Centractin (ARP1) Associates with Spectrin Revealing a Potential Mechanism to Link Dynactin to Intracellular Organelles

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Abstract. Centractin (Arp1), an actin-related protein, is a component of the dynactin complex. To investigate potential functions of the protein, we used transient transfections to overexpress centractin in mammalian cells. We observed that the overexpressed polypeptide formed filamentous structures that were significantly longer and more variable in length than those observed in the native dynactin complex. The centractin filaments were distinct from conventional actin in subunit composition and pharmacology as demonstrated by the absence of immunoreactivity of these filaments with an actin-specific antibody, by resistance to treatment with the drug cytochalasin D, and by the inability to bind phalloidin. We examined the transfected cells for evidence of specific associations of the novel centractin filaments with cellular organelles or cytoskeletal proteins. Using immunocytochemistry we observed the colocalization of Golgi marker proteins with the centractin polymers. Additional immunocytochemical analysis using antibodies to non-erythroid spectrin (fodrin) and Golgi-spectrin ($\beta I\Sigma^*$) revealed that spectrin colocalized with the centractin filaments in transfected cells. Biochemical assays demonstrated that spectrin was present in dynactin-enriched cellular fractions, was coimmunoprecipitated from rat brain cytosol using antibodies to dynactin subunits, and was coeluted with dynactin using affinity chromatography. Immunoprecipitations and affinity chromatography also revealed that actin is not a *bona fide* component of dynactin. Our results indicate that spectrin is associated with the dynactin complex. We suggest a model in which dynactin associates with the Golgi through an interaction between the centractin filament of the dynactin complex and a spectrin-linked cytoskeletal network.

CENTRACTIN is an actin-related protein (Arp)[1] that has been defined as a member of the Arp1 class (Frankel et al., 1994; Fyrberg et al., 1994; Schroer et al., 1994). Actin-related proteins share 30–62% sequence identity with conventional actin and are predicted to have similar core structures for nucleotide binding (Kabsch and Holmes, 1995; Kelleher et al., 1995). The actin-related proteins have been grouped into three classes (Schroer et al., 1994), however, the extent of the actin-related protein family is not yet clear. To date, over 20 actin-related proteins have been identified in eukaryotes; an actin-related protein in protists has also been reported (Guerrero et al., 1995). The actin-related proteins appear to have important, but diverse cellular functions. Arp1 null mutants in *S. cerevisiae* have defects in nuclear migration and mitotic spindle orientation (Clark and Meyer, 1994; Muhua et al., 1994). In filamentous fungi, nuclear distribution is also affected by Arp1 disruption (Plamman et al., 1994; Robb et al., 1995; Xiang et al., 1994; Tinsley et al., 1996; Bruno et al., 1996). Actin-related proteins 2 and 3 have been demonstrated to be essential in *S. cerevisiae* and *S. pombe*, respectively (Schwob and Martin, 1992; Harata et al., 1994; Lees-Miller et al., 1992*b*). In *Acanthamoeba*, Arps 2 and 3 are subunits of an 8.5-S oligomeric profilin-binding complex which localizes to the filopodia and cell cortex (Mullins, 1996; Kelleher et al., 1995; Machesky et al., 1994). The most divergent actin-related proteins, Act3p from *S. cerevisiae* and Arp13E from *Drosophila*, have been localized to the nucleus and may play a role in chromatin packaging (Weber et al., 1995).

Centractin (Arp 1), named for its apparent localization to the centrosome, was the first actin-related protein identified in vertebrates (Lees-Miller et al., 1992*a*; Clark and Meyer, 1992). Centractin has been found exclusively as a subunit of a 20-S macromolecular complex, dynactin, that consists of at least seven different polypeptides (Lees-Miller et al., 1992*a*; Paschal et al., 1993; Schafer et al., 1994; Tokito et al., 1996). Ultra-structural analysis of dynactin

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1. *Abbreviations used in this paper*: Arp, actin-related protein; DIC, dynein intermediate chain.

by rotary shadowing electron microscopy and antibody decoration revealed that centractin forms a 37-nm filament of 8 to 13 monomers at the base of the complex, which resembles a short actin filament (Schafer et al., 1994). The actin capping protein, CapZ, and the p62 subunit of dynactin were localized to opposite ends of the centractin filament. The p150^{Glued} subunit of dynactin binds to microtubules and extends from the 37-nm centractin filament to form a 24-nm sidearm (Waterman-Storer et al., 1995; Schafer et al., 1994). The location of the p50 subunit within the complex remains to be determined and other potential components are still being characterized.

Dynactin copurifies with the microtubule-based motor cytoplasmic dynein in preparations from numerous sources (Collins and Vallee, 1989; Steuer et al., 1990; Holzbaur et al., 1991; Gill et al., 1991). Genetic studies have revealed that cytoplasmic dynein and dynactin are involved in similar cellular processes (Plamman et al., 1994; Muhua et al., 1994; Eschel et al., 1993; Li et al., 1993; McGrail et al., 1995; Clark and Meyer, 1994; Robb et al., 1995) and biochemical evidence indicates that the intermediate chain of dynein binds directly to the p150^{Glued} subunit of dynactin (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995).

Cytoplasmic dynein has been implicated in the maintenance of the perinuclear localization of the Golgi (Courthesy-Theulaz et al., 1992), in the transport of lysosomes and endocytic vesicles (Lin and Collins, 1992; Aniento et al., 1993), and in the retrograde transport of organelles along the axon (Schnapp and Reese, 1989; Hirokawa et al., 1990). Dynein and dynactin are also involved in the processes of mitosis and nuclear migration (Vaisberg et al., 1993; Eshel et al., 1993; Li et al., 1993; Muhua et al., 1994; Plamann et al., 1994; Xiang et al., 1994; Saunders et al., 1995; Yeh et al., 1995; Robb et al., 1995; Echeverri et al., 1996). While the role of cytoplasmic dynein is clearly to generate force, the details of the specific role of dynactin in these processes remains to be resolved. The dynactin complex has been shown to be required for cytoplasmic dynein-mediated organelle motility along microtubules in an in vitro assay (Gill et al., 1991), and antibodies that inhibit the dynactin/dynein interaction block fast axonal transport of organelles in extruded squid axoplasm (Waterman-Storer, C.M., S. Kvznetsov, S. Karki, D. Weiss, G. Langford, E.L.F. Holzbaur, manuscript submitted for publication).

Although dynactin and dynein have been demonstrated to be involved in disparate membrane-associated cellular processes, the nature of the interaction of these proteins with the membrane is not yet clear. Both dynein and dynactin may be isolated from membrane-associated as well as soluble cellular fractions (Gill et al., 1991; Holzbaur et al., 1991; Lacey and Haimo, 1992; Tokito et al., 1996). Cytoplasmic dynein and dynactin have been shown to copurify with Golgi-derived membrane fractions (Fath et al., 1994). Immunocytochemistry with antibodies raised to the dynactin subunits p150^{Glued}, centractin, and p50 yields a punctate vesicular staining of the cell, suggesting the association of dynactin with membranous vesicles (Gill et al., 1991; Clark and Meyer, 1992; Paschal et al., 1993; Fath et al., 1994; Echeverri et al., 1996). The association with the membrane appears to be peripheral (Gill et al., 1991; Yu et al., 1992; Fath et al., 1994), yet no membrane receptor or mechanism to tether dynactin and/or dynein to the membrane has been identified.

In this study we used cellular and biochemical analyses to address questions regarding the nature and function of the actin-related protein, centractin. With transfection assays, we demonstrate that overexpression of centractin in mammalian cells results in the assembly of novel filaments. These filaments have morphological and pharmacological properties distinct from F-actin. Affinity chromatography and immunoprecipitations reveal that conventional actin is not a component of the dynactin complex. Coprecipitation assays demonstrate that spectrin and adducin associate with the dynactin complex. In transfected cells, centractin is associated with Golgi membrane structures and Golgi-spectrin ($\beta I\Sigma^*$). Our results suggest a mechanism for attachment of the dynactin complex to the Golgi organelle. We propose that the 37-nm centractin filament, which resembles the short actin mini-filaments associated with the spectrin-membrane network of erythrocyte cells, incorporates into an organelle-spectrin cytoskeleton to tether dynactin to the membrane. In this manner, in conjunction with dynein, dynactin may perform a role in organelle sorting and trafficking.

Materials and Methods

Antibody Production

Polyclonal rabbit antibodies were raised against centractin and p150^{Glued}, expressed as fusion proteins in *E. coli* using the pET system (Novagen, Madison, WI). The centractin antibody UP385 (rabbit) was raised against a bacterially expressed fragment of the human cDNA clone corresponding to amino acids 3 to 376 (Waterman-Storer et al., 1995; and this study). UP454 (rabbit) and UPGP21 (guinea pig) anti-centractin antibodies were raised against amino acids 216 to 376. The anti-p150^{Glued} antibody UP235 (rabbit) was raised against amino acids 39-1325 of the rat polypeptide (Holzbaur et al., 1991). These antibodies were affinity purified against the respective antigen immobilized on a CH-Sepharose 4B matrix (Pharmacia LKB Biotechnology, Piscataway, NJ). The centractin and actin antibodies used in this study were probed against purified actin, purified centractin, and cellular extract fractions to ensure that the antibodies to centractin (UPGP21, UP454, and UP385) and actin (C4) did not cross-react (see Fig. 1).

A monoclonal antibody to 58K, a resident Golgi protein, was a generous gift from Dr. George Bloom, University of Texas Southwestern, Dallas, TX (Bloom and Brashear, 1989). A polyclonal antibody to chicken red blood cell spectrin, MH-1 9B, was a generous gift from Dr. Elizabeth Repasky, Roswell Park Cancer Institute, Buffalo, NY (Black et al., 1988). A monoclonal antibody, VIIIC7, to $\beta I\Sigma I$ spectrin was a generous gift from Drs. Prasad Devarajan and Jon Morrow (Yale University, New Haven, CT) (Harris et al., 1986). A polyclonal antibody raised against canine erythrocyte spectrin, which recognizes $\beta I\Sigma^*$ Golgi-spectrin was a generous gift from Drs. Kenneth Beck (Stanford University, Stanford, CA) and W. James Nelson (Beck et al., 1994). A polyclonal antibody to α-adducin was a generous gift from Dr. Vann Bennett. All other antibodies were obtained from commercial suppliers: mouse monoclonal antibodies to α-fodrin (clone AA6; ICN Biomedicals, Costa Mesa, CA), β-COP (M3A5; Sigma Chem. Co., St. Louis, MO), γ-adaptin (Robinson and Kreis, 1992; A100/3; Sigma) and actin (Cao et al., 1993; clone C4; Boehringer Mannheim Corp., Indianapolis, IN) and a rat monoclonal antibody to tubulin (clone YL1/2; Serotec, Washington, DC).

Cell Culture, Transient Transfections, Immunocytochemistry, and Drug Treatment

Mammalian PtK2 cells were maintained in E-MEM supplemented with 2 mM glutamine, 10% heat-treated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin, and were split twice weekly using trypsin-EDTA (Gibco LTI, Grand Island, NY). Cells were transiently transfected by the calcium phosphate precipitation method (Sambrook et al., 1989) with a construct

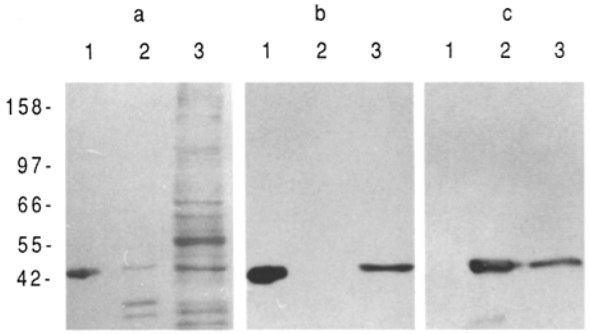


Figure 1. Specificity of antibodies for centractin and actin. Protein samples were resolved on a 10% SDS-PAGE gel, electroblotted to Immobilon, and the blot was (*a*) Coomassie stained. (Lane *1*) Actin purified from acetone powder; (lane *2*) recombinant centractin purified from *E. coli*; (lane *3*), a high salt extract of taxol-stabilized microtubules prepared from rat brain cytosol. (*b*) Corresponding immunoblot of *a* probed with the monoclonal antibody to actin, C4. (*c*) Corresponding immunoblot of *a* probed with a polyclonal antibody to centractin, UP454. The second immunogenic band in *c* (lane *2*) is a breakdown product of recombinant centractin. Note that the two antibodies, C4 and UP454, are not only specific for purified proteins, but are specific in recognition of the respective proteins within a cellular extract fraction (lane *3*). The centractin specific antibodies, UP385 and UPGP21, also used in this study, show no cross-reactivity with actin (not shown).

(pcC) of the human cDNA encoding centractin cloned into pcDNA3 vector (Invitrogen, San Diego, CA) in which expression is under the control of the human cytomegalovirus promoter. After transfection, cells were fixed at time points ranging from 18 to 24 h after washout using either −20°C MeOH with 1 mM EGTA or at 37°C for 5 min in 2% formaldehyde/1% Triton-X 100 (in MES buffer: 0.1 M MES, 1 mM $MgCl_2$, 1 mM EGTA, 0.05 mM EDTA, 4% PEG_{8000}, pH 6.75), followed by 5 min in 0.125% glutaraldehyde in MES, 5 min in MES, 5 min in 2% formaldehyde in MES, and finally two 5-min changes in $NaBH_4$/PBS. The slides were rinsed in PBS and blocked in 5% goat serum containing 1% BSA and 0.05% sodium azide in PBS, pH 7.4. For some experiments the cells were placed on ice for 1–2 h before fixation as noted.

The fixed cells were assayed by immunofluorescence with primary antibodies as noted, either at 4°C overnight or at 30°C for 2 h. Fluorescein-labeled goat anti–mouse and donkey anti–rabbit and/or Texas red–labeled donkey anti–rabbit and donkey anti–guinea pig secondary antibodies were used to detect the primary antibodies. Slides were mounted using Mowial 4-88 (Calbiochem Corp., La Jolla, CA), viewed using a Leica DMRB epifluorescence microscope and 100 × objective (0.6 NA) with FITC and Texas red filters, and photographed using a Leica photoautomat system. In all epifluorescence double-labeled immunocytochemistry experiments, the primary antibody to centractin was followed with Texas red labeling and the primary antibody for the other protein of interest was assayed using fluorescein labeling. This procedure eliminates bleed-through of the centractin signal from one channel to the other (experimental controls reviewed but not shown).

Cytosol Preparation and Immunoprecipitations

Cytosol was prepared by rinsing frozen rat brains in cold 0.9% saline to wash away blood vessels followed by rinsing in PHEM (50 mM Pipes, 50 mM Hepes, 1 mM EDTA, 2 mM $MgCl_2$, pH 6.9). Ice cold PHEM buffer containing leupeptin (10 μg/ml), pepstatin (1 μg/ml), *n*-tosyl-L-arginine methylester (10 μg/ml) and 1 mM phenylmethylsulfonyl fluoride was added to the brain tissue at a 1:1 (wt/vol) ratio. The tissue was Dounce homogenized and the resulting homogenate was clarified by centrifugation at 39,000 *g* for 30 min at 4°C. The supernatant was further clarified at 100,000 *g* for 1 h to obtain cytosol. For immunoprecipitations, 1 ml of a resuspended slurry of protein A agarose beads (GIBCO-BRL, Gaithersburg, MD) was washed in phosphate-buffered saline (PBS, pH 7.4). Antibodies to either centractin (UP454) or $p150^{Glued}$ (UP235) were loaded onto the protein A agarose in separate tubes, rocked overnight at 4°C, and then washed in PBS to remove any unbound antibody. Cytosol was preadsorbed with protein A agarose without antibody for 30 min at 4°C before incubation with antibody-bound beads. The two tubes with antibody (UP454 and UP235) and control protein A agarose beads without antibody were incubated with equal amounts of cytosol with rocking at 4°C for 3 h and then washed extensively in nondenaturing 1× RIPA (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate). The protein A beads were boiled in sample buffer (Laemmli, 1970) and the eluted samples were analyzed by SDS-PAGE and immunoblotting.

ATP Extract of Microtubules and Sucrose Gradients

An ATP extract of microtubules was prepared from cytosol (described above) according to the method of Paschal et al. (1991). The resulting ATP extract from the microtubule affinity preparation was then loaded onto a 5–20% sucrose gradient and centrifuged at 150,000 *g* for 16 h at 4°C. The gradient was then fractionated into 1-ml fractions and analyzed by SDS-PAGE followed by Western blotting.

Affinity Chromatography

Cytosol was prepared as described above. The dynein intermediate chain (DIC) affinity beads and the BSA control beads were prepared as described in Karki and Holzbaur (1995). Briefly, 1 ml of rat brain cytosol was loaded onto an affinity column with a bed volume of 0.4 ml of packed beads and washed with 2 column volumes of 50 mM NaCl in HEM (50 mM Hepes, 1 mM EDTA, 2 mM $MgCl_2$, pH 6.9), followed by 18 column volumes of HEM. The beads were eluted with 1 M NaCl in HEM. The proteins in each sample were precipitated with TCA, resuspended in gel sample buffer and analyzed by SDS-PAGE and Western blotting.

SDS-PAGE and Immunoblotting

Gel samples were resolved by electrophoresis through SDS-PAGE (Laemmli, 1970) and electroblotted onto Immobilon-P membranes (Millipore Corp., Bedford, MA). After Coomassie Brilliant blue staining, the blots were blocked using 5% nonfat milk in Tris-buffered saline, pH 8.0, with 0.05% Tween-20 and 0.05% sodium azide and incubated with primary antibodies overnight at 4°C. Detection was accomplished by means of horseradish peroxidase (Jackson Immunoresearch Labs, West Grove, PA) and Renaissance Western blot chemiluminescence reagent (Dupont New England Nuclear, Boston, MA).

Results

Overexpression of Centractin Results in the Formation of Novel Filaments That Do Not Colocalize with Actin or Microtubules

To investigate the intracellular role of centractin, we began our analysis using transient transfection assays. Transient transfection assays allowed us to create a system in which we could increase the concentration of centractin above normally low cellular levels (α-centractin has been estimated to represent 0.006% of total protein in HeLa cells; Clark et al., 1994), in the presence of any interacting proteins and folding machinery. Transfection assays have been a powerful tool in the study of other cytoskeletal proteins such as in the analysis of functional differences among actin isoforms in mouse myoblasts and cardiomyocytes (Schevzov et al., 1992; Lloyd et al., 1992; Lloyd and Gunning, 1993; von-Arx, 1995) and in the characterization of the pharmacology, self-association, and nucleation characteristics of γ-tubulin (Shu and Joshi, 1995).

Mammalian PtK2 cells were transiently transfected with a construct in which expression of the human cDNA encoding centractin (pcC) is regulated by a strong promoter (CMV). Immunoblot analysis of the transfected cell lysate

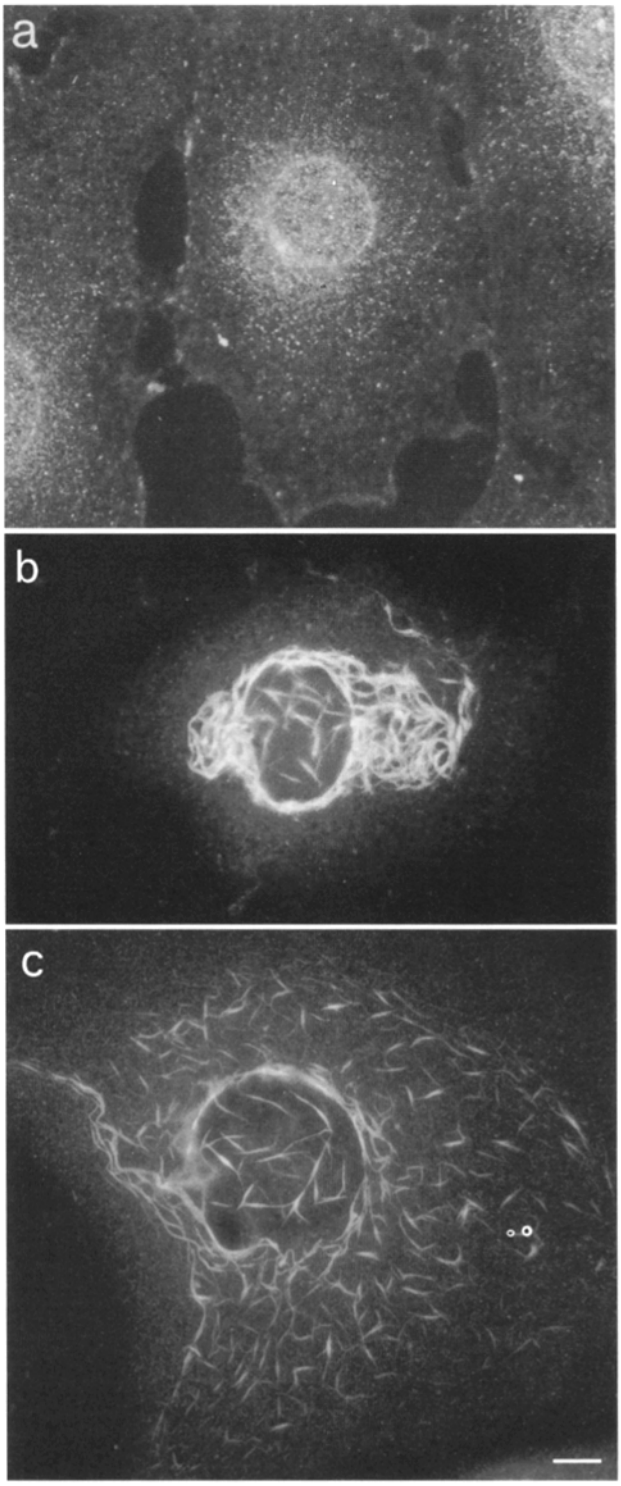


Figure 2. Epifluorescence micrographs of cultured PtK2 cells overexpressing centractin. Cells were transfected with a plasmid encoding human centractin and stained with the centractin-specific polyclonal antibody UP385. (*a*) Untransfected cells reveal centrosomal staining as well as punctate staining indicative of vesicular association. Immunocytochemistry of transfected cells revealed two major phenotypes: (*b*) highly three dimensional extended structures, mainly perinuclear in origin and (*c*) short centractin filaments distributed throughout the cytoplasm. Centractin-specific antibodies UP454 and UPGP21, showed similar results. Bar, 2 μM.

confirmed an increase in the expression level of centractin (data not shown). The transfection frequencies observed in these experiments ranged from 20% to 79% as determined by direct counting of numerous fields of immunostained cells.

In untransfected cells, centractin is localized to punctate vesicular structures as well as to the centrosome (Fig. 2 *a*; and see Clark and Meyer, 1992). In transfected cells overexpressing centractin, we observed novel filamentous structures which reacted strongly with affinity-purified antibodies to centractin. The filamentous structures ranged in length from ~0.63 μm to ~22 μm. Two distinct patterns were consistently observed: cells with centractin filaments localized throughout the cytoplasm (Fig. 2 *c*), and cells with a more perinuclear accumulation of centractin filaments (Fig. 2 *b*). The perinuclear filaments appeared to be highly three dimensional in structure, as confirmed by serial optical sectioning using a confocal microscope (data not shown). In some transfected cells the filaments formed a weblike structure (Fig. 7 *g*) and in other transfected cells we observed single strands of centractin overlying the nucleus or adjacent to the nucleus (Fig. 5 *a*). REF52 and NIH3T3 cells were also transfected with the pcC construct, and identical results were observed. Cells transfected with the vector alone displayed normal punctate vesicular staining of centractin indistinguishable from that seen in untransfected cells.

We performed double-label immunofluorescence with antibodies to either of the cytoskeletal proteins tubulin (YL1/2) or actin (C4) along with antibodies to centractin (UP385 or UP454). In both untransfected and transfected cells, centractin did not colocalize with microtubules (Fig. 3). This is in contrast to the observations made from transfection assays of the p150^{Glued} component of the dynactin complex, which contains a microtubule-binding domain and binds to microtubules in vitro and when overexpressed in transfected cells (Waterman-Storer et al., 1995).

Although the predicted protein sequences of actin and centractin share ~50% identity (Clark and Meyer, 1992), antibodies we have raised to centractin (UP385, UP454, and UPGP21) and a commercial pan-actin monoclonal antibody (C4) were observed to not cross-react as assessed by immunoblots (Fig. 1), or by immunocytochemistry in untransfected cells. Our immunofluorescence data indicate that in transfected cells, overexpressed centractin was not incorporated into conventional actin filaments and actin was not incorporated into the novel centractin filaments (Fig. 4, *a* and *b*).

Pharmacological Properties of Centractin Polymers Are Distinct from Actin

Two characteristic properties of conventional actin are the tight binding of phalloidin to F-actin and the disruption of actin filaments by cytochalasin D (for review see Cooper, 1987). To compare these properties of conventional actin with those of centractin, we treated cells with phalloidin or with cytochalasin D, followed by immunocytochemical analysis.

Phalloidin staining was performed three ways to prevent any competition between the centractin antibody and phalloidin: (*1*) transfected cells were incubated with phalloidin

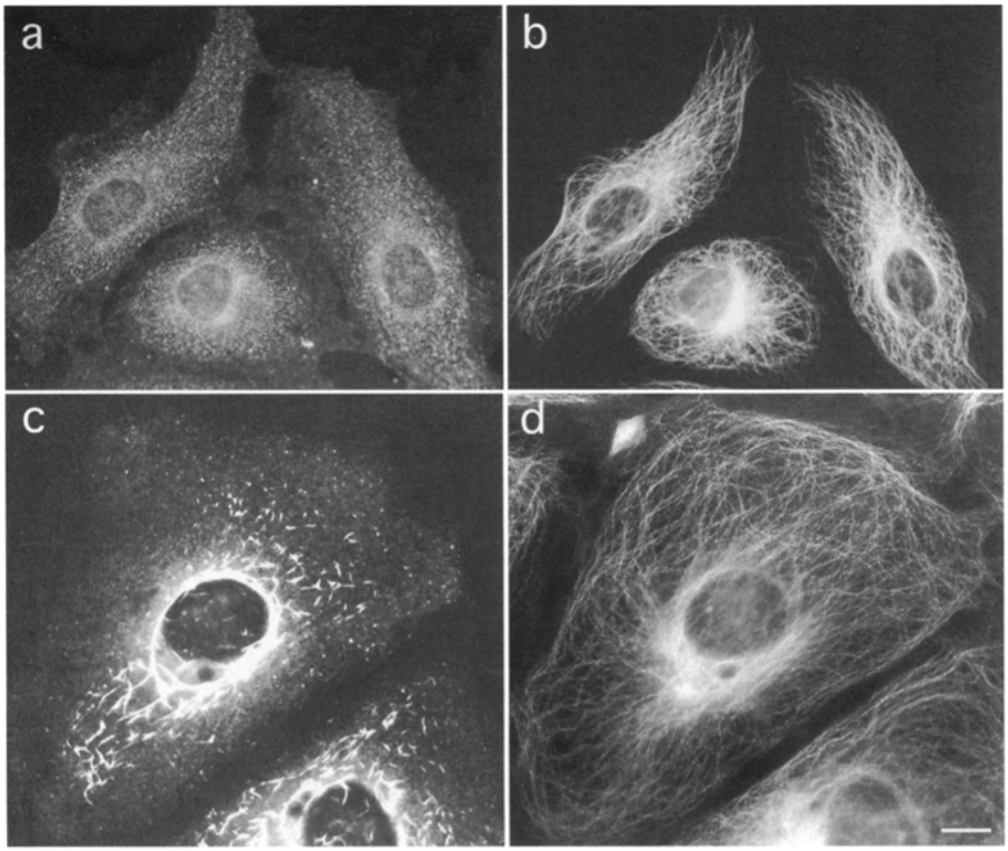


Figure 3. Epifluorescence micrographs demonstrating that overexpressed centractin and microtubules do not colocalize. Cells were fixed in 1 mM EGTA in MeOH and examined by epifluorescence microscopy using double-label immunocytochemistry with affinity-purified anti-centractin antibody, UP385 (*a* and *c*), and a tubulin-specific rat monoclonal antibody, YL1/2 (*b* and *d*). Untransfected PtK2 cells are shown in *a* and *b* and cells transfected with a cDNA construct encoding centractin are shown in *c* and *d*. Bar, 3 μM.

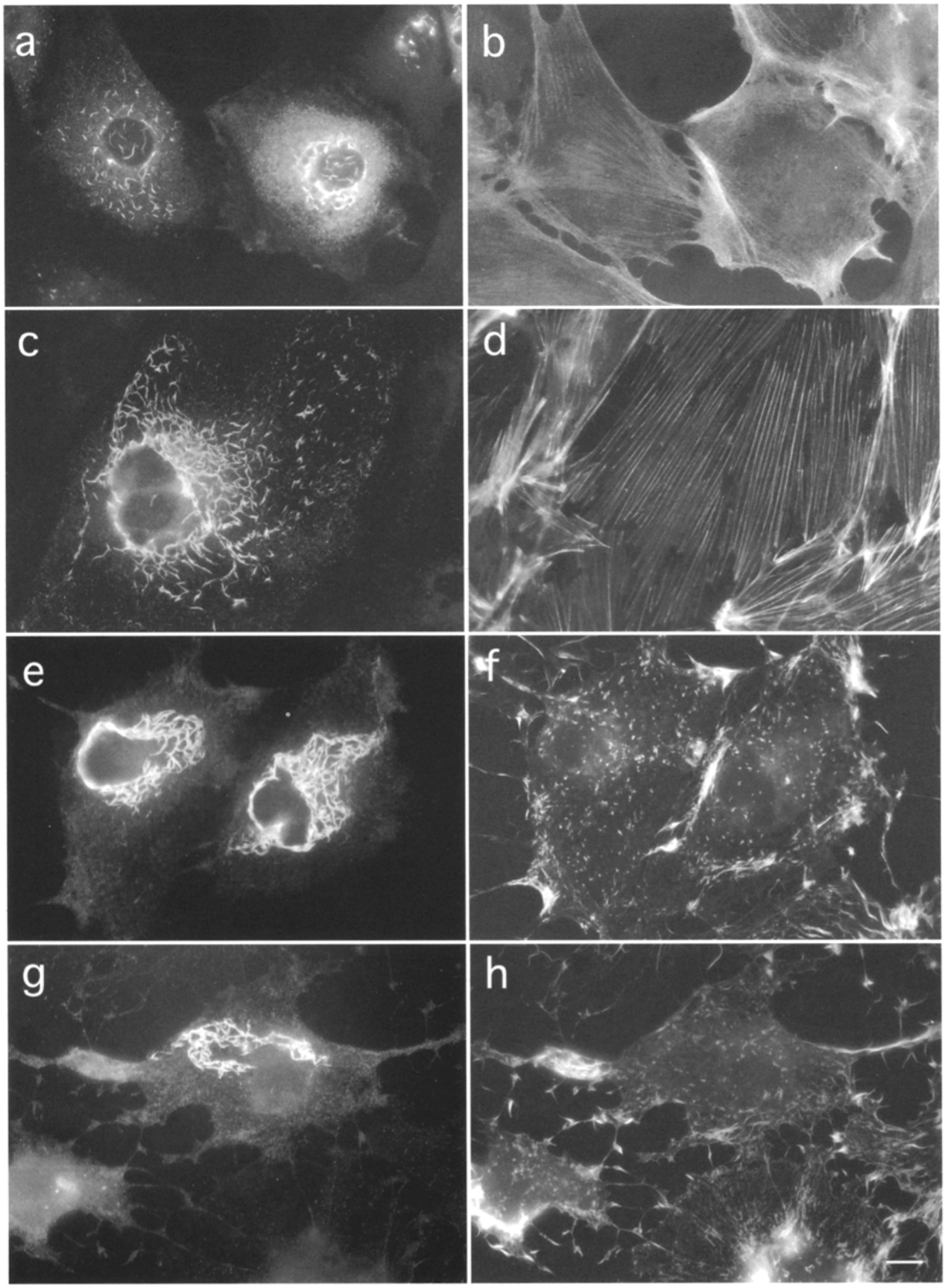
before incubation with the anti-centractin antibody, UP385; (*2*) transfected cells were stained with phalloidin only; and (*3*) phalloidin and UP385 were mixed in solution together and then added to the coverslips. In all three protocols, while conventional actin filament staining was observed, in no case were the centractin filaments observed to label with rhodamine or FITC-conjugated phalloidin (Fig. 4, *c* and *d*).

To analyze the effect of cytochalasin D upon the centractin filaments in transfected cells, we treated the cells with a 2-μM solution for 30 min. While cytochalasin D treatment resulted in the complete disruption of the actin cytoskeleton, the centractin filaments remained unaffected (Fig. 4, *e–h*). In further experiments, following a 30-min incubation with cytochalasin D, the cells were washed and incubated in fresh medium to allow for actin stress fiber regeneration. The cells were fixed at 0.5-, 1-, 2-, 3-, and 24-h time points after treatment with cytochalasin D and assayed with the anti-centractin antibody UP385 and with fluorescein-labeled phalloidin. In these regeneration experiments centractin was not incorporated into stress fibers (data not shown). Our results suggest that centractin and actin have different pharmacological and dynamic properties and that centractin and actin do not copolymerize even under conditions where the actin cytoskeleton is disrupted and allowed to reform at a relatively high cellular concentration of centractin.

Golgi Membrane Markers Colocalize with the Novel Centractin Filaments

Dynactin has been shown to copurify along with cytoplasmic dynein in Golgi-derived membrane fractions (Fath et al., 1994) and immunocytochemistry using antibodies raised against the dynactin subunits p150^{Glued}, centractin, and p50 yields a punctate vesicular staining of the cell, implying an association of dynactin with membranous vesicles (Gill et al., 1991; Clark and Meyer, 1992; Paschal et al., 1993; Fath et al., 1994; Echeverri et al., 1996). In addition, dynactin can be immunoprecipitated from both soluble and membrane fractions (Tokito et al., 1996). These results and our observations of the often perinuclear localization of centractin observed in transfected cells led us to perform double-label immunocytochemistry using antibodies to Golgi proteins and centractin.

Antibodies to 58K, a resident Golgi protein (Bloom and Brashear, 1989), to γ-adaptin (Robinson and Kreis, 1992) and to β-COP (Allen and Kreis, 1986), were used to visualize the Golgi. Using the anti–β-COP antibody, a reticular perinuclear staining pattern typical of Golgi was observed, in addition to the expected β-COP staining of a population of vesicles throughout the cytoplasm in untransfected cells. The anti-58K and γ-adaptin antibodies also revealed an intact Golgi apparatus in untransfected cells. In transfected cells, the antibodies to all three of these Golgi-specific proteins produced a staining pattern coincident with the localization observed with antibodies to centractin. In these transfected cells the Golgi staining pattern lacked the characteristic perinuclear punctate appearance observed in untransfected cells and appeared highly disrupted (Fig. 5). In some transfected cells, the Golgi proteins that we used as markers colocalized with centractin filaments distributed throughout the cytoplasm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and therefore appeared fragmented (Fig. 5 *d*); in others the colocalization with centractin was mainly perinuclear (Fig. 5 *b*).

We investigated the effect of cold treatment and nocodozole on the cells by incubation of the cells with or without 33 μM nocodozole at 4°C for 1–2 h. Interestingly, upon cold treatment (in the absence or presence of nocodozole) the centractin structures became thinner and more extended than was observed at 37°C (Fig. 5 *e*). This effect was reversible upon shifting the temperature from 4°C back to 37°C before fixation. Double immunocytochemical analysis using antibodies to the 58K Golgi resident protein and to centractin revealed an apparent dissociation of the Golgi from the cold-treated cytoplasmic centractin filaments in transfected cells (Fig. 5 *f*). While the exact mechanism for this dissociation is unknown, this observation eliminates the possibility that the integrity of the centractin filament is dependent on an association with tubulovesicular membranes which might act as a template for the formation of centractin polymers. This result also excludes the possibility that centractin associates with the Golgi only because it is being retained by this organelle. We are currently using electron microscopy to determine the structure and composition of these centractin filaments at a higher resolution (Holleran, E.A., G. Grayboard, L.D. Peachey, and E.L.F. Holzbaur, manuscript in preparation).

Non-erythroid Spectrin and Golgi-Spectrin (βIΣ) Colocalize with Centractin Filaments*

Structural and primary sequence comparisons of centractin with conventional actin predict the conservation of surface residues that are known to be involved in interactions of actin with several actin-binding proteins (reviewed in Mullins et al., 1996). The spectrin super-family binding site which has been identified in actin (reviewed in Hartwig, 1994; Matsudaira, 1994) is well-conserved in centractin (Fig. 6). We used a rabbit polyclonal antibody to βIΣ* Golgi-specific spectrin (Beck et al., 1994) and a mouse monoclonal antibody to a broad range of non-erythroid spectrin isoforms, AA6, to perform double-label immunocytochemistry in transfected cells to assay for an association between spectrin and centractin. While untransfected cells showed a perinuclear punctate localization of Golgi-spectrin (βIΣ*) that corresponded to a punctate centractin pattern, in transfected cells the Golgi-specific spectrin colocalized with the centractin filaments (Fig. 7, *c–f*). The transfected cells double-labeled with non-erythroid spectrin and centractin revealed that in addition to the endogenous spectrin localization, spectrin was observed to colocalize with the novel centractin filaments (Fig. 7, *g* and *h*). These unique structures which reacted with a monoclonal antibody to spectrin were absent from control slides of untransfected cells. Our results indicate that while actin and tubulin do not colocalize with the novel centractin filaments observed in transfected cells, Golgi-spectrin and Golgi proteins do, suggesting a close cellular association between the centractin filaments, spectrin, and the Golgi.

Spectrin Is Present in Dynactin-enriched Cellular Fractions

The immunocytochemical results described above suggested a potential association of spectrin with dynactin. We therefore examined cellular fractions in which the dynactin complex is enriched by probing a high salt extract and an ATP extract of taxol-stabilized microtubules prepared from rat brain cytosol with antibodies to spectrin (Fig. 8, *a* and *b*). We observed that spectrin is present within these dynactin-enriched fractions, using both a monoclonal antibody (AA6) which recognizes α-fodrin (α-spectrin) and a polyclonal antibody (MH-1 9B) raised against chicken red blood cell spectrin (Fig. 8 *b*, lanes *3* and *4*).

The dynactin complex sediments at 20 S when loaded onto a 5–20% sucrose gradient (Holzbaur et al., 1991; Gill et al., 1991). We investigated further if there was any spectrin present in the 20-S dynactin-enriched sucrose fraction. We fractionated an ATP-extract of microtubules on a sucrose gradient and analyzed the fractions by immunoblot using antibodies to centractin and spectrin. While the peak of dynactin was found in fractions 4 and 5 and the peak of spectrin was found at a lower S value in fractions 6–8, there was a significant amount of spectrin which comigrated with the dynactin complex (Fig. 8, *c* and *d*).

The Dynactin Complex and Spectrin Coimmunoprecipitate

To examine the possibility of a direct interaction between centractin filaments and spectrin, we performed immunoprecipitations from rat brain cytosol using affinity-purified antibodies to centractin and to p150^{Glued}. These immunoprecipitations were performed under nondenaturing conditions which have been previously demonstrated to result in the coprecipitation of the subunit polypeptides of dynactin (Tokito et al., 1996). As a control for nonspecific interactions, cytosol was also incubated with protein A agarose in the absence of antibodies; these beads were washed and eluted in parallel with the antibody-bound beads.

Analysis of the resulting protein samples by Coomassie blue protein staining and by immunoblotting with antibod-

Figure 4. Centractin in transfected cells does not colocalize with the actin cytoskeleton and is morphologically and pharmacologically distinct from actin. (*a–h*) Cells were fixed in formaldehyde with Triton X-100 and glutaraldehyde and examined by epifluorescence microscopy using double-label immunocytochemistry. (*a* and *b*) Transfected cells double-labeled with the centractin-specific antibody, UP454 (*a*) and a monoclonal actin-specific antibody, C4 (*b*). (*c* and *d*) Fluorescein-labeled phalloidin (final concentration of 0.005 U/μl) was mixed with anti-centractin antibody, UP385 (Molecular Probes, Inc.) and placed on the coverslips for 1 h at room temperature. UP385 staining of centractin in a transfected cell (*c*) and the same cell stained with fluorescein-labeled phalloidin (*d*). (*e–h*) Transfected PtK2 cells were treated with a final concentration of 2 μM cytochalasin D for 30 min at 37°C (Bonder and Mooseker, 1986) before permeabilization and fixation and examined by double-label immunocytochemistry. The cells were processed using UP385 (*e* and *g*) and FITC-phalloidin (*f* and *h*). Under conditions where actin filaments were completely disrupted (*g* and *h*), the novel centractin filaments remained intact (*e* and *g*). Bar, 5 μM.

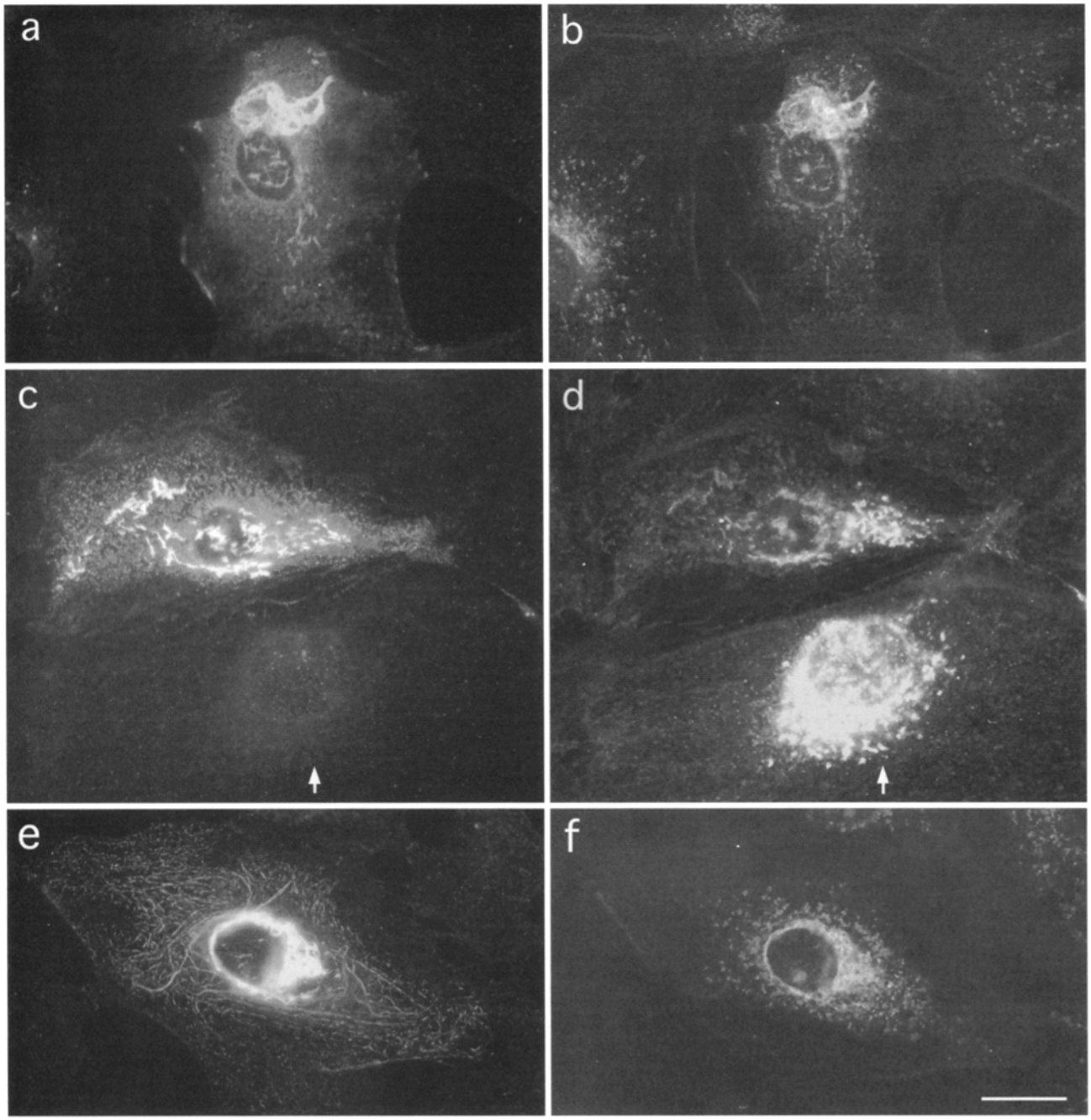


Figure 5. Epifluorescence micrographs demonstrating the colocalization of centractin filaments with the Golgi in transfected cells. (*a–d*) After MeOH fixation, transfected cells were labeled with anti-centractin antibody, UP385 (*a*, *c*, and *e*) and antibodies to Golgi marker proteins β-COP (*b*) and 58K (*d* and *f*). The Golgi is disrupted, thicker and less punctate and follows the centractin pattern, indicating disruption of this organelle from its normal configuration and recruitment to novel centractin filaments in transfected cells, in contrast to the morphology observed in untransfected cells (*arrow*). (*e* and *f*) Cells were treated at 4°C for 1 h before fixation. Upon immunocytochemical analysis using antibodies to centractin and 58K, centractin was observed to form highly extended, thinner filaments that were mainly dissociated from the Golgi. Although we do not know the mechanism, this result suggests that the integrity of the centractin filaments in transfected cells is independent of an association with tubulovesicular membranes, and the association of centractin with the Golgi is not due to retention of excess polypeptide in the organelle. Bar, 5 μM.

ies to centractin and to p150^{Glued} demonstrated that the dynactin complex was immunoprecipitated with either antibody (Fig. 9). We then probed the immunoblots with a monoclonal antibody to spectrin (clone AA6). As shown in Fig. 9 *b* lanes *3* and *4*, significant immunoreactivity with spectrin was observed in the immunoprecipitated dynactin, but not in the control precipitate (Fig. 9 *b*, lane *2*). We then probed the immunoblots with an antibody to adducin, and observed that this polypeptide also coimmunoprecipitated with dynactin (Fig. 9 *b*, lanes *3* and *4*). In contrast, conventional actin remained in the cytosol and was absent from immunoprecipitations performed with antibodies di-

Figure 6. The spectrin-binding site in conventional actin is conserved in centractin. The binding site for spectrin and related family members overlays subdomains 1 and 2 of the conventional actin subunit and subdomain 1 of the adjacent subunit in the actin filament. The residues in actin which bind to the spectrin superfamily of proteins are conserved in the primary sequence of centractin (reviewed in Hartwig, 1994; reviewed in Matsudaira, 1994).

rected against either the p150^{Glued} or centractin subunits of dynactin (Fig. 9 *b*, lane *1*). Control precipitates with protein A agarose beads showed no significant reactivity with antibodies to centractin, p150^{Glued}, actin, spectrin, or adducin (Fig. 9 *b*, lane *2*). In addition to the monoclonal antispectrin AA6 antibody, we also probed the immunoprecipitates with other spectrin antibodies: a monoclonal antibody to the spectrin isoform, βIΣI (Harris et al., 1986) and a polyclonal spectrin antibody, MH-1 9B (Black et al., 1988). These antibodies all recognized similar sized bands slightly above the 212-kD molecular weight marker (Fig. 9 *c*). The results of the immunoprecipitations indicate that spectrin, adducin, and dynactin associate.

Affinity Chromatography Also Demonstrates an Interaction between Dynactin and Spectrin

Previously we have shown that the dynactin complex can be retained on a column of CH-Sepharose beads crosslinked to the dynein intermediate chain (DIC; Karki and Holzbaur, 1995). In this study we observed that in addition to the elution of dynactin components from a DIC column, spectrin also co-elutes (Fig. 10, *a* and *b*). These results in conjunction with the immunoprecipitations described above reveal that dynactin and spectrin associate. Affinity chromatography of dynactin also demonstrates that actin does not co-elute consistently with the complex (data not shown). Our set of experiments excludes the possibility that conventional actin is a *bona fide* dynactin component in contrast to the results of Schafer et al. (1994).

Taken together, our data demonstrate that centractin can form novel filamentous structures in transfected cells which are distinct from conventional actin filaments in composition, morphology and pharmacology, as determined by a resistance to cytochalasin D and an inability to bind phalloidin. These filaments colocalize with Golgi membrane markers and Golgi-spectrin. Analysis of cellular fractions enriched for dynactin revealed that spectrin comigrates with dynactin and affinity chromatography and coimmunoprecipitations demonstrate an association between spectrin, adducin, and the dynactin complex.

Discussion

Centractin has been identified as a member of a growing family of actin-related proteins. Centractin shares 53% identity with conventional actin and forms a 37-nm filament of apparently invariant length in the context of native dynactin. The ultrastructure of this centractin mini-filament reveals similarity to actin and indicates that centractin can self associate to form a polymer of 8 to 13 monomers (Schafer et al., 1994). Our transient transfection studies revealed that centractin can form novel macromolecular filaments of varying lengths (0.62–22 μm), if the protein is expressed significantly above normal cellular levels. The difference in lengths suggests that the mechanism of length regulation is not inherent to the centractin molecule. The length regulation, whether it is due to an association of "ruler proteins" or other processes, is therefore not tightly coupled to the overexpression of centractin protein. At this time, we cannot distinguish whether these filaments are extended polymers, bundles, or associations of shorter centractin filaments. Further analysis using electron microscopy to examine thin sections may clarify these possibilities. However, we have excluded the possibility that the formation of the centractin filaments in transfected cells is due to copolymerization with actin. The integrity of these filaments is also independent of an association with a tubulovesicular membrane template, and is not due to the sequestration of the overexpressed protein in the Golgi apparatus.

The pharmacological properties and cellular distribution of the novel centractin filaments in transfected cells indicate that F-actin and centractin do not incorporate into a common structure. It has been reported that centractin synthesized in an in vitro transcription/translation system was observed to copellet with F-actin in a cosedimentation assay (Melki et al., 1993). It is unclear if this cosedimentation reflects a direct association or if it is mediated by other proteins present within the reticulocyte lysate. In our transient transfection assays, the centractin filaments do not colocalize with actin, do not bind phalloidin, and are not disrupted by cytochalasin D. While it is possible that actin and centractin may interact in vivo by a lateral or capping association which might be below the limits of detection of our transfection assay, our results demonstrate that in transfected cells overexpressed centractin and endogenous actin do not copolymerize to a measurable extent. Even in experiments where we disrupt the filamentous actin with cytochalasin D and then allow the F-actin to reform, we observe no coincorporation of actin into the novel centractin filaments or of centractin into the reforming F-actin. We cannot exclude the possibility that the novel centractin filaments may be stable in the presence of cytochalasin D possibly because they are capped at both ends.

Biochemical analysis revealed that actin was absent from immunoprecipitations of the dynactin complex which effectively concentrate the dynactin complex subunits from cytosol. Actin is also absent from dynactin preparations purified by affinity chromatography. These results contradict a previous study which reported that conventional actin is a *bona fide* component of dynactin (Schafer et al., 1994). We suggest that actin is not an integral subunit of dynactin. Instead, actin may be more loosely associated with dynactin, potentially through a link mediated by other polypeptides.

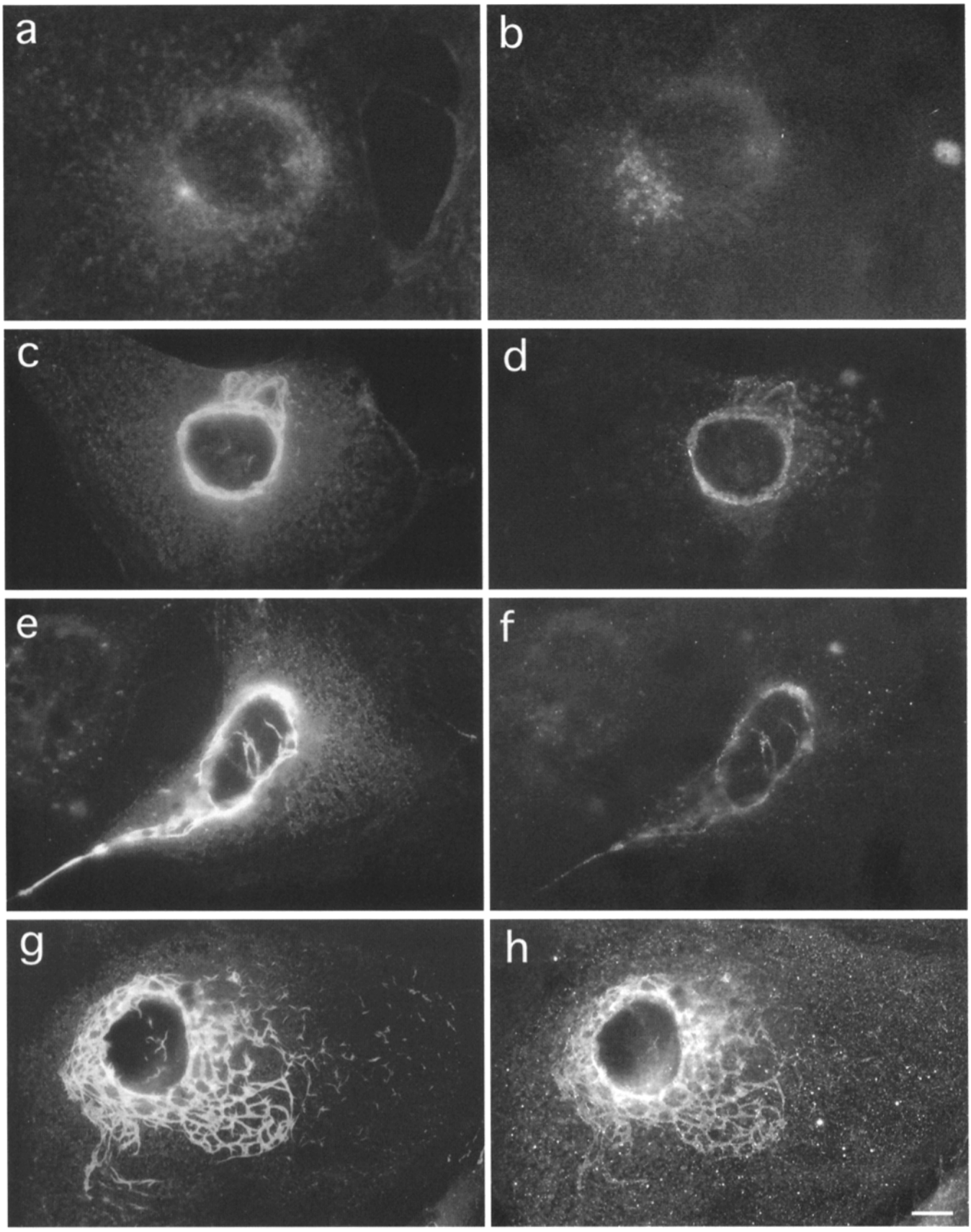


Figure 7. Epifluorescence micrographs demonstrating the colocalization of spectrin and Golgi-spectrin (βIΣ*) with centractin filaments in transfected cells. Cells were processed for IMF after fixation in 1 mM EGTA in MeOH. (*a* and *b*) Double-label immunocytochemistry using the anti-centractin antibody UPGP21 and the anti-Golgi specific antibody, βIΣ*, reveals the colocalization of centractin (*a*) with Golgi-specific spectrin (*b*) in untransfected cells. Analysis of transfected cells using the anti-centractin antibody, UPGP21 (*c* and *e*), and the antibody to the Golgi-specific spectrin, βIΣ* (*b* and *d*), reveals that the two proteins colocalize in transfected cells. Analysis of transfected cells using anti-centractin UP385 antibodies (*g*) and a broad range of anti–non-erythroid spectrin isoform antibody, AA6 (*h*) also demonstrates an association of overexpressed centractin polypeptide with non-erythroid spectrin. Bar, 5 μM.

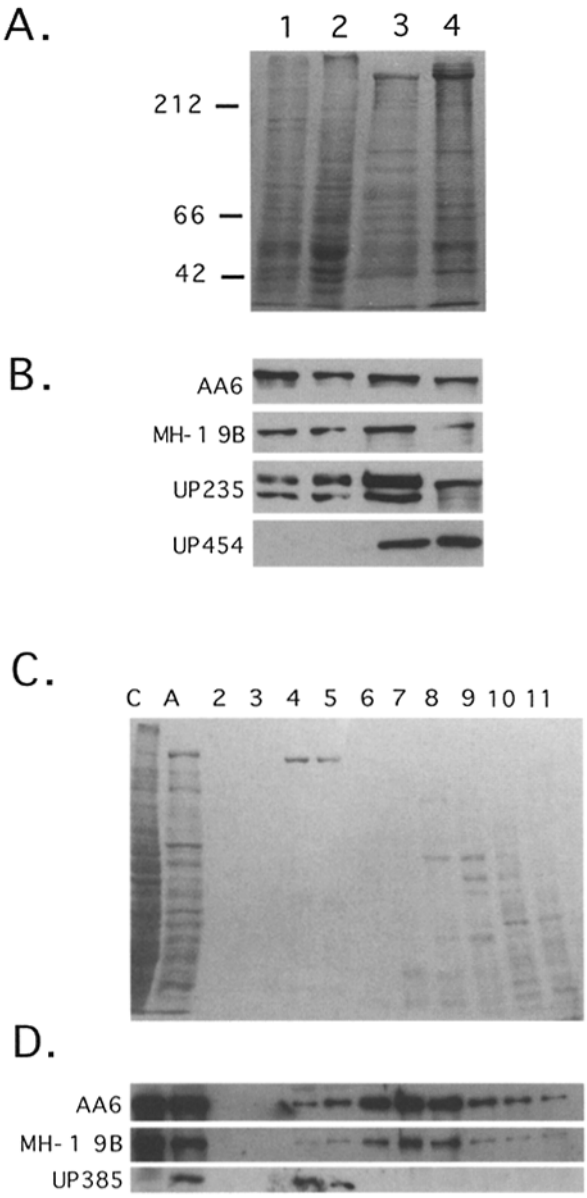


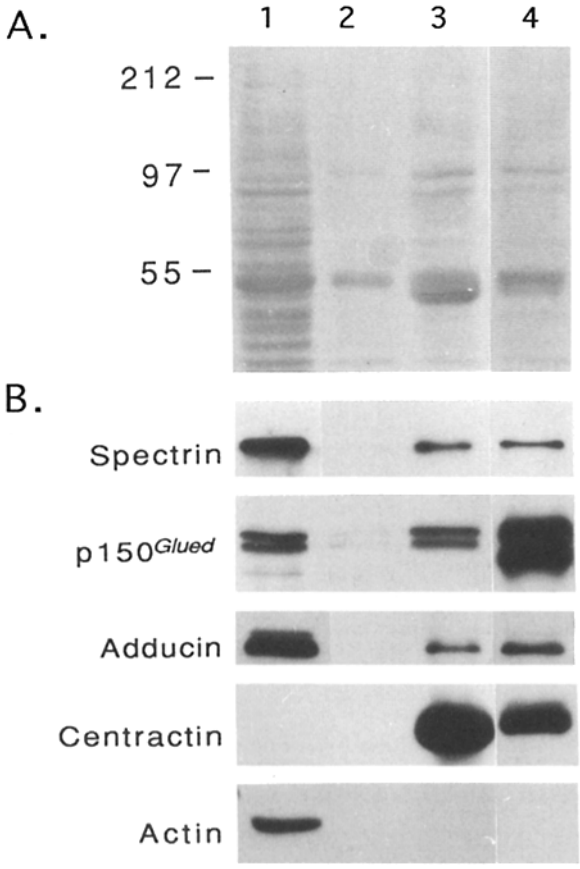
Figure 8. Spectrin is present in fractions in which dynactin is enriched and comigrates with dynactin fractions on a sucrose gradient. (*A*) Protein samples of cellular fractions were resolved on a 7% SDS-PAGE gel, electroblotted to Immobilon, and stained with Coomassie. Lanes: (*1*) Whole brain extract, (*2*) brain cytosol, (*3*) ATP extract, (*4*) NaCl extract. (*B*) Corresponding immunoblots of the blot shown in *A* probed with two anti-spectrin antibodies, AA6 and MH-1 9B, anti-p150^{Glued} antibody UP235 and anti-centractin antibody, UP454. (*C*) Coomassie stained blot of ATP extract of microtubules fractionated over a 5–20% sucrose gradient. (*D*) Corresponding immunoblot in *C* probed with antibodies to spectrin (AA6 and MH-1 9B) and centractin (UP385). While the peak of spectrin is in fractions 6–8, there is a considerable amount of spectrin that comigrates with the dynactin complex in fractions 4 and 5. Although our antibodies to centractin (UP454 and UP385) lack the sensitivity to recognize centractin within the whole brain extract and the cytosol, these two antibodies recognize centractin when it is concentrated in the ATP and high salt extracts of taxol-stabilized microtubules prepared from rat brain cytosol (*B*, lanes *3* and *4*).

In this study we have observed that in cells transfected with centractin, the Golgi is disrupted. These results are intriguing since dynactin appears to play a fundamental role in the processes of nuclear migration and vesicle motility in conjunction with cytoplasmic dynein (reviewed in Holzbaur and Vallee, 1994; Sweeney and Holzbaur, 1996). The colocalization of Golgi marker proteins with centractin filaments revealed that in many transfected cells the organelle becomes elongated in contrast to the normal punctate perinuclear distribution of the Golgi in untransfected control cells. In other transfected cells, the Golgi appears to be fragmented and associated with numerous short centractin filaments. We suggest that as overexpressed centractin forms a filament, fragmented Golgi vesicles are recruited along its length. This would result in "stretching" the Golgi along the side of the forming filament. We speculate that the difference in morphology in transfected cells may be due to differences in the status of the Golgi at the time of centractin overexpression. The Golgi apparatus becomes disrupted during mitosis and is then reformed during interphase (Lucocq et al., 1989). It may be that the cells with shorter filaments were transfected after cell division and the overexpressed centractin filaments become associated with Golgi elements, disrupting the reforming Golgi stacks.

The transfection results revealing Golgi disruption and colocalization of Golgi markers with centractin, the structure of the centractin mini-filament within the native dynactin complex and the primary sequence of centractin, led us to look for potential mediator proteins, like spectrin, in this association of centractin with the Golgi organelle.

In non-erythroid cells spectrin is thought to play a role in membrane compartmentalization and sorting through the differential localization of functionally specific isoforms (reviewed in Goodman et al., 1988; Winkelmann and Forget, 1993). The β non-erythroid spectrins are not as highly conserved as the α spectrins in sequence and structure (Dubreuil et al., 1989; Wasenius et al., 1989; Sahr et al., 1990), and this diversity has been implicated in the functional specialization of individual spectrin subtypes. For example, in addition to the identification of a Golgi-specific β-spectrin isoform (Beck et al., 1994), spectrin isoforms expressed in the brain are associated with "distinct" cytoplasmic organelles and membrane compartments (Lazerides and Nelson, 1983; Zagon et al., 1986). Assigning distinct subtypes of spectrins to different membrane populations would allow directed vesicle trafficking within the cell. Zagon et al. (1986) have determined that in brain, a tissue in which dynactin is highly enriched (Melloni et al., 1995), spectrin localizes to the plasma membrane, the cytoplasmic surface of the ER, the nuclear envelope, the mitochondria, in the cytoplasm of neuronal soma and dendrites and the glial cell body, and synaptic vesicles. Spectrin which is associated with intracellular organelles may play an important role in axonal organelle and vesicle transport possibly by linking membrane proteins and soluble proteins to the microtubules and aiding in their transport (Fach et al., 1985).

Within the membrane network of erythrocyte and non-erythrocyte cells, spectrin associates with short actin oligomers of 10–14 subunits in length (for review see Hartwig, 1994). Primary sequence analysis indicates that those residues in actin which have been shown to participate in



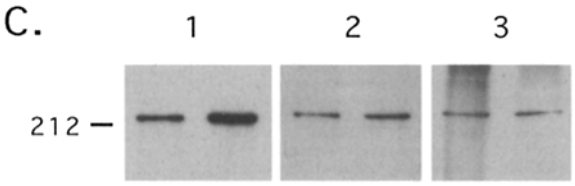


Figure 9. Spectrin coprecipitates with the dynactin complex under nondenaturing conditions. (*A*) Protein samples of immunoprecipitations from rat brain cytosol were resolved on a 7% SDS-PAGE gel, electroblotted to Immobilon, and stained with Coomassie blue. Lanes: (*1*) Rat brain cytosol before immunoprecipitation; (*2*) control precipitation with protein A–agarose with no antibody bound; (*3*) immunoprecipitation with anti-centractin antibody UP454; (*4*) immunoprecipitation with anti-p150^{Glued} antibody UP235. (*B*) Corresponding immunoblots of the blot shown in *A* probed with antibodies to spectrin, p150^{Glued}, adducin, centractin, and conventional actin. These immunoprecipitations demonstrate that the dynactin complex coprecipitates with spectrin and α adducin. In contrast, actin was not observed in the immunoprecipitate. Although our antibodies to centractin (UP454 and UP385) lack the sensitivity to recognize centractin within the cytosol, these two antibodies recognize centractin when it is concentrated in extracts, such as recombinant purified centractin (Fig. 3 *C*, lane *2*), a high salt extract of taxol-stabilized microtubules prepared from rat brain cytosol (Fig. 1 *c*, lane *3* and Fig. 8 *B*, lane *4*) or in an immunoprecipitate with antibodies to dynactin subunits (Fig. 9 *B*, lanes *3* and *4*). (*C*) Immunoblots of the immunoprecipitated samples, UP454 (*left lane*) and UP235 (*right lane*) were probed with three independent anti-sera to spectrin, (*1*) AA6 monoclonal antibody, (*2*) anti-spectrin antibody, VIIIC7 (Harris et al., 1986), and (*3*) polyclonal anti-spectrin MH-1 9B antibody (Black et al., 1988). All three anti-spectrin antibodies recognize a similar protein band migrating just above the 212-kD marker confirming that the band recognized by the AA6 antibody is indeed spectrin.

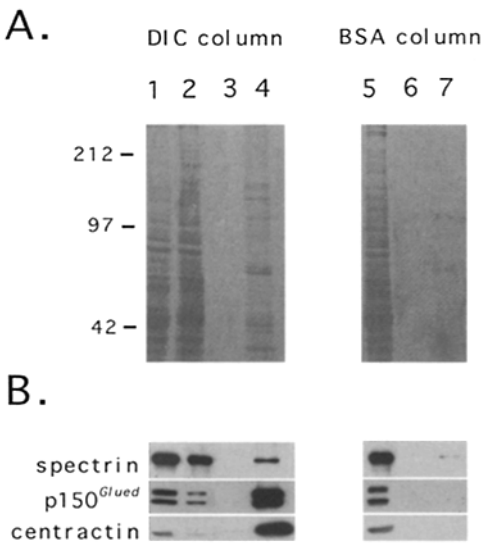


Figure 10. Affinity chromatography reveals that spectrin associates with dynactin. (*A*) Protein samples were resolved on a 7% SDS-PAGE gel, electroblotted to Immobilon, and stained with Coomassie blue. Lanes: (*1*) rat brain cytosol, (*2*) the flow-through of the DIC column, (*3*) the final wash fraction before elution, (*4*) the 1 M NaCl eluate of the DIC column, (*5*) the flow-through of the BSA control column, (*6*) the final wash fraction before elution, and (*7*) 1 M eluate of the BSA control column. (*B*) Corresponding immunoblots of *A* probed with antibodies to spectrin (AA6), p150^{Glued} (UP235), centractin (UP454) demonstrating an association between spectrin and dynactin.

spectrin binding (reviewed by Hartwig, 1994; Matsudaira, 1994) are conserved in centractin (Fig. 6). Stoichiometric analysis of purified dynactin complex has led to estimates of between 8 and 13 subunits of centractin per mini-filament (Gill et al., 1991; Paschal et al., 1993; Schafer et al., 1994) and structural analysis of dynactin reveals that the 37-nm centractin filament resembles actin (Schafer et al., 1994) and can be compared to the short actin oligomers that associate with spectrin in the membrane. In this study we demonstrate that Golgi-spectrin (βIΣ*) and centractin colocalize in transfected cells. The colocalization of centractin with spectrin observed by immunocytochemistry combined with the comigration of dynactin and spectrin in dynactin preparations, affinity chromatography, and coimmunoprecipitations of these two polypeptides suggest that spectrin may associate with dynactin via the centractin filament of the complex. With the results of our study, we suggest a model for an association of dynactin with the vesicle.

We are now designing experiments to probe for direct binding between spectrin and centractin, but we propose that the attachment of dynactin to the membrane cargo in interphase cells may be mediated by the short 37-nm centractin filament in native dynactin through an interaction with a spectrin meshwork associated with the membrane of the Golgi organelle. Because spectrin has many functionally distinct subunit types and splice forms and cen-

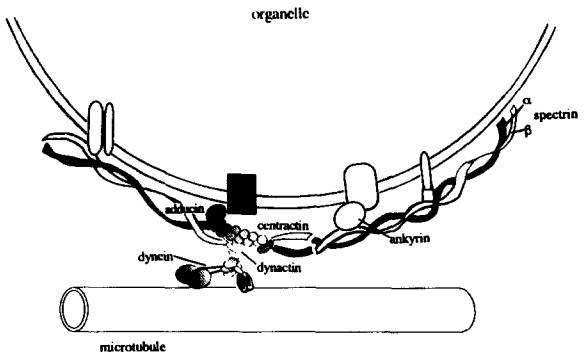


Figure 11. Model depicting the interaction of dynactin with the organelle based on (*1*) the conservation in structure of the centractin mini-filament in the native dynactin complex with the short actin mini-filaments that associate with spectrin in erythrocyte cells, (*2*) the conservation of the spectrin-binding site in conventional actin with the centractin sequence, (*3*) the colocalization of spectrin and Golgi protein antibodies with centractin antibodies using immunocytochemistry, and (*4*) the affinity chromatography of dynactin in conjunction with the coimmunoprecipitation of spectrin and adducin in the absence of actin. We propose that the centractin mini-filament of the dynactin complex incorporates into the spectrin-membrane associated cytoskeleton. The attachment of centractin to spectrin may be strengthened by proteins such as adducin (as denoted by the hatched circle). The specificity of targeting of dynactin to organelles for minus end directed transport along microtubules driven by cytoplasmic dynein may be mediated by organelle-specific isoforms of membrane cytoskeletal linker proteins such as spectrin, ankyrin, or adducin which may then associate with an integral membrane protein (denoted by the black box). Additional proteins may also be involved but are not shown. Plasma membrane-specific isoforms of spectrin may mediate a similar type of attachment of dynactin to the cell membrane. This association might anchor dynactin and dynein to the cortex and allow dynein to produce a force on the cell microtubules of the mitotic apparatus during cell division. (Dynactin in the model is adapted from Karki and Holzbaur, 1995.)

tractin has at least two isoforms (Clark et al., 1994), we suggest that the association of centractin with the Golgi in transfected cells may be mediated by a non-erythroid spectrin isoform which can associate with the Golgi membrane as well as interact with centractin. A candidate is the recently characterized Golgi-spectrin (βIΣ*, Beck et al., 1994). In this way, like erythroid spectrin, which secures the spectrin-actin scaffold to the plasma membrane in red blood cells (Morrow, 1989; Bennett, 1990), an organelle specific isoform of spectrin may link centractin and therefore dynactin to the membrane of the Golgi (Fig. 11). It remains to be determined if other proteins would mediate or reinforce the association of dynactin with the Golgi and/or other cellular organelles. Using immunoprecipitations, we show that α-adducin, in addition to spectrin, coprecipitates with the dynactin complex. Erythrocyte adducin promotes spectrin-actin binding in vitro (Gardner and Bennett, 1986; Li and Bennett, 1996). In non-erythrocyte cells, adducin has multiple isoforms that vary in their associations and properties (Joshi et al., 1990; Bennett et al., 1988; Waseem and Palfrey, 1990). The complexity of adducin isoforms and subunit associations makes it tempting to speculate that there may exist an isoform specific for promoting an association between dynactin and spectrin and a membrane receptor. The identification of multiple isoforms of β-spectrin, α and β centractin isoforms, and isoforms of other proteins such as adducin suggests that together these interactions can provide multiple combinations and may be important for vesicle targeting and sorting and Golgi maintenance by motor proteins and their associated accessory complexes such as dynactin.

Mutations in subunits of either dynein or dynactin in yeast, *Aspergillus*, *Neurospora*, and cultured cells have indicated a role for these complexes in nuclear migration and spindle maintenance during mitosis (Eshel et al., 1993; Li et al., 1993; Muhua et al., 1994; Plamann et al., 1994; Xiang et al., 1994; Saunders et al., 1995; Yeh et al., 1995). Overexpression of the dynactin subunit, p50, resulted in aberrant spindle pole formation in some transfected cells (Echeverri et al., 1996). Vaisberg et al. (1993) observed that micro-injection of antibodies raised against the ATP-binding site of the cytoplasmic dynein heavy chain led to a collapse of the interpolar distance of the spindle. These results demonstrate that dynein and dynactin may be involved in the maintenance of centrosome separation early in mitosis. A minus-end directed motor such as dynein may contribute to spindle pole separation by providing a pulling force via the astral microtubules from an anchorage in the cell cortex (Vaisberg et al., 1993; Waters et al., 1993). In this model, the tethering of dynein to the cortex would provide support for the motor so that an effective pulling force could be produced on the microtubules of the spindle apparatus. Such an interaction between dynein and the cell cortex may be mediated by dynactin. We propose that in a manner similar to our model for the association of dynactin with the vesicle (Fig. 11), dynactin may also associate with the plasma membrane via the incorporation of the centractin mini-filament into a membrane-associated spectrin meshwork. In this manner, dynactin would link dynein to the cell cortex while allowing it to interact with the microtubules emanating from the spindle poles.

In summary, when expressed significantly above endogenous levels, centractin can form filamentous structures within the cell. Actin is not coincorporated into the centractin filaments, and the novel filaments are pharmacologically distinct from F-actin. The cellular analysis of centractin in transfected cells coupled with biochemical analysis using sucrose gradients, immunoprecipitations, and affinity chromatography has yielded results which enable us to propose a specific mechanism for dynactin attachment to the vesicle/membrane. While it has been speculated that both dynactin and dynein may be linked to the vesicle via a cytoskeletal membrane-associated network (Vallee and Sheetz, 1996), our study provides the first specific evidence for such an interaction. We suggest that association of the dynactin complex with its membrane target can be mediated by spectrin, possibly by incorporation of the 37-nm centractin filament within the complex into the spectrin network that associates with membranes and vesicles. In this model, centractin would serve as the structural element of dynactin that links dynactin to its membrane target or vesicle cargo.

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