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The p150^{Glued} component of the dynactin complex binds to both microtubules and the actin-related protein centractin (Arp-1)

(actin-related protein/cytoplasmic dynein/microtubule-binding domain/Glued/microtubule motor)

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ABSTRACT **p150^{Glued} was first identified as a polypeptide that copurifies with cytoplasmic dynein, the minus-end-directed microtubule-based motor protein, and has more recently been shown to be present as a member of the oligomeric dynactin complex, which includes the actin-related protein centractin (Arp-1). Dynactin is thought to mediate dynein-driven vesicle motility, as well as nuclear transport, in lower eukaryotes. The mechanism by which dynactin may function in these cellular processes is unknown. To examine the role of the dynactin complex *in vivo*, we overexpressed the rat cDNA encoding p150^{Glued} in Rat-2 fibroblasts. Overexpression of full-length, as well as C-terminal deletion, constructs resulted in the decoration of microtubules with the p150^{Glued} polypeptides. This cellular evidence for microtubule association was corroborated by *in vitro* microtubule-binding assays. Amino acids 39–150 of p150^{Glued} were determined to be sufficient for microtubule association. We also tested for a direct interaction between p150^{Glued} and centractin. *In vitro* translated centractin was specifically retained by a p150^{Glued} affinity column, and this interaction was blocked by a synthetic peptide which corresponds to a highly conserved motif from the C terminus of p150^{Glued}. These results demonstrate that p150^{Glued}, a protein implicated in cytoplasmic dynein-based microtubule motility, is capable of direct binding to both microtubules and centractin.**

p150^{Glued} was first identified as a polypeptide of 150 kDa, which copurifies with the microtubule-activated mechanochemical ATPase, cytoplasmic dynein (1). Subsequent biochemical characterization indicated that p150^{Glued} exists in cells as part of a stable macromolecular complex (2), termed the dynactin complex (3), together with nine other polypeptides (4). The major components of the dynactin complex include p150^{Glued}, a 50-kDa polypeptide, and the actin-related protein centractin or Arp-1 (4–6), in a stoichiometry of 2:6:10, respectively (2, 4). Immunoprecipitation and sucrose gradient sedimentation studies demonstrated that the dynactin, or Glued, complex is distinct from cytoplasmic dynein in the cytosol (2); however, dynactin was found to be required to obtain cytoplasmic dynein-mediated vesicle motility along microtubules in an *in vitro* assay (3). These data suggested a role for the dynactin complex in cytoplasmic dynein-driven, microtubule-based vesicle motility.

The two best-characterized members of the dynactin complex are p150^{Glued} and centractin. Molecular analysis of rat cDNAs encoding p150^{Glued} revealed 32% amino acid sequence identity to the product of the *Drosophila* gene Glued (7). A dominant mutation at the Glued locus of *Drosophila* results in pleiotropic developmental defects in the eye and optic lobe in heterozygotes and an embryonic lethal phenotype in homozygotes (8). Centractin is a recently characterized actin-related protein, which shares 53% sequence identity to human α-actin (5), compared with the >70% sequence identity found among conventional actins from divergent sources. Recent genetic analyses of centractin mutants identified in *Neurospora crassa* (9) and *Saccharomyces cerevisiae* (10, 11) have pointed to roles for this protein in nuclear migration and mitotic spindle positioning. As mutations in the heavy chain of cytoplasmic dynein in both of these organisms also give rise to similar phenotypes (10–13), an interaction between cytoplasmic dynein and dynactin *in vivo* is suggested.

The mechanism by which the dynactin complex may potentiate the dynein-driven processes of vesicular transport, nuclear migration, and spindle orientation is not yet known. Here, we have used overexpression studies and *in vitro* binding assays to establish that a member of the dynactin complex, p150^{Glued}, is capable of binding directly both to microtubules and to centractin. This result provides a mechanistic insight into how dynactin may mediate the interactions among cytoplasmic dynein, its cellular cargo, and the cytoskeleton *in vivo*.

MATERIALS AND METHODS

Cellular Transfection and Immunocytochemistry. The plasmid pc150 was constructed by subcloning the full-length rat cDNA encoding p150^{Glued} (7) by inserting it into the eukaryotic expression vector pcDNA1/neo under the control of the human cytomegalovirus promoter (Invitrogen). Deletion constructs of pc150 were engineered at appropriate sites to generate C-terminal truncations of p150^{Glued} at amino acids 1082, 942, 811, and 305. All constructs were verified by analyzing their DNA sequence by using the Sequenase system (United States Biochemical).

These constructs were used to transfect Rat-2 fibroblasts [American Type Culture Collection (ATCC)] growing on coverslips by calcium phosphate precipitation (14). Forty-eight hours after transfection, cells were fixed at −20°C in methanol with 1 mM EGTA and processed for indirect immunofluorescence microscopy with antibodies to p150^{Glued} and tubulin. The polyclonal rabbit anti-p150^{Glued} antibody was raised against a peptide fragment of the rat p150^{Glued} protein expressed in *Escherichia coli* from a 2.1-kb fragment of rat p150^{Glued} cDNA in the expression vector pET-15b (Novagen) and purified by means of the His-tag system by affinity chromatography on a Ni^{2+} column. Rabbit serum against this fusion protein was affinity purified by binding to the immunogen polypeptide immobilized on cyanogen bromide-activated Sepharose 4B (Pharmacia). The purified antibodies reacted solely with a doublet of 150/135 kDa on immunoblots of whole Rat-2 cell lysate (not shown). Microtubules were localized with the rat monoclonal antibody YL1/2 to tubulin (Serotec), and cytoplasmic dynein was localized by using the mouse monoclonal anti-cytoplasmic dynein heavy chain antibody 440.1 (Sigma). In some experiments, to depolymerize microtubules cells were treated with 10 μM nocodazole for 45 min at 37°C prior to fixation. Immunofluorescence microscopy was performed on a Zeiss Photomicroscope III.

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Abbreviation: BSA, bovine serum albumin.

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Microtubule-Binding Assays. C-terminal deletion constructs of p150^{Glued} for *in vitro* transcription and translation were generated by linearizing the original pBluescript template at appropriate restriction sites. [^{35}S]methionine-labeled protein was synthesized by using a coupled transcription/translation rabbit reticulocyte lysate system (Promega). Following a 1:3 dilution in PEMT buffer (100 mM NaPipes/2 mM $MgCl_2$/1 mM EGTA/0.2% Triton X-100, pH 6.8), translation products were incubated for 15 min at 20°C and then centrifuged at 35,000 × *g* for 1 h at 4°C. Eight microliters of the supernatant was then incubated for 20 min at 20°C with buffer only, with 20 μg of DEAE-purified, taxol-stabilized bovine brain microtubules, or with 20 μg of the purified and stabilized microtubules and 1 M NaCl. Microtubules were then sedimented through a cushion of 30% sucrose in PEMT buffer in a microcentrifuge, and both supernatants and pellets (washed once with PEMT buffer) were analyzed by SDS/PAGE and autoradiography. The binding affinity of p150^{Glued} for microtubules was determined as described (15).

Construction of the p150^{Glued} Affinity Column and the Centractin-Binding Assay. A polypeptide corresponding to the C-terminal 1286 amino acids of p150^{Glued} was expressed in bacteria, purified by Ni^{2+}-affinity chromatography, and bound to Affi-Gel 10 (Bio-Rad) in PBS buffer (50 mM sodium phosphate/150 mM NaCl, pH 6.4). The affinity matrix was blocked with 1.0 M ethanolamine·HCl at pH 8.0 and equilibrated in PHEM buffer (50 mM NaPipes/50 mM Hepes/2 mM $MgCl_2$/1 mM EDTA, pH 7.0). Affinity columns containing a 0.5 ml bed volume of p150^{Glued} affinity matrix were used to assay centractin binding. The 2.9-kb cDNA encoding human centractin was obtained by screening a human fetal brain cDNA library (Stratagene) by hybridization with a 777-bp probe (clone EST00370; ref. 16; obtained from ATCC) and verified by sequencing the DNA. The centractin cDNA was inserted into pET-15b, which provided a promoter for *in vitro* transcription and translation. The [^{35}S]methionine-labeled translation products were diluted 1:20 in PHEM buffer and applied to the p150^{Glued} affinity column, as well as to a column that contained an equivalent amount of bound bovine serum albumin (BSA). Columns were washed extensively with PHEM buffer, and protein was then eluted with a step gradient of 0.5, 1.0, and 1.5 M NaCl. Eluates were concentrated by methanol precipitation, analyzed by SDS/PAGE, and processed for autoradiography. For peptide-block experiments, diluted centractin translation products were incubated with a 50-fold molar excess of the synthetic peptide MKKEKEFEETMDALQA or EAKCDTKAEMKLEQFE or with BSA for 1 h at room temperature with agitation prior to being applied to the affinity column.

RESULTS AND DISCUSSION

To examine the role of the dynactin complex *in vivo*, we transiently overexpressed the p150^{Glued} polypeptide in Rat-2 fibroblasts. Transfected cells were assayed by double immunofluorescence labeling with an affinity-purified rabbit polyclonal antibody to p150^{Glued} and a rat monoclonal anti-tubulin antibody (Fig. 1). Untransfected control Rat-2 cells displayed centrosomal and punctate cytoplasmic staining as previously described (2, 3). Overexpression of the full-length p150^{Glued} polypeptide (1325 amino acids) in transfected cells resulted in p150^{Glued} decoration along the entire length of interphase microtubules (Fig. 1*A*). In many transfected cells, overexpression of p150^{Glued} altered the normal distribution of microtubules, such that they no longer emanated from a single focal point at the centrosome but appeared to be grouped into thick, wavy bundles (Fig. 2*C*).

To determine the domain within p150^{Glued} responsible for microtubule binding, C-terminal deletion constructs for overexpression were engineered that encoded polypeptides 305, 811, 942, and 1082 amino acids in length. Microtubule decoration and morphological alterations identical to those seen in full-length p150^{Glued} transfectants were observed upon overexpression of each of these deletion constructs (Figs. 2 and 3). This indicates that p150^{Glued} contains a microtubule-binding motif within the N-terminal 305 amino acids of the polypeptide and that this region may be capable of inducing microtubule bundling.

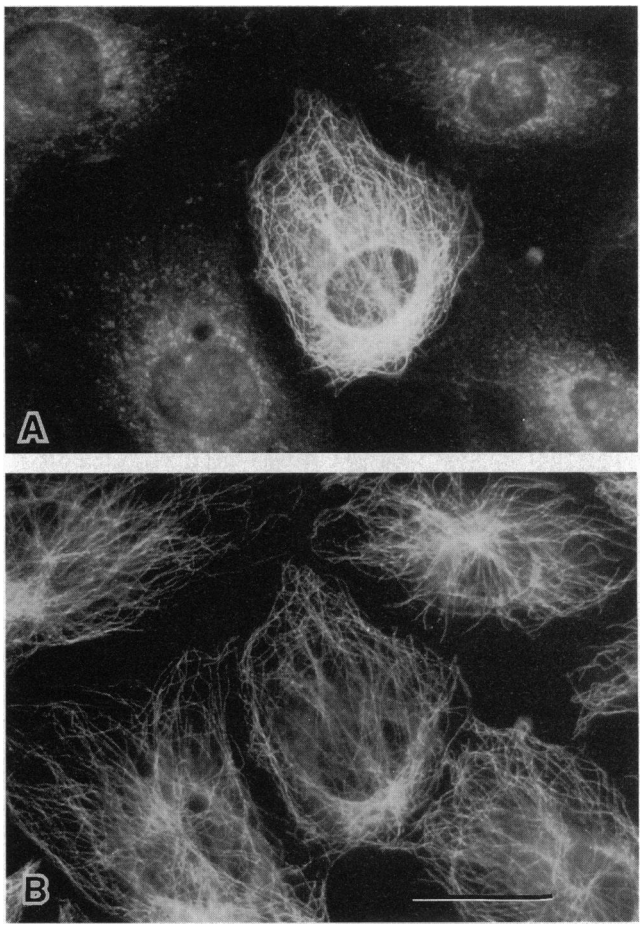

FIG. 1. Overexpression of full-length p150^{Glued} in Rat-2 fibroblasts. Cells were processed for double-label immunofluorescence with antibodies directed against p150^{Glued} (*A*) and tubulin (*B*). (Bar = 10 μm.)

p150^{Glued} and cytoplasmic dynein colocalize in cells (17) and apparently interact during vesicle transport *in vitro* (3). In addition, the centrosomal distribution of p150^{Glued} is microtubule dependent (2) and is believed to be a result of an accumulation of the polypeptide at the centrosome induced by association of dynactin with the minus-end-directed motor, cytoplasmic dynein. To approach the question of whether p150^{Glued} and cytoplasmic dynein interact *in vivo*, we examined the effect of overexpression of p150^{Glued} on the localization of cytoplasmic dynein in transfected cells. The distribution of cytoplasmic dynein heavy chain in cells overexpressing the full-length p150^{Glued} was unaltered compared with the distribution in untransfected interphase cells (Fig. 2 *C* and *D*). Thus, although colocalization is observed in untransfected cells, overexpression of p150^{Glued} did not induce recruitment of cytoplasmic dynein to cellular microtubules. This suggests that p150^{Glued} alone may not be capable of direct interaction with cytoplasmic dynein heavy chain; this interaction may require other members of the dynactin complex. Alternatively, the interaction may be transient or rely on posttranslational modifications such as phosphorylation, which may not be sufficiently regulated in the overexpressed protein.

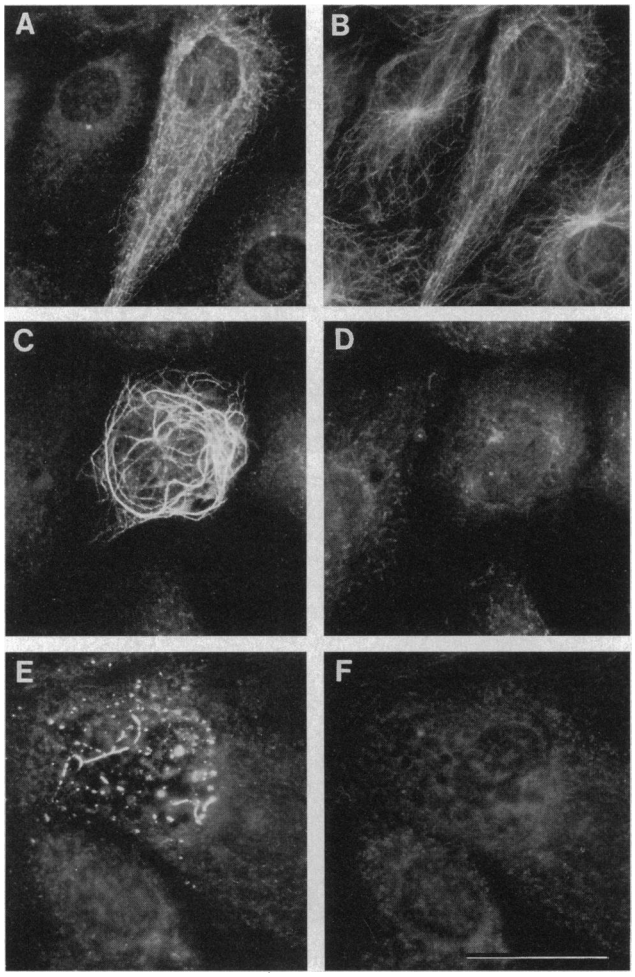

FIG. 2. (*A* and *B*) Localization of p150^{Glued} (*A*) and tubulin (*B*) in Rat-2 cells overexpressing the first 305 amino acids of p150^{Glued}. Immunolocalization of p150^{Glued} (*C*) and cytoplasmic dynein heavy chain (*D*) in transfected cells expressing full-length p150^{Glued}. (*E* and *F*) Localization of p150^{Glued} (*E*) and tubulin (*F*) in nocodazole-treated cells that were overexpressing full-length p150^{Glued}. (Bar = 10 μm.)

To test whether the removal of cellular microtubules would cause overexpressed p150^{Glued} to collect on any other cellular structures, such as vesicles or the actin cytoskeleton, cells transfected with the plasmid encoding the full-length p150^{Glued} polypeptide were treated with nocodazole to depolymerize microtubules. This treatment resulted in intense decoration of apparent microtubule remnants by p150^{Glued} (Fig. 2 *E* and *F*). Double labeling with anti-tubulin could not confirm the identity of these structures, but lack of antibody labeling may be due to steric hindrance imposed by the large excess of p150^{Glued} on the surface of the few microtubules that remained (18). These results demonstrate that depolymerization of microtubules does not cause overexpressed p150^{Glued} to reassociate with other intracellular structures, and decoration of microtubules by p150^{Glued} does not induce significant resistance to nocodazole treatment, as has been observed in the overexpression of other microtubule-associated proteins (18, 19).

In vitro microtubule binding and sedimentation assays were then used to define more precisely the molecular domain involved in the p150^{Glued}–microtubule interaction, as well as to corroborate the cellular evidence for this interaction. p150^{Glued} constructs were translated *in vitro*, incubated with taxol-stabilized microtubules, and tested for salt-dependent cosedimentation. In this assay, full-length p150^{Glued} cosedimented completely with microtubules (Fig. 3*A*). Among the constructs with C-terminal deletions tested using this assay, a fragment containing the first 76 amino acids of p150^{Glued} was the shortest polypeptide capable of at least partial cosedimentation with microtubules (Fig. 3*A*). In addition, a construct lacking the first 38 amino acids cosedimented with microtubules completely, while no cosedimentation was observed for a construct in which the first 150 amino acids were deleted (Fig. 3*A*). All other constructs tested bound to microtubules in a salt-dependent manner (Fig. 3*B*). The binding affinity of p150^{Glued} expressed *in vitro* for microtubules was determined by assaying the extent of binding as a function of the concentration of polymerized tubulin (15). A dissociation constant (K_d) of 10 μM (n = 2) was determined by densitometry of the resulting autoradiographs.

These results illustrate that a microtubule-binding domain lies between amino acids 39 and 150 of p150^{Glued}. It was recently shown that the N termini of p150^{Glued} and the yeast microtubule-associated protein BIK-1 (20) share a homologous motif with CLIP-170, a protein believed to link endocytic carrier vesicles to microtubules (21). This motif, present as a tandem repeat in CLIP-170, was shown to be necessary and sufficient for microtubule binding. CLIP-170 and p150^{Glued} share no significant relatedness outside of this motif, except for predicted α-helical coiled coils in regions C-terminal to their microtubule-binding domains. CLIP-170 has been shown to form a dimer *in vivo* (21). The observation of microtubule bundling in cells overexpressing p150^{Glued} constructs suggests that in this experiment, p150^{Glued} may form antiparallel dimers capable of cross-linking microtubules. Even the shortest C-terminal deletion constructs used for transfection encoded a portion of the predicted coiled-coil domain, and cells expressing these constructs also displayed the microtubule bundling phenotype. The stoichiometry of the dynactin complex suggests the presence of two molecules of p150^{Glued} per complex (4). These polypeptides may also self-associate in the native complex. Alternatively, the microtubule-bundling phenotype seen in transfected cells may be an artifact of the high levels of p150^{Glued} expression induced in transfected cells, and at normal levels of expression, the C-terminal coiled coils of p150^{Glued} may take part in heterotypic interactions with other members of the dynactin complex or with subunits of cytoplasmic dynein.

The recent demonstration that p150^{Glued} is a member of a multisubunit complex that includes the actin-related protein centractin (2, 6) prompted us to investigate whether these two polypeptides interacted directly. Binding of centractin by p150^{Glued} was demonstrated by constructing an affinity column of bacterially expressed p150^{Glued} immobilized on agarose beads and then applying [^{35}S]methionine-labeled human centractin, which had been synthesized by *in vitro* translation of centractin cDNA. Centractin was specifically retained on the p150^{Glued} column through extensive washing and was eluted by high-salt buffer (Fig. 4 *A* and *B*). Centractin was not specifically retained on a control BSA column (Fig. 4*C*) or by agarose beads alone (not shown).

Examination of predicted amino acid sequences of p150^{Glued} from rat (7), chicken (3), and *Drosophila* (22) revealed a highly conserved, C-terminal cluster of charged amino acids (KKEK) that is identical to a motif implicated in actin binding by the headpiece domain of villin, an actin-bundling protein of the epithelial brush border (23). To test the hypothesis that centractin binds to p150^{Glued} by means of the conserved KKEK motif in a manner analogous to the binding of conventional actin by villin, *in vitro* translated centractin was incubated with a 50-fold molar excess of the peptide MKKEKEFEETMDALQA (corresponding to amino acids 1005–1019 of p150^{Glued}) prior to its application to the p150^{Glued} affinity column. This peptide effectively blocked a majority of the binding activity that could be eluted by 0.5 M NaCl (88% reduction in signal compared with unblocked control, n = 2; Fig. 4*D*), while preincubation of translation product with BSA had no effect on the binding of centractin to the p150^{Glued}

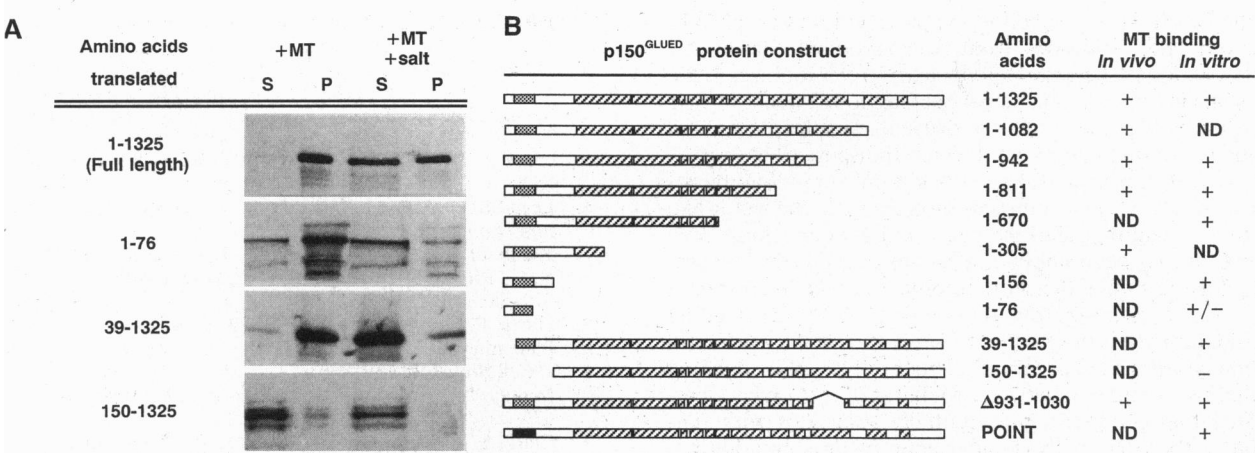

FIG. 3. (*A*) Autoradiograms of *in vitro* microtubule-binding assays with selected constructs of p150^{Glued}. *In vitro* translated p150^{Glued} constructs were incubated with microtubules (+MT) or with microtubules plus 1 M NaCl (+MT, +salt), and the microtubules were sedimented. Microtubule binding is demonstrated by cosedimentation of the construct with microtubules into the pellet (P), while lack of association would result in the protein remaining soluble in the supernatant (S). (*B*) Summary of the results of transfection and microtubule-binding experiments. For transfection experiments, + signifies interphase microtubule decoration by p150^{Glued} in Rat-2 fibroblasts (*in vivo* column). For *in vitro* microtubule binding experiments, + signifies complete cosedimentation of the translated construct, +/− signifies partial cosedimentation (>50%) and − signifies no cosedimentation (*in vitro* column). Stippled area represents the putative microtubule-binding domain, hatched areas represent regions of predicted α-helical coiled coil, and the black area represents a region of the construct containing the point mutations Lys-65 to Glu and Lys-67 to Glu. ND, not determined.

column (Fig. 4*E*). A peptide composed of identical amino acids but in a randomized order (EAKCDTKAEMKLEQFE) was similarly tested for its ability to block the interaction between centractin and p150^{Glued}. Preincubation of centractin translation product with the randomized peptide reduced the amount of centractin retained by the p150^{Glued} column by 43% ($n = 2$) compared with an unblocked control (data not shown). As both the native and random sequence peptides are primarily composed of charged amino acids, the partial disruption of binding observed with the randomized peptide suggests that ionic interactions are key to the p150^{Glued}–centractin binding interaction. However, because more complete disruption of binding was seen with the native peptide, it is likely that the sequence, as well as the composition of the unscrambled peptide, was important in disrupting the binding of centractin to p150^{Glued}.

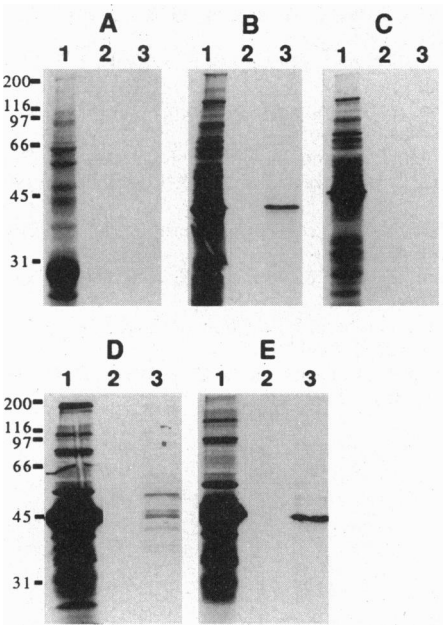

FIG. 4. Specific binding of centractin to p150^{Glued}. Labeled centractin generated by *in vitro* translation of cloned cDNA in the presence of [^{35}S]methionine was applied to a p150^{Glued} (*A* and *B*) or BSA (*C*) affinity column, and the flow-through (lane 1 in *A–C*), final buffer wash (lane 2 in *A–C*), and 0.5 M NaCl eluate (lane 3 in *A–C*) were analyzed by SDS/PAGE (*A*) and autoradiography (*B* and *C*). Centractin was eluted from the p150^{Glued} column by 0.5 M NaCl, while there was no salt-dependent retention of centractin on the BSA column. (*D*) Specific binding of centractin to p150^{Glued} was blocked by incubation with the synthetic peptide of p150^{Glued} MKKEKEFEET-MDALQA. (*E*) Same experiment as in *D*, except with BSA block. Lanes 1 in *D* and *E* are column flow-through; lane 2, final buffer wash, and lane 3, 0.5 M NaCl eluate. Numbers on the left represent the positions of molecular mass standards in kDa.

Taken together, these data demonstrate that p150^{Glued} is capable of specific binding to centractin, which may be mediated by a conserved cluster of charged residues. A comparison of the amino acids immediately adjacent to the KKEK motif of either p150^{Glued} or villin demonstrates no apparent primary sequence homology. This suggests that the residues neighboring the putative binding motifs in these two proteins may confer specificity for centractin or conventional actin binding *in vivo*. However, in both polypeptides, the binding motif is localized to the middle of a predicted α-helix; a common feature of the actin-binding domains of several actin-binding proteins (reviewed in ref. 24).

We have demonstrated that p150^{Glued}, a member of the dynactin complex, binds to both microtubules and centractin. As the dynactin complex is believed to be involved in mediating cytoplasmic dynein-based vesicular transport, the microtubule-binding property of p150^{Glued} suggests that p150^{Glued} may function *in vivo* as part of a vesicle docking complex, mediating the link between vesicles and microtubules. A similar function has been proposed for CLIP-170 (21).

An alternative model may also be proposed in which the dynactin complex may be continuously required during vesicular transport. Following the vesicle docking event, association between the dynactin complex and cytoplasmic dynein may occur. During active transport of the vesicle, the dynactin complex may act as a tether among cytoplasmic dynein, the microtubule, and the cellular cargo through the phase of the mechanochemical ATPase cycle where the dynein heads are dissociated from the microtubule. Kinetic analysis of the axonemal dynein ATPase suggests that both heads of the enzyme are likely to be dissociated from the microtubule for a significant fraction of the ATP hydrolysis cycle at physiological ATP con-

centrations (25). Thus, a tethering complex could serve to prevent diffusion of the cargo away from the microtubule during the enzyme's off time. The observation that p150^{Glued} localizes to the centrosome in a microtubule-dependent fashion (2) lends further support to the hypothesis that the dynactin complex is required continuously during minus-end-directed transport.

We have demonstrated that, in addition to binding microtubules, p150^{Glued} also interacts directly with the actin-like protein centractin. The interaction between these two polypeptides has been implied by means of an ultrastructural analysis of the native dynactin complex, in which centractin forms a short, actin-like filament, with p150^{Glued} protruding like a sidearm from the filament (4). Functionally, centractin may simply serve as a structural element of a vesicle docking or tethering complex. However, Melki *et al.* (26) have demonstrated that centractin preferentially associates with filamentous rather than with globular actin *in vitro*. In addition, Schafer *et al.* (4) have shown that one molecule of conventional actin was present per dynactin complex as a *bona fide* subunit. This single actin monomer could serve to link the dynactin complex to the conventional actin cytoskeleton. In the cell, the association of the complex with the actin cytoskeleton could allow direct interaction of dynactin with both the microtubule and actin cytoskeletal networks during cytoplasmic dynein-based motility. Potentially, a simultaneous link between filament systems could allow switching of vesicular cargo between the actin/myosin and microtubule/dynein motility systems in processes such as axonal transport, as has been suggested by the work of Kuznetsov *et al.* (27). Alternatively, the dynactin complex could serve to anchor cytoplasmic dynein to the cortical actin cytoskeleton during the microtubule-based motile processes of nuclear migration or spindle orientation.

While the mechanism by which dynein and dynactin drive such apparently disparate cellular processes as mitosis, vesicular transport, and nuclear migration remains to be determined, our observation that the p150^{Glued} component of the dynactin complex binds directly to microtubules independent of cytoplasmic dynein provides insight into this mechanism at the molecular level.

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