays and immuno-affinity column chromatography, it was found that dynein IC interacted with dynactin and the interaction was mediated via the $p150^{Glued}$ subunit (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). To determine whether the ICs play an important part in the function of cytoplasmic dynein, we investigated the formation of membrane networks in cytoplasmic extracts of *Xenopus* eggs by using our monoclonal antibodies (m74–1 and m74–2) specific for the 74-kDa dynein IC. We demonstrate that IC-specific antibodies prevented the interaction between dynactin and dynein IC in vitro and disrupted the dynein–membrane interaction. Our data, therefore, indicate that the IC–$p150^{Glued}$ interaction plays a critical role in binding the motor complex to membranous organelles.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies m74–1 and m74–2 specific for dynein IC that had been characterized previously (Steffen *et al.*, 1996) were obtained from cell culture supernatant. Monoclonal cell lines were cultured in Ultrodoma medium (BioWittaker, Bender, Vienna, Austria) containing 2% fetal calf serum depleted of IgGs at 5% CO_2. Antibodies were isolated from cell culture supernatant by protein G column chromatography. Purified antibodies were dialyzed into phosphate-buffered saline (PBS: 10 mM sodium phosphate, 150 mM NaCl, 2.7 mM KCl, pH 7.4), concentrated to about 10 mg/ml with Centricon 10 filters (Amicon, Witten, Germany), and stored in aliquots at −80°C. Fab fragments were generated according to Mage (1980) by incubating protein G-purified antibody with 50 mM cysteine, 1 mM EDTA, and 3 U/ml papain attached to agarose beads for 90 min at 37°C. The completion of the digest was monitored by SDS-PAGE.

Monoclonal antibody LEP100 developed by Dr. D.M. Fambrough was obtained from the Developmental Studies Hybridoma Bank (maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biological Science, University of Iowa, Iowa City, IA, under contract N01-HD-6–2915 from the National Institute of Child Health and Human Development). Monoclonal antibody m150–1 specific for $p150^{Glued}$ was raised against ATP-extracted microtubule-associated proteins from bovine brain as described earlier (Steffen *et al.*, 1996). An affinity-purified polyclonal antibody raised against bacterially expressed heavy chain from *Dictyostelium* was obtained from E. Vaisberg (Vaisberg *et al.*, 1993).

Isolation of Xenopus Egg Cytosol

Interphase cytoplasmic extract was isolated from *Xenopus* oocytes according to Allan (1993) and Murray (1991). The eggs were washed in MMR/4 buffer [MMR buffer: 100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), 0.1 mM EDTA, pH 7.8]. The jelly coats were removed with freshly prepared 2% cysteine (pH 7.8). The eggs were washed again three times with MMR/4 buffer. Interphase eggs were obtained by activation in the presence of 0.4 μg/ml ionophore A23187 for 5 min, washing in MMR/4 buffer containing 100 μg/ml cycloheximide, and incubation at room temperature for 20 min. The eggs were washed twice in ice-cold acetate buffer [100 mM potassium acetate, 2.5 mM magnesium acetate, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 mM HEPES, 50 mM sucrose, 1 mM dithiothreitol (DTT), pH 7.2]. Cytochalasin B (50 μg/ml) and protease inhibitors (10 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml N^{α}-*p*-tosyl-L-arginine methyl ester) were added to the last washing step. Eggs were settled by centrifugation at 150 × *g* for 1 min followed by a centrifugation at 600 × *g* for 30 s. Excess buffer was removed and eggs were crushed by centrifugation at 10,000

rpm in a Beckman SW60 rotor for 20 min. Cytosol was removed, 0.05 volume of the energy mixture (150 mM creatine phosphate, 20 mM Mg-ATP, 2 mM EGTA), protease inhibitors, and cytochalasin B (50 μg/ml) were added. The cytosol was frozen as 50- to 100-μl aliquots in liquid N_2 and stored at −80°C.

Fractionation of Cytoplasm and SDS-PAGE

Cytoplasmic extract was fractionated by flotation through a sucrose density gradient. Twenty microliters of cytosol was mixed with 0.5 ml of 60% sucrose in acetate buffer, placed at the bottom of a step gradient (0.5 ml of 58% sucrose, 1 ml of 50% sucrose, and 0.5 ml of 15% sucrose in acetate buffer) in a Beckman TLS-55 centrifuge tube, and centrifuged at 55,000 rpm for 30 min at 4°C. The membrane fraction at the 15–50% sucrose interface was removed from the top with a hypodermic needle and a peristaltic pump. The membrane fractions were precipitated with ethanol or pelleted at 100,000 × *g* and then analyzed by SDS-PAGE and immunoblot. Electrotransfer to nitrocellulose was carried out according to Towbin *et al.* (1979) at 70 V for 3 h by using a Tris(hydroxymethyl)aminomethane (Tris)/glycine buffer containing 25 mM Tris, 192 mM glycine, and 10% methanol. The antigens were detected with a goat anti-mouse and a goat anti-rabbit peroxidase-conjugated antibody (Bio-Rad, Richmond, CA) and visualized using a luminescence assay (Pierce, Rockford, IL). In some experiments cytoplasmic extracts were incubated for 3 h on ice with 0.5 mg/ml antibody prior to flotation in the presence or absence of protein kinase inhibitors (150 μM H7, 10 μM staurosporin) and/or phosphatase inhibitors (1 μM calyculin, 10 μM microcystein, 1 μM okadaic acid).

Quantification of SDS-PAGE and SDS-PAGE Immunoblot

Membrane fractions were isolated from equal volumes of cytoplasmic extracts. The relative proportion of the membrane fraction was determined by gel densitometry using a flat bed scanner HP Scanjet IIp (Hewlett-Packard, Vienna, Austria) and the NIH Image software (National Institutes of Health, Bethesda, MD). The SDS-PAGE immunoblots were normalized to the major membrane proteins on corresponding SDS-PAGE gels to compensate for slight variations in loading between lanes. Also, where equal loading was necessary, the protein loading was adjusted prior to SDS-PAGE immunoblot.

Isolation and Purification of Cytoplasmic Dynein and Tubulin

Cytoplasmic dynein was isolated via its affinity to taxol-stabilized microtubules and purified by sucrose density gradient (5–25%) centrifugation (Paschal *et al.* 1987). Taxol was kindly provided to us by Jill Johnson (National Cancer Institute, Bethesda, MD). Brain stem (100–150 g) from bovine brain was homogenized in an equal volume of PEM buffer [100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 7.3, 1 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF, 10 μg/ml N^{α}-*p*-tosyl-L-arginine methyl ester] and then centrifuged at 10,000 × *g* for 15 min and 80,000 × *g* for 30 min at 4°C. Microtubules were polymerized at 37°C in the presence of 1 U/ml hexokinase and 10 mg/ml glucose by adding 10 μM taxol and then centrifuged through a 10% sucrose cushion in PEM at 80,000 × *g* for 40 min at 25°C. Dynein was released by resuspending taxol-stabilized microtubules in 0.05 tissue volume of extraction buffer (PEM supplemented with 20 mM Mg-ATP and 5 μM taxol) followed by an incubation at room temperature for 30 min. Released microtubule-associated proteins were separated from microtubules by centrifugation at 100,000 × *g* for 30 min at 25°C. The supernatant was then centrifuged through a 5–25% continuous sucrose density gradient in a Beckman SW-28 rotor at 100,000 × *g* (27,000 rpm) for 18 to 19 h. Up to 0.1 mM Mg-ATP was included in the sucrose gradient to enhance the stability of cytoplasmic dynein. Fractions of about 0.8 ml were collected from the bottom of the gradient.

Taxol-stabilized microtubules obtained as leftover from the dynein preparation were used as a source of tubulin. Microtubules were depolymerized by resuspending and incubating them in PEM buffer containing a final concentration of 300 mM NaCl and 3 mM $CaCl_2$ for 90 min on ice according to Collins (1991). The depolymerized microtubules were centrifuged at 100,000 × *g* for 60 min. The supernatant was adjusted to 250 mM NaCl and 0.2 mM GTP and then further purified by DEAE-Sephadex column chromatography. DEAE-Sephadex A-50 was equilibrated in PEM buffer containing 250 mM NaCl. Tubulin was bound to the column, washed with 250 mM NaCl in PEM buffer, eluted with 500 mM NaCl in PEM buffer, and then dialyzed against PEM containing 0.1 mM GTP and 0.5 mM DTT. Aliquots were frozen in liquid N_2 and stored at −80°C. The purity of cytoplasmic dynein and tubulin was examined by SDS-PAGE.

Video Microscopy

Microscope flow chambers were built as described by Blocker *et al.* (1996). An 11-mm circular cover glass (Menzel, Braunschweig, Germany) was placed onto a glass microscope slide with two pieces of double-sided tape (Scotch, 3 M, St. Paul, MN) forming a 2- to 3-μl chamber. Chambers were perfused with *Xenopus* cytoplasmic extracts diluted 1:4 (vol/vol) with acetate buffer containing 2 mM ATP. Preparations were observed with video-enhanced differential interference contrast (VEC-DIC) microscopy (Allen *et al.*, 1981, 1985; Weiss *et al.*, 1989). A Nikon Diaphot 300 inverted microscope (Nikon, Düsseldorf, Germany) equipped with an oil-immersion condenser (numerical aperature 1.4), 100× DIC PlanApo oil objective (numerical aperature 1.4), and xenon lamp (XBO 100). A Hamamatsu C2400–07 Newvicon camera was used for acquiring DIC images (Hamamatsu Photonics Deutschland, Herrsching, Germany). The video signals were first subjected to analogue contrast enhancement (Allen *et al.*, 1981, 1985; Weiss *et al.*, 1989), followed by real-time digital image processing in the following steps: subtraction of an out-of-focus background (mottle pattern), accumulation or averaging of images to increase signal to noise ratio, and finally selection of the desired range of gray levels (histogram stretching or digital contrast enhancement; Allen and Allen, 1983; Weiss and Maile, 1993). The analogue and digital processing of VEC-DIC signals was performed by using the ARGUS 20 real-time image processor (Hamamatsu Photonics Deutschland). All observations were recorded in real time with a Panasonic AG-6730 SVHS video recorder. Single-frame images were captured from video tape or directly from ARGUS 20 image processor onto a Power MacIntosh 8500 (Apple Computer, Cupertino CA) equipped with a LG-3 frame grabber (Scion, Frederick, MD) using IPLab Spectrum software (Scanalytics, Vienna, VA).

Affinity Column Binding Assay

The affinity column binding assay was performed essentially as described by Karki and Holzbaur (1995). Three identical columns were constructed by packing 200 μl of CH-Sepharose 4B (Pharmacia, Uppsala, Sweden) beads cross-linked with purified recombinant dynein IC at a concentration of 2 mg/ml. After thorough equilibration of the columns with PHEM buffer [50 mM sodium piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), 50 mM sodium HEPES, 1 mM EDTA, 2 mM $MgCl_2$, pH 6.9] 2 mg bovine serum albumin (BSA) was added to the first column, 700 μg of antibody m74–1 was added to the second column, and 700 μg of antibody m74–2 was added to the third column. After washing with 10 column volumes of PHEM, 500 μl of rat brain cytosol was loaded onto each column. After thorough washing (100 column volumes) with HEM buffer (50 mM sodium HEPES, 1 mM EDTA, 2 mM $MgCl_2$, pH 6.9), the columns were eluted with 500 μl of 1 M NaCl, and the resulting fractions were precipitated with trichloroacetic acid. The precipitates were each

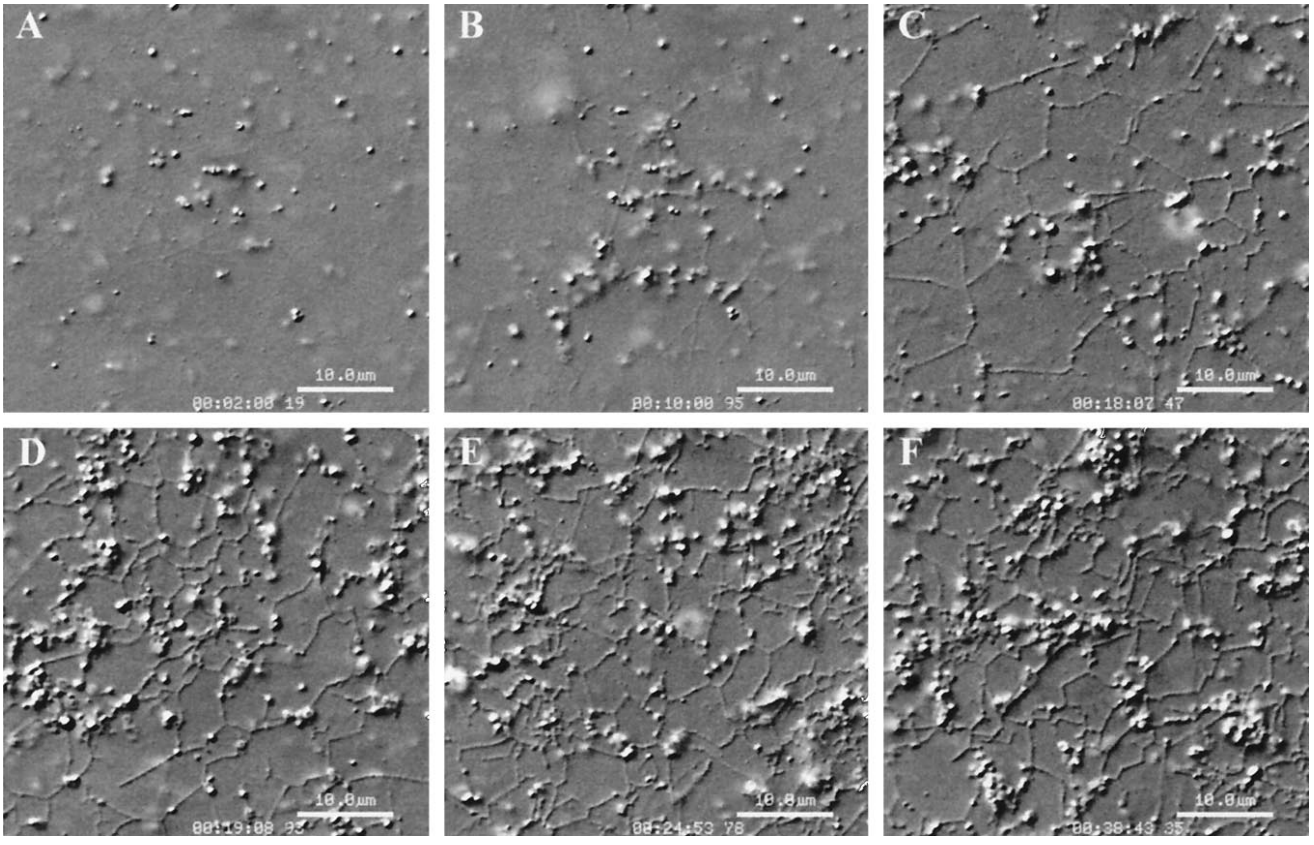

Figure 1. Formation of a tubular network in crude cytoplasmic extracts of *Xenopus* oocytes. Cytoplasmic extract was diluted 1:4 with acetate buffer containing 2 mM ATP and placed in a microscope flow chamber. The same area was monitored over a period of 60 min, and photos were taken at intervals from the video recording. After 2 to 5 min, microtubules became visible at the surface of the cover glass (A) and some membrane tubules appeared about 10 min after incubation (B). An evenly spread membrane network was already established after about 20 min (C). (D–F) The density of the membrane networks increased and underwent constant rearrangements. Bar, 10 μm.

resuspended in 30 μl of 1× Laemmli sample buffer and neutralized with saturated Tris. A 15-μl aliquot of the precipitate was loaded and resolved by SDS-PAGE. The proteins were transferred onto Immobilon-P (Millipore, Bedford, MA), Coomassie-stained, and subsequently probed with affinity-purified rabbit polyclonal antibodies to 150^{Glued} and centractin (Holleran *et al.*, 1996; Tokito *et al.*, 1996).

Expression of Dynein IC cDNA

The coding region of IC-1a (Paschal *et al.*, 1992) was subcloned into the pET-14b expression vector (Novagene, Madison, WI) by using standard cloning techniques (Vaughan and Vallee, 1995). Truncation mutants of IC-1a were prepared in pET-14b by using a combination of internal restriction sites and site-directed mutagenesis (Vaughan and Vallee, 1995). After transformation into the expression strain BL21(DE3) and induction with 0.4 mM isopropyl β-D-thiogalactoside, bacteria were lysed in a French press and expressed proteins were purified by using nickel-affinity chromatography. Purified recombinant polypeptides were immobilized on Immobilon-P (Millipore) membranes by using a slot-blot apparatus (BRL, Gaithersburg, MD). Immunoblots were performed by standard techniques and results were visualized by using ECL (Kirkegaard & Perry, Gaithersburg, MD). A pan-IC antiserum was generated against full-length IC-1a and shown to react with all truncation mutant polypeptides (Vaughan and Vallee, 1995).

RESULTS

ER Network Formation and Maintenance

Incubation of cytoplasmic extract from *Xenopus* oocytes leads to the formation of tubular membrane networks (Allan and Vale, 1991). The formation of these networks is depended on the attachment of microtubules to the glass surface, the extension of membrane tubules along these microtubules, and the fusion of these membrane tubules (Allan and Vale, 1994). Figure 1 demonstrates the formation of a typical network of cytoplasmic extracts isolated from interphase *Xenopus* oocytes. Polymerized microtubules became visible at the surface of the cover glass within minutes of placing the diluted cytoplasmic extract in a microscope flow chamber. Shortly thereafter, membrane plaques became attached to the surface and membrane tubules were pulled out by moving along microtubule tracks. Evenly spread membrane networks were established within 20 min and the density of the networks continued to increase over time. The

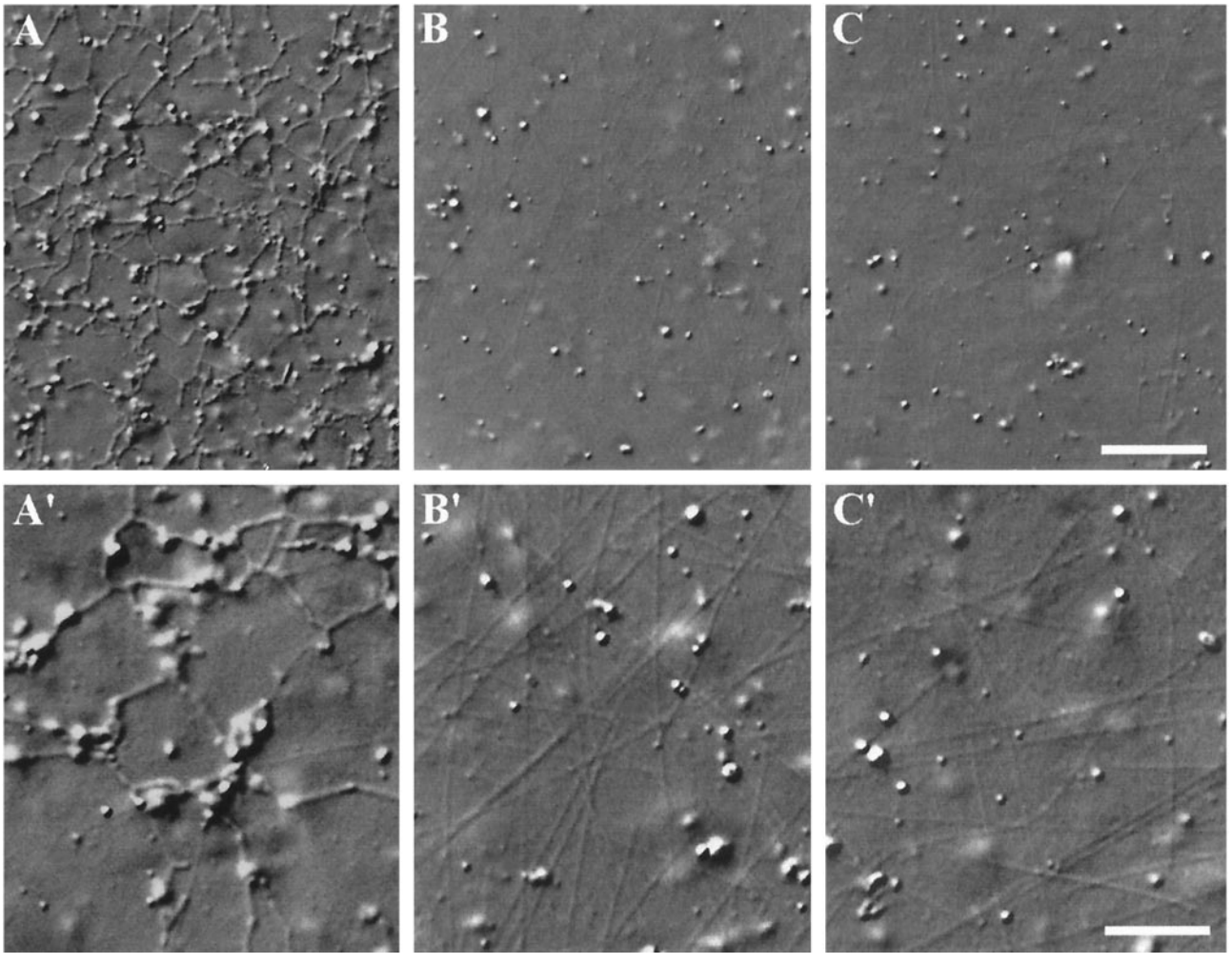

Figure 2. Effect of anti-dynein antibodies on the formation of membrane networks in cytoplasmic extracts of *Xenopus* oocytes. VEC-DIC images of cytoplasmic extracts supplemented 0.5 mg/ml antibody after an incubation for 60 min in a microscope flow chamber. (A and A′) Monoclonal antibody LEP100 specific for a lysosomal protein. (B and B′) Monoclonal antibody m74–1 specific for the 74-kDa dynein IC. (C and C′) Monoclonal antibody m74–2 also specific for the 74-kDa dynein IC. (A) A normal formation of polygonal network could be observed in control experiments, but the samples containing either of the monoclonal anti-dyneins (B and C) lacked any tubular networks. The anti-dyneins did not effect the polymerization of microtubules and their attachment to the cover glass. Bars: C, 10 μm; C′, 5 μm.

membrane networks underwent constant rearrangements and stayed motile for at least 3 h (period of observation). During the whole period, movement of membrane tubules along microtubules and vesicle motility could be observed.

Importance of Cytoplasmic Dynein for ER Network Formation

In a previous study, the 74-kDa intermediate chain of bovine cytoplasmic dynein was localized to the base of the motor complex by direct immunogold labeling (Steffen *et al.*, 1996). To investigate the role of the dynein IC in docking the motor complex onto the membrane and thereby influencing the formation of membranous networks, monoclonal antibodies specific for dynein IC (m74–1 and m74–2) were added to cytoplasmic extract prior to network formation. The cytoplasmic extract was placed in a microscope flow chamber and monitored by VEC-DIC microscopy for up to 1 h after the addition of the antibodies. A normal membrane network formed in control samples containing 0.5 mg/ml mouse antibody LEP100 (Figure 2). The motile activity of the membrane tubules was indistinguishable from samples lacking control antibodies. In contrast, no membrane networks were observed in the presence of 0.5 mg/ml of either monoclonal antibody m74–1 or m74–2 (Figure 2). Although the antibody m74–1 and m74–2 interfered with the for-

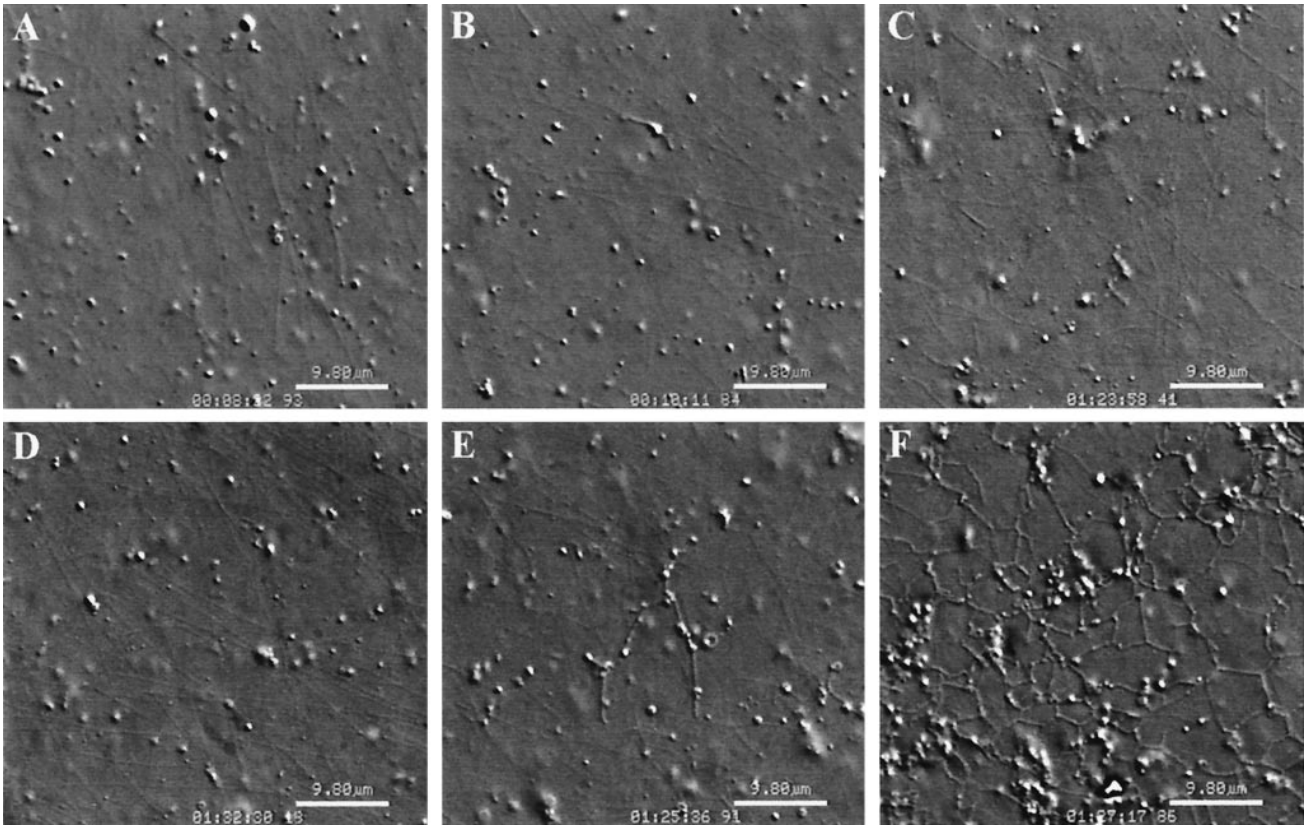

Figure 3. Determination of minimal concentration of antibody m74–2 for blocking the formation of tubular networks. VEC-DIC images of cytoplasmic extract supplemented with 125 μg/ml (A), 62.5 μg/ml (B), 31.5 μg/ml (C), 15.5 μg/ml (D), 7.5 μg/ml (E), and 0 μg/ml (F) monoclonal antibody m74–2. Tubular network formation was completely blocked at an antibody concentration of 15.5 μg/ml (D). Short membrane tubules were occasionally visible at an antibody concentration of 7.5 μg/ml (E), and a fully formed network was present in the control samples (F).

mation of membrane networks, the polymerization of microtubules and their attachment to the glass surface remained unaffected (Figure 2). Because both monoclonal antibodies m74–1 and m74–2 appeared to have identical effects in blocking the formation of membrane networks, only m74–2 was used in the follow up experiments.

As described above, the ER network formation was inhibited with an antibody concentration of 0.5 mg/ml. To determine the efficiency of the antibodies in interfering with the network formation, the titer of the antibodies was gradually reduced from 125 μg/ml to 7.5 μg/ml (Figure 3). Complete inhibition of membrane network formation was observed at an antibody concentration of ~15 μg/ml. At an antibody concentrations of ~7 μg/ml, short membrane tubules could be observed occasionally (Figure 3E).

Allan (1995) has reported that a large portion of the cytoplasmic dynein is found in a nonmembrane bound pool. It is therefore conceivable that the divalent IgG might deplete the pool of soluble dynein by immunoprecipitation, thereby removing the motor protein from the membrane fraction. To determine whether the effect seen by the antibody was due to an interference with a specific function of dynein IC rather than due to a mere immunoprecipitation of the soluble cytoplasmic dynein, protein G-purified antibody m74–2 was fragmented into Fab fragments by Sephadex-immobilized papain. When Fab fragments of m74–2 were added to the cytoplasmic extract, identical results were obtained as had been obtained for whole IgGs; the formation of membrane networks was blocked (our unpublished results).

The lack of network formation caused by the IC-specific antibodies could be interpreted either by an interference with the motor activity (ATPase activity) or by an interference with the dynein–membrane interaction. Dynein-dependent microtubule gliding was investigated in the presence of IC-specific antibodies to determine whether the antibodies might interfere with the motor activity. As demonstrated in Figure 2, the antibodies m74–1 and m74–2 did not affect the polymerization of microtubules or their attachment onto the glass surface. Furthermore, microtubule glid-

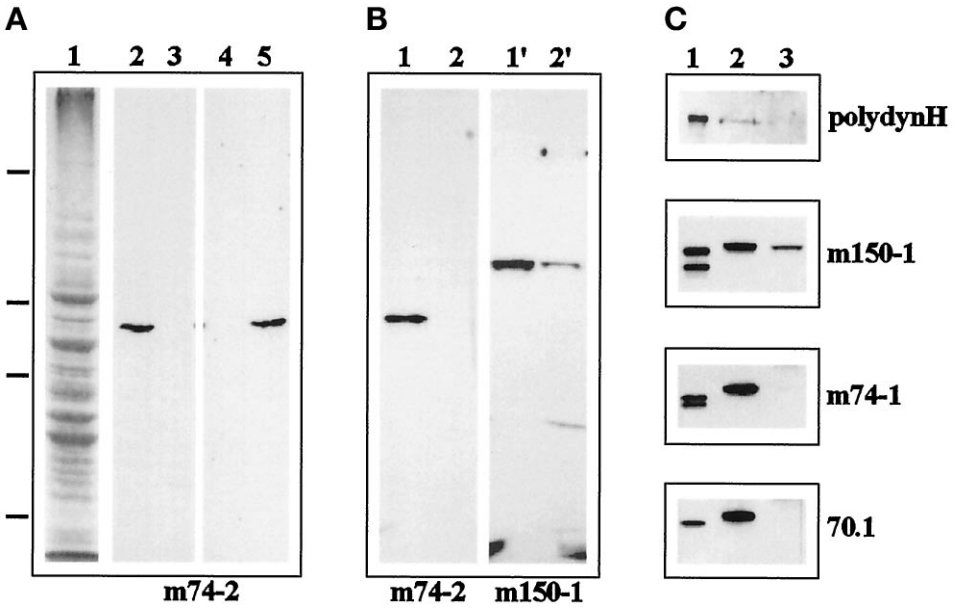

Figure 4. Dissociation of cytoplasmic dynein from membranes of cytoplasmic extracts of *Xenopus* oocytes analyzed by SDS-PAGE immunoblot. (A) Undiluted cytoplasmic extracts of interphase oocytes were supplemented with 0.5 mg/ml monoclonal antibody and incubated on ice for 3 h before resuspending the extract into 58% sucrose acetate buffer. The membrane fractions were isolated by flotation and collected at the 15–50% sucrose interphase. Lane 1, membrane fraction stained with Coomassie; lanes 2–5, immunoblot of membrane fractions stained with dynein IC-specific monoclonal antibody m74–2. Lane 2, membrane fractions of untreated cytoplasmic extract; lane 3, membrane fractions of cytoplasmic extract treated with 0.5 mg/ml m74–2; lane 4, membrane fraction of cytoplasmic extract treated with 0.5 mg/ml m74–1; lane 5, membrane fraction of cytoplasmic extract treated with 0.5 mg/ml control antibody LEP100. Molecular weight markers at left are as follows from top to bottom: 205,000, 97,000, 66,000, and 45,000. (B) Isolated membranes of *Xenopus* extracts were incubated with antibodies for 3 h on ice and then isolated by centrifugation through a 20% sucrose cushion. Lanes 1 and 1′, membrane fraction treated with 0.5 mg/ml control antibody; lanes 2 and 2′, membrane fraction treated with 0.5 mg/ml m74–2; lanes 1 and 2, stained with dynein IC-specific antibody m74–2; lane 1′ and 2′, stained with monoclonal antibody m150–1 specific for dynactin $p150^{Glued}$. (C) Immunoblot of antibody-treated membrane fractions from *Xenopus* extracts stained with polyclonal antibody (polydynH) raised against bacterially expressed dynein heavy chain from *Dictyostelium* (Vaisberg *et al.*, 1993), dynactin $p150^{Glued}$-specific monoclonal antibody m150–1, dynein IC-specific monoclonal antibody m74–1, and dynein IC specific monoclonal antibody 70.1 (Steuer *et al.*, 1990). Lane 1, microsomes isolated from bovine brain were used to demonstrate the specificity of the antibodies; lane 2, membrane fraction of cytoplasmic extract from *Xenopus* oocytes treated with control antibody; lane 3, membrane fraction of cytoplasmic extract of *Xenopus* oocytes treated with 0.5 mg/ml m74–2.

ing was observed as well. In cytoplasmic extracts of *Xenopus* oocytes, the directionality of microtubule movement could not be determined due to the high density of microtubules and most importantly due the lack of an appropriate marker of microtubule polarity. To determine whether m74–2 interfered with dynein-dependent microtubule motility, microtubule gliding was analyzed by using taxol-stabilized microtubules and cytoplasmic dynein from bovine brain. In this assay, the antibody m74–2 did not cause an inhibition of microtubule gliding. Instead, the velocity of gliding microtubules was slightly elevated in the presence of the antibody. In control samples microtubules moved with a velocity of 0.62 ± 0.14 μm/sec (mean ± SD; n = 51), and they moved with a velocity of 0.82 ± 0.2 μm/sec (mean ± SD; n = 46) in samples treated with m74–2, indicating that the antibody did not block the ATPase activity.

Blocking of Dynein–Membrane Interaction

The lack of network formation in the presence of IC-specific antibodies appeared to be due to interference with the dynein–membrane interaction. To gain a better understanding of how the antibody m74–2 might interfere with the formation of membrane networks, the effect of the antibodies on the binding of cytoplasmic dynein to membranous organelles was investigated. The monoclonal antibodies m74–2 (see above) and 70.1 (Steuer *et al.*, 1990) were used to identify cytoplasmic dynein within the membrane fractions. On the basis of immunoblot data obtained from bovine brain microsomes, these two antibodies recognized different epitopes. The monoclonal antibody m74–2 raised against the 74-kDa intermediate chain of sucrose-density-gradient–purified cytoplasmic dynein (Steffen *et al.*, 1996) detected a doublet band of 72/74-kDa in bovine brain microsomes. In contrast, monoclonal antibody 70.1 detected just the 74-kDa polypeptide band in bovine brain microsomes. In membrane fractions from *Xenopus* oocytes, both antibodies (m74–2 and 70.1) detected a single polypeptide band of about 83-kDa (Figure 4C). As pointed out by Allan (1995), the 83-kDa polypeptide band represents dynein IC in *Xenopus* oocytes.

Undiluted cytoplasmic extracts were incubated with 0.5 mg/ml monoclonal antibody for 3 h on ice in the presence of protease inhibitors. To obtain a membrane fraction free of soluble proteins or cosedimenting pro-

tein complexes, the membrane fractions were isolated by flotation through a sucrose step gradient (Niclas *et al.*, 1996) and then analyzed by SDS-PAGE immunoblot. Although the 83-kDa IC was observed in membrane fractions of untreated samples and samples treated with a control antibody (Figure 4A, lanes 2 and 5), it was no longer detectable in membrane fractions of samples treated with antibodies m74–1 and m74–2 (Figure 4B, lanes 3 and 4). Identical results were obtained, if the immunoblot was carried out with the monoclonal antibodies m74–1 and 70.1 (Figure 4C). The absence of the 83-kDa antigen in m74–2-treated membrane fractions indicated a dissociation of cytoplasmic dynein from the membranes. To determine, therefore, whether the loss of the m74–2 antigen from the membrane fraction had been caused by blocking the rebinding of cytoplasmic dynein due to the presence of the monoclonal antibody m74–2, membranes were isolated by flotation prior to incubation with m74–2. The treatment of isolated membrane fractions with the monoclonal antibody m74–2 resulted again in a complete loss of the 83-kDa IC (Figure 4B). Immunoblot replicas of antibody-treated membrane samples were also probed with a polyclonal antibody against the heavy chain and two different monoclonal antibodies against the intermediate chain, to determine whether the whole motor complex was removed from the membrane (Figure 4C). By using the luminescence immunoblot assay, neither of these antibodies detected subcomponents of cytoplasmic dynein in m74–2-treated membrane fractions.

As demonstrated by video microscopy, 15 μg/ml m74–2 was sufficient to block ER network formation (Figure 3). For the network formation assay, the cytoplasmic extract was diluted 1:5, and undiluted extract was used for the membrane binding study. To compare the data from motility assay and membrane binding study, we determined the molar ratio of the antibody per 1 ml of undiluted cytoplasmic extract and found that 0.39 nmol of m74–2 was sufficient to block ER network formation. Because the biochemical experiments were carried out on ice to prevent microtubule polymerization, an ~10-fold excess of antibody was used to disrupt the dynein–membrane interaction (Figure 4). To be able to compare both sets of experiment, cytoplasmic extract was incubated with 0.66 nmol/ml of m74-2 and analyzed after 5, 10, and 30 min of incubation. A loss of ~90% of dynein IC was already observed after an incubation of 10 min (our unpublished observation), indicating a similar activity of the antibody in the membrane binding studies.

Interaction of Cytoplasmic Dynein with Dynactin

It has been demonstrated that dynactin, a regulator for the dynein-dependent membrane transport (Gill *et al.*, 1991), can bind to dynein IC via the p150^{Glued} subcomponent (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). Because the IC-specific antibody m74–2 was able to influence the dynein–membrane interaction, the association of the regulatory component dynactin with the membrane fractions was also investigated. The monoclonal antibody m150–1 raised against p150^{Glued} from bovine brain recognized two polypeptide bands of ~150 kDa and ~160 kDa in microsomes of bovine brain (Figure 4C, lane 1). In membrane fractions from *Xenopus* oocytes, the antibody m150–1 detected only a single polypeptide band of about 165-kDa. A significant reduction in the association of dynactin with membrane was observed in m74–2 treated cytoplasmic extracts by using the monoclonal antibody m150–1 to detect dynactin (Figure 4B, lanes 1′ and 2′). When compared with untreated membranes, only about 20% of the dynactin p150^{Glued} remained with the membranes after treatment with the antibody m74–2. The use of phosphatase inhibitors and protein kinase inhibitors did not influence the dissociation of dynactin caused by m74–2 (our unpublished results).

The effect of the antibody m74–2 on the dynein–dynactin interaction was further investigated by an affinity column binding assay. Immobilized bacterially expressed dynein IC was employed as affinity matrix. The column matrix was equilibrated with BSA, m74–1, or m74–2 before loading whole brain extract. The wash fractions and the eluted fractions were analyzed by SDS-PAGE immunoblot using antibodies to dynactin p150^{Glued} and centractin. As shown in Figure 5, both antibodies m74–1 and m74–2 prevented the binding of dynactin to the column matrix, whereas in the presence of BSA, the dynactin p150^{Glued} and centractin were retained by the dynein IC, demonstrating that both antibodies m74–1 and m74–2 interfered with the dynein–dynactin interaction.

Determination of Epitopes for m74–1 and m74–2

Binding studies using the IC affinity column indicate that both antibodies interfere with the interaction of dynein IC with dynactin p150^{Glued}. This interaction has been mapped to the N-terminal domain of the ICs containing a coiled-coil structure (Vaughan and Vallee, 1995). To test this possibility, we mapped the epitopes of the two monoclonal antibodies by using a panel of IC truncation mutants. A pan-IC antibody generated against full-length IC-1A detected all peptide fragments (Figure 6), as reported previously (Vaughan and Vallee, 1995). Both monoclonal antibodies detected all fragments down to amino acids 1–66 yet failed to detect a fragment containing amino acids 61–643 (Figure 6). These findings suggest that the epitopes for the monoclonal antibodies m74–1 and m74–2 lie within the N-terminal coiled-coil domain

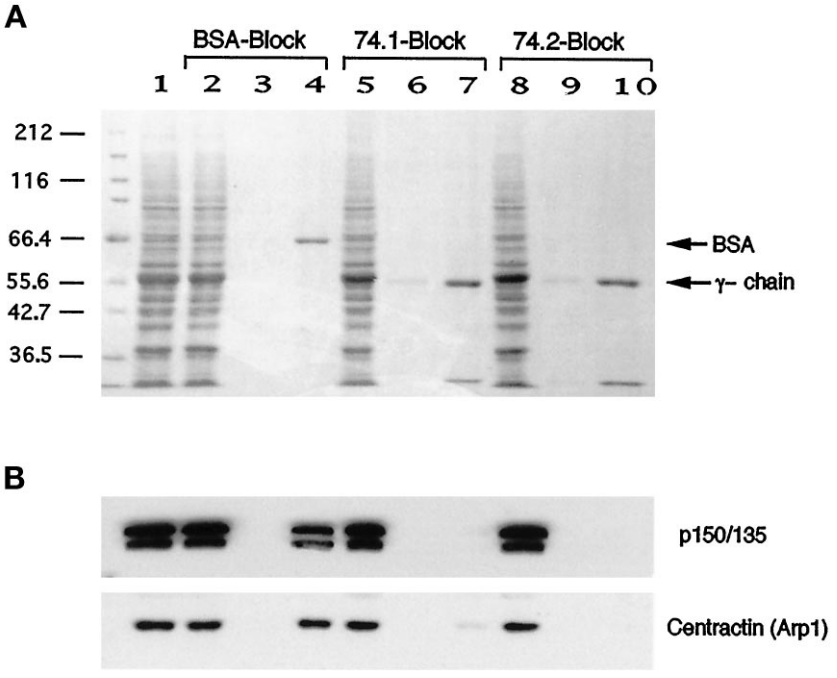

Figure 5. Monoclonal antibodies 74-1 and 74-2 block dynein IC–dynactin interaction in vitro. Rat brain cytosol (lane 1) was loaded onto a dynein IC affinity column (see MATERIALS AND METHODS) that was pretreated with BSA (lanes 2–4), m74–1 (lanes 5–7), or m74–2 (lanes 8–10). The columns were extensively washed and eluted with 1 M NaCl. The fractions were resolved by SDS-PAGE followed by transfer onto Immobilon and stained with Coomassie brilliant blue (A) and subsequently probed with antibodies to $p150^{Glued}$ and centractin (B). Lane 1, cytosol; lanes 2, 5, and 8, flow-through fractions; lanes 3, 6, and 9, final wash fractions; lanes 4, 7, and 10, fractions eluted with 1 M NaCl.

overlapping with the region dictating the IC–$p150^{Glued}$ interaction.

DISCUSSION

We have demonstrated here for the first time that monoclonal antibodies specific for dynein IC are able to block the function of cytoplasmic dynein to form ER networks in cytoplasmic extracts of *Xenopus* oocytes in vitro. The dynein IC-specific antibodies caused a dissociation of cytoplasmic dynein from the membrane surface and thereby resulted in a disruption of the function of cytoplasmic dynein to generate membrane networks in vitro. These observations, therefore, support the hypothesis that dynein IC plays an essential part in docking the motor complex on to its target; a model which had been supported by the immunogold localization of the intermediate chain to the base of the motor complex (Steffen *et al.*, 1996).

To address the question of how the monoclonal antibodies m74–1 and m74–2 might be able to disrupt the association of cytoplasmic dynein with membranes, we discuss first the possible interaction of cytoplasmic dynein with membranous organelles. Two models are still under discussion, a binding of the motor complex onto a dynein-specific receptor or a charge interaction of cytoplasmic dynein with the membrane surface (Lacey and Haimo, 1992). The model of an electrostatic interaction of cytoplasmic dynein with membranes has been supported by a binding study using purified cytoplasmic dynein and phospholipid vesicles (Lacey and Haimo, 1994) and the model was, furthermore, substantiated by the presence of a lysine-rich region at the N-terminal end of dynein IC (Paschal *et al.*, 1992; Trivinos-Lagos *et al.*, 1993). Electrostatic interaction with the surface of membranes has also been proposed for myosin (Adams and Pollard, 1989; Hayden *et al.*, 1990; Doberstein and Pollard, 1992; Li *et al.*, 1994). On the other hand, conflicting data concerning a possible dynein receptor were obtained from binding studies of cytoplasmic dynein to protease-treated vesicles. Muresan *et al.* (1996) demonstrated that cytoplasmic dynein can bind to trypsin-treated KI-extracted vesicles from squid axoplasm. In contrast, Yu *et al.* (1992) and Blocker *et al.*, (1997) found the binding of cytoplasmic dynein to membrane was α-chymotrypsin-sensitive.

Little is known about the exchange between soluble- and membrane-bound cytoplasmic dynein. In our studies of membranes isolated from *Xenopus* oocytes, the antibody m74–2 caused a dissociation of cytoplasmic dynein from the membrane. If we assume that the soluble and membrane-bound dyneins are constantly exchanged within the cytoplasm, then the loss of membrane-bound cytoplasmic dynein due to the presence of m74–2 could very well be explained by blocking the binding sites needed for an electrostatic interaction with phospholipids. The antibody m74–2 also caused a loss of cytoplasmic dynein from isolated membranes, suggesting a more active role of the antibody in membrane–dynein interaction. However, we cannot completely rule out the possibility that the

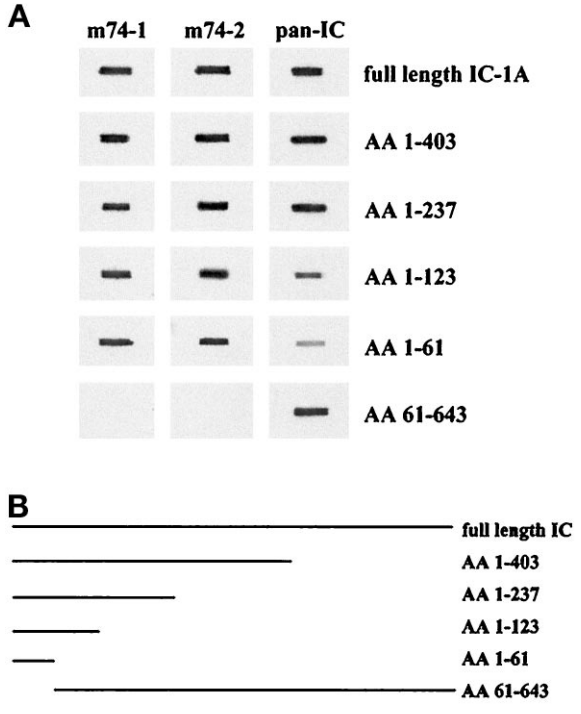

Figure 6. Identification of the binding site of monoclonal antibodies m74–1 and m74–2 on dynein IC. (A) Immunoblot of truncation mutants of expressed dynein IC labeled with monoclonal antibody m74–1 and m74–2 or polyclonal antibody pan-IC specific for the whole dynein IC polypeptide. (B) Line diagram depicting dynein IC fragments used for mapping.

antibody blocked the rebinding of dynein to the membrane.

Recently it had been proposed that dynactin might represent the "receptor" for the cytoplasmic dynein (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). The dynactin complex was initially described as an activator for dynein-mediated vesicle transport (Gill *et al.*, 1991; Schroer and Sheetz, 1991). The dynactin p150^{Glued} contains a binding site for microtubules (Waterman-Storer *et al.*, 1995) and for dynein IC (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). As demonstrated herein, dynein IC-specific antibodies displaced the cytoplasmic dynein from the membrane and also blocked the in vitro binding of dynactin onto immobilized dynein IC. By epitope mapping, the binding sites for the monoclonal antibodies m74–1 and m74–2 have been localized to the N terminus of the dynein IC, in close proximity to the binding site for dynactin p150^{Glued}. Because of the close proximity of binding sites for antibodies and dynactin p150^{Glued}, it is not surprising that m74–1 and m74–2 disrupt the dynein–dynactin interaction. A dissociation of cytoplasmic dynein from squid membrane vesicles has also been achieved with monoclonal dynein IC antibody m74-1 and a polyclonal antibody against p150^{Glued} (Waterman-Storer *et al.*, 1997). Binding of cytoplasmic dynein to dynactin as a primary target does not, however, exclude an additional electrostatic interaction of dynein with negatively charged membrane regions as has been suggested by binding studies with phospholipids (Lacey and Haimo, 1994). A treatment of *Xenopus* membranes with the antibody m74–2 revealed that the membrane association of dynactin was affected as well, indicating that both molecules, cytoplasmic dynein and dynactin, are needed for a proper membrane interaction. Although dynactin might provide the primary target for cytoplasmic dynein (Vaughan and Vallee, 1995), an additional stabilization of the binding could be achieved by an electrostatic interaction of cytoplasmic dynein with the membrane.

In summary, our data indicate that the ICs play an important role in docking cytoplasmic dynein onto membranous organelles. The use of dynein IC-specific antibodies that interfered with the dynein–dynactin interaction resulted in a dissociation/loss of cytoplasmic dynein from the membrane, indicating that the dynactin complex represents the primary target for cytoplasmic dynein. Because the anti-dynein monoclonal antibody m74–2 also affected the membrane binding of the dynactin complex, we propose that both complexes, dynactin and cytoplasmic dynein, are needed for a proper attachment to the membrane surface.

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