Analysis of Dynactin Subcomplexes Reveals a Novel Actin-related Protein Associated with the Arp1 Minifilament Pointed End

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Abstract. The multisubunit protein, dynactin, is a critical component of the cytoplasmic dynein motor machinery. Dynactin contains two distinct structural domains: a projecting sidearm that interacts with dynein and an actin-like minifilament backbone that is thought to bind cargo. Here, we use biochemical, ultrastructural, and molecular cloning techniques to obtain a comprehensive picture of dynactin composition and structure. Treatment of purified dynactin with recombinant dynamitin yields two assemblies: the actin-related protein, Arp1, minifilament and the p150Glued sidearm. Both contain dynamitin. Treatment of dynactin with the chaotropic salt, potassium iodide, completely depolymerizes the Arp1 minifilament to reveal multiple protein complexes that contain the remaining dynactin subunits. The shoulder/sidearm complex contains p150Glued, dynamitin, and p24 subunits and is ultrastructurally similar to dynactin's flexible projecting sidearm. The dynactin shoulder complex, which contains dynamitin and p24, is an elongated, flexible assembly that may link the shoulder/sidearm complex to the Arp1 minifilament. Pointed-end complex contains p62, p27, and p25 subunits, plus a novel actin-related protein, Arp11. p62, p27, and p25 contain predicted cargo-binding motifs, while the Arp11 sequence suggests a pointed-end capping activity. These isolated dynactin subdomains will be useful tools for further analysis of dynactin assembly and function.

Key words: dynein • ultrastructure

The minus-end directed, microtubule-based motor, cytoplasmic dynein, drives a wide range of cytoplasmic motor activities. Dynein is required for the steady state localization of the Golgi complex and endosomal membranes near the center of the cell (Burkhardt et al., 1997; Harada et al., 1998) and for movement of ER-Golgi transport complexes (Presley et al., 1997), late endosomes, and lipid droplets (V aletti et al., 1999). The centripetal movement of virus capsids also appears to use the dynein motor (Sodeik et al., 1997; Suomalainen et al., 1999). Dynein contributes in a major way to microtubule organization during interphase (Q uintyne et al., 1999) and mitosis (Compton, 1998). Perturbation of dynein function during mitosis results in defective spindle pole separation (Vaisberg et al., 1993) and chromosome congression (Echeverri et al., 1996), causing pseudoprometaphase arrest. Assays of microtubule aster and spindle formation in vitro have shown dynein to be necessary for microtubule focusing and pole maintenance in these systems as well (Verde et al., 1991; Gaglio et al., 1996; Echeverri et al., 1996). In some cells, such as budding yeast (Carminati and Stearns, 1997), polarized epithelia (Busson et al., 1998) and Caenorhabditis elegans early embryos (Skop and White, 1998; Gönçzy et al., 1999), dynein also powers movements of the entire mitotic spindle and/or centrosomes relative to the cell cortex. In syncytial Drosophila embryos, dynein helps anchor and move centrosomes relative to nuclei (Robinson et al., 1999).

To drive such a variety of subcellular motile functions, cytoplasmic dynein works in concert with an accessory factor, dynactin. Like dynein (Holzbaur and Vallee, 1994), dynactin is a massive protein complex that contains multiple polypeptide subunits (Schroer, 1996). First discovered as an activator of cytoplasmic dynein-based vesicle motility in vitro (Gill et al., 1991; Schroer and Sheetz, 1991), dynactin is now known to be required for all forms of dynein-based motility (A llan, 1996; V allee et al., 1996; Holleran et al., 1998). Dynemin provides the “brawn” for movement, while dynactin appears to serve as the “brain” that directs
and coordinates the activities of the dynein motor. Dynactin is thought to do this in two ways. First, it is proposed to function as an adapter that binds dynein to cargo structures. Second, dynactin may increase the efficiency of the cytoplasmic dynein motor by enhancing processivity (King, S.J., and T.A. Schroer, manuscript submitted for publication). Dynactin functions that are independent of cytoplasmic dynein, such as microtubule anchoring at centrosomes, have also been discovered (Quintyne et al., 1999).

In keeping with this diverse array of functions, dynactin has a unique and complicated structure. Previous biochemical and ultrastructural analyses (Schafer et al., 1994a) showed dynactin to contain at least nine different polypeptide species that are organized into two distinct structural domains, an actin-like minifilament and a pleomorphic projecting sidearm. The actin-like minifilament is a short, uniformly sized polymer of the actin-related protein (A rp). A Arp1. This structure is capped on one end by capZ, a heterodimeric, barbed-end actin capping protein, and carries the p62 subunit at the other. The flexible dynactin sidearm contains the p150$_{Glued}$ subunit. In intact cells and subcellular fractions, exposure of dynactin to an excess of the dynactin subunit dynamitin (p50) causes both p150$_{Glued}$ (E Cheverri et al., 1996; Wittman and Hyman, 1999) and p24 (Karki et al., 1998) to be released from the A rp1 minifilament, suggesting that dynactin’s two prominent structural domains are held together by dynamitin.

The dynactin structure has prompted much speculation and discussion as to how its different parts might contribute to function. A large body of evidence (Karki and H olzbaur, 1995; V Vaughan and V allee, 1995; E Cheverri et al., 1996; Steffen et al., 1997; Quintyne et al., 1999) suggests that dynactin’s 74-kD intermediate chains bind directly to the projecting p150$_{Glued}$ sidearm. p150$_{Glued}$ can also bind microtubules (Waterman-Storer et al., 1995), an activity that appears to be important for dynactin’s effects on dynein processivity (King, S.J., and T.A. Schroer, manuscript submitted for publication) and microtubule anchoring at centrosomes (Quintyne et al., 1999). Dynamitin can bind directly to the chromosomal protein zw10, which may provide a mechanism for dynactin binding to kinetochores (Starr et al., 1998). Dynamitin’s A rp1 minifilament is thought to be the domain that interacts with most cargoes. Golgi membrane binding is proposed to occur via a spectrin-like membrane skeleton (Schafer et al., 1994a; H olzer et al., 1996; Schroer et al., 1996; V allee and S heetz, 1996; H olzer and H olzbaur, 1998), while other cargoes use as yet undefined binding mechanisms.

As a first step toward analyzing binding interactions between specific dynactin subunits, dynein, and cargo organelles, we have isolated and characterized several protein subcomplexes that comprise dynactin. Treatment of bovine brain dynactin with recombinant human dynamitin (Wittman and Hyman, 1999) in 0.5 ml disruption buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA plus protease inhibitors) and incubated for 30 min on ice. Control dynactin samples that did not contain added dynamitin were incubated in parallel. Samples were diluted to 1.5 ml and the proteins were sedimented into 5–20% sucrose gradients at 35,000 rpm in an SW-41 rotor for ~17 h. 1-m1 fractions were collected and the proteins were chloroform/methanol precipitated and subjected to SDS-PAGE (80% of the sample) or immunoblotting (20% of the sample).

For quantification of the stoichiometry of subunits released by dynamitin, 200 g of dynactin was disrupted with 1.4 mg dynamitin as described above. Sucrose gradient fractions 6 and 7, which contained the peak of p150$_{Glued}$ and p24, were combined and resedimented into a second 5–20% sucrose gradient. Fractions 6 and 7 from the second gradient were combined, precipitated, and subjected to SDS-PAGE followed by Coomassie blue staining. Shoulder/sidearm subunit stoichiometry was determined by densitometry (see below).

**Isolation of Dynactin Subcomplexes**

5 mg of bovine dynactin was disrupted by adding freshly prepared 6 M KI in H$_2$O, to yield a final concentration of 0.7 M. The sample was incubated on ice for 25 min, and then chromatographed on a Supersose12 (Pharmacia LKB Biotechnology Inc.) gel filtration column preequilibrated in column buffer (2 mM Tris-Cl, pH 8, 0.2 mM ATP, 0.2 mM CaCl$_2$, 0.5 mM DTT) containing 0.7 M KI. Individual column fractions (0.5 ml) were aliquoted and analyzed by SDS-PAGE followed by Coomassie blue staining. Shoulder/sidearm subunit stoichiometry was determined by densitometry (see below).

**Materials and Methods**

**Dynactin Purification**

Dynactin isolated from bovine brain and purified to 95% homogeneity as previously described (Bingham et al., 1998) was used in all experiments.

**Dynamin-mediated Disruption of Dynactin**

20 g of dynactin was combined with 144 g of recombinant human dynamitin (Wittman and Hyman, 1999) in 0.5 ml disruption buffer (30 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA plus protease inhibitors) and incubated for 30 min on ice. Control dynactin samples that did not contain added dynamitin were incubated in parallel. Samples were diluted to 1.5 ml and the proteins were sedimented into 5–20% sucrose gradients at 35,000 rpm in an SW-41 rotor for ~17 h. 1-m1 fractions were collected and the proteins were chloroform/methanol precipitated and subjected to SDS-PAGE followed by Coomassie blue staining (80% of the sample) or immunoblotting (20% of the sample).

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matography (pointed-end complex). The MonoS column was eluted with a linear gradient of 0-500 mM KCl over 20-column volumes. Efforts to improve the resolution of pointed-end complex subunits were unsuccessful; these proteins eluted as a very broad peak under all conditions.

**Ultrastructural Analysis**

Samples (2.5 µg protein in 0.5 ml column buffer) were adsorbed to freshly cleaved mica, rapidly pressure frozen, freeze fractured, and deep etched as previously described (H euser, 1983). Images of fields of molecules were made on conventional EM negatives; then molecules of interest were captured from projections of the negatives using a digital camera. Final galleries were assembled using A dobe Photoshop®, but the images were not digitally image processed except to add pseudocolor.

**Antibodies**

Monoclonal antibodies to p150

Arp1, p62, and actin were previously described (anti-p150

-actin mAb: C4 (Lessard, 1988).

**Gels, Blots, Densitometry, and Peptide Fingerprinting**

PAGE was carried out as described (Laemmli, 1970). Immunoblotting was performed as described (Towbin et al., 1979); appropriate alkaline phosphatase-conjugated secondary antibodies were detected by chemiluminescence (Tropix). For densitometry, gels were stained with Coomassie blue and scanned at room temperature. For sequencing at EMBL (p62), gels were subjected to trypsin digestion. p62 and p27 tryptic fragments were isolated by HPLC and compared with a BSA standard dilution series using N ational Institutes of Health Image software. Molar ratios were determined based on cloned subunit Mr. Peptide fingerprints were determined by in-gel proteolysis of gel-purified proteins, as described (Cleveland, 1983).

**Peptide Microsequencing**

Dynactin subunits were separated by SDS-PAGE (12.5% acrylamide) and Coomassie stained at room temperature. For sequencing at EMBL (p62 and p27), stained gels were sent intact. For sequencing at H arvard U niversity M icrochemistry Facility (p25 and Arp11), gels were briefly destained in 10% acetic acid and individual bands were excised, washed twice in 50% acetonitrile, and frozen at −80°C. At both facilities, the proteins were subjected to trypsin digestion. p62 and p27 tryptic fragments were isolated by HPLC and their NH2-terminal sequences determined by Ed man degradation. Arp11 and p25 sequences were obtained by in-line, tandem liquid chromatography and mass spectrometry, yielding multiple peptide sequences from a single digest.

**p62 Cloning**

A λ ZAP II rat brain library (Stratagene Inc.) was screened using mAb 62B (Schafer et al., 1994a). Immunopositive lambda ZAP clones were selected in a complex that sedimented 19S suggested that p62 (1.80:1) was significantly greater than the 17S ratio observed in intact dynactin (Wittman and Hyman, 1999).

**Isolation of full-length cDNAs**

p62. Rapid amplification of cDNA ends (RACE)–PCR was used to obtain the putative ATG start codon of the cDNA. A gene-specific primer, 62.05: 5’-TGGGACCCAAGCTGTTCTGATGGAAG-3’ was used to prime the synthesis of first-strand cDNA using poly A + mRNA from adult rat brains. The reverse transcription was done at 42°C with Superscript reverse transcriptase (Life Technologies, Inc.). A first dA tailing, the PCR reactions were performed essentially as described (Frohman et al., 1988). A primary PCR reaction was performed with the gene-specific primer, 62.03: 5’-TCCACCATCATGATATGTTGTCG-3’, and a primer matching the synthesized dA tail: 5’ A MP, 5’-GATCCTGAGTCTGA- CATCATTTTTTTTTTTTTTTTT-3’. The PCR products from the primary reaction were reamplified with a nested gene-specific primer, 62.06: 5’-CAAGGATAGTAAATTTCTTCTCGC-3’ and 5’ A MP. All PCR reactions were 30 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min using AmpliTaq (Perkin-Elmer Cetus Corp.). All PCR products were gel purified and ligated into the pcRII TA cloning vector (Invitrogen Corp.). The deduced amino acid sequence was verified by comparison with sequences obtained from tryptic peptides prepared from bovine brain p62 (available from GenBank/EMBL/DDJB) under accession number A F190798.

Arp11, p25, and p27 cDNAs were obtained by searching the EST database (NCBI) using peptide sequences obtained from the native bovine proteins. Full-length mouse EST sequences containing the required in-frame stop upstream of the initiator methionines and polyadenylation sites were obtained from the American Type Culture Collection. Mouse p25 and p27 were encoded by single ESTs (A A869597 and A A073653, respectively). pEGFP-p25 was constructed from A A869597 BamHI/M tag and pEGFP-C2 BglIII/M tag. p62 was constructed from an EcoRI fragment containing the rat p27 open reading frame (clone 27.004) and pEGFP-C3 EcoRI. The Arp11 open reading frame was assembled from nonredundant fragments of three overlapping ESTs (the SalI/BstXI fragment from A A109989, the BstXI/EcoRI fragment from A A72131, and the EcoRI/HindIII fragment from A A475054) in three separate constructs (C, B, and A). Construct A contains the EcoRI/HindIII fragment of A A475054 in pEGFP-C2 HindIII/EcoRI. The EcoRI fragment of A A27131 was cloned into the EcoRI site of construct A to yield construct B (correct orientation was confirmed by restriction digest). Construct C (A p11 ORF reverse) was finally assembled by ligating construct B digested with XhoI and BstXI with the SalI/BstXI fragment of A A109989. Bacterial strains were obtained from Genome Systems and large-scale plasmid preparations were CsCl-gradient purified before digestions and subcloning. A dimer cloning into the pEGFP vectors, plasmid inserts were completely sequenced from the EGFPC primer (Clontech) and internal primers designed by the Johns Hopkins DNA Facility (Baltimore, MD). Sequences were visually confirmed from E dman sequencing gel output. The sequences of all three E STs reported in the database were found to be correct (sequences are available from GenBank/EMBL/DBJ under accession numbers: Dynactin p25, A F190795; Dynactin p27, A F190796; Arp11, A F190797).

**Results**

**Dynamitin Disruption of Dynactin In Vitro**

Overexpression of the dynactin subunit, dynamitin (p50), in cultured cells induces release of p150

-actin, but not p62, from the dynactin Arp1 minimifilament (Echeverri et al., 1996). This effect can be mimicked in vitro by adding an excess of recombinant dynamitin to a X enopus egg cell extract (Wittman and H yman, 1999) or partially purified dynactin preparation (K arki et al., 1998). Because all these studies were performed in complex protein mixtures, it was not clear whether dynamitin alone was sufficient to disrupt dynactin or whether additional cellular factors were required. To explore this question further, and to characterize in more detail the dynactin components that were released by dynamitin, we mixed highly purified bovine brain dynactin with a 30-fold excess of purified, recombinant human dynamitin (Wittman and H yman, 1999). Sedimentation of the mixture into a sucrose density gradient revealed two major peaks of protein, one at 175 kDa and one at 95 kDa (Fig. 1). The subunit compositions of both pools were evaluated by SDS-PAGE staining (Fig. 1, A and C) and immunoblot analysis with Abs to p150

Dynamitin Disruption of Dynactin In Vitro
in the 19S complex). This finding suggests that dynamitin may contribute to the stability of the Arp1 minifilament.

Dynamitin treatment of dynactin released a 9S protein complex containing p150 Glued and p24 (Fig. 1 C, fractions 6 and 7), as expected from previous studies (Echeverri et al., 1996; Karki et al., 1998; Wittman and Hyman, 1999). This material also appeared to contain dynamitin. However, the fractions most enriched in p150 Glued and p24 (Fig. 1 C, lanes 6 and 7) were not well resolved from the bulk of free recombinant dynamitin (Fig. 1 C, lanes 8 and 9), making it impossible to estimate relative stoichiometries. To further characterize the sidearm structure released by dynamitin, 9S fractions (equivalent to fractions 6 and 7 in Fig. 1 C) were pooled, dialyzed, and sedimented into a second 5–20% sucrose gradient. p150 Glued, p24, and some dynamitin cosedimented at <9S, while the residual free dynamitin sedimented at <7S (Fig. 1 E). Quantitative densitometry revealed the subunit stoichiometry of the 9S pool to be 2:4:2 p150 Glued:dynamitin:p24, which is the same as in intact dynactin (see also Bingham et al., 1998). This result suggests that exogenous dynamitin effects disruption by introducing four additional dynamitin monomers at the Arp1/p150 Glued junction. This generates an Arp1 minifilament and a complex of p150 Glued and p24 subunits, both of which have four associated dynamitin subunits.

**KI Disruption of Dynactin Reveals Additional Stable Subcomplexes**

Dynactin's Arp1 minifilament can be depolymerized by treatment with the chaotropic salt, KI (Bingham and Schroer, 1999). Gel filtration chromatography in the presence of 0.7 M KI yields primarily monomeric Arp1 and actin (Bingham and Schroer, 1999), but other dynactin subunits elute earlier from the sizing column, suggesting they might be organized into higher order complexes (Fig. 2 A). Although the elution profile was complex, five overlapping peaks of protein (Fig. 2 A, A–E) were obvious. In highly concentrated samples, small amounts of Arp1 could be seen across the entire column profile, suggesting that some Arp1 might still be assembled into small multimers or associated with other dynactin subunits. Peak A contained p150 Glued, dynamitin, and p24 and eluted with an approximate Stokes' radius of 10.7 nm. Peak B, which contained dynamitin and p24, but little or no p150 Glued, eluted broadly from the column (mean Stokes' radius ~6 nm). Peak C was enriched for the p62 and p27 subunits (Schafer et al., 1994a) and also contained a doublet of proteins at ~43 kD, the larger of which comigrated with Arp1, and a protein of ~25 kD that electrophoresed slightly slower than p24. Peak C eluted from the column with a Stokes' radius of 4.7 nm. Peak D was highly enriched for capZ. Arp1 was most concentrated in peak E, which eluted with the expected Stokes' radius of monomers.

The complex of p150 Glued, dynamitin, and p24 that was liberated from dynactin by KI (Fig. 1 A, peak A) appeared similar in composition to the p150/dynamitin/p24 complex released by excess soluble dynamitin. However, KI treat-
ment also appeared to yield a second, distinct subcomplex containing dynamitin and p24 subunits (peak B). To gain a better understanding of the relationship between these three protein complexes, peaks A and B were dialyzed to remove KI, and then subjected to velocity sedimentation into a sucrose gradient. The three polypeptides present in peak A (p150<sub>Glued</sub>, dynamitin, and p24) cosedimented at \( \approx 9S \) (Fig. 2 B), much like the p150/dynamitin/p24 complex released by dynamitin. The small amount of Arp1 that was sometimes present in peak A did not sediment at 9S, but instead spread between 11S and >205, demonstrating that Arp1 is not an integral component or necessary for the stability of this assembly. Densitometric analysis of Coomassie blue–stained gels suggested that each 9S complex contained p150<sub>Glued</sub>, dynamitin, and p24 in a ratio of 2:2:1 (Table I). This stoichiometry, together with the predicted molecular weight of each subunit from the cloned cDNAs, predicts a subcomplex of 401.5 kD. In good agreement with this estimate, a subcomplex of 396.6 kD was predicted from the Svedberg equation solved with sedimentation coefficient and Stokes’ radius values (Table I).

A parallel analysis of peak B (Fig. 2 C) revealed that dynamitin and p24 cosedimented at \( \approx 5S \). As was seen for peak A, the Arp1 in peak B sedimented cleanly away from the other polypeptides. The stoichiometric ratio of dynamitin to p24 in the 5S pool was estimated by densitometry to be 2:1 (Table I). Subunit molecular weights deduced from cloned cDNA's predict a complex mass of 110.9 kD, in good agreement with the 110.8 kD mass predicted from hydrodynamic measurements. Together, the complexes in peaks A and B sum to yield p150<sub>Glued</sub>, dynamitin, and p24 subunits in a ratio of 2:4:2, which is the same as in native dynactin.

The biochemical composition and hydrodynamic properties of the p150<sub>Glued</sub>/dynamitin/p24 assembly isolated by KI treatment strongly suggested that it corresponded to the flexible p150<sub>Glued</sub> sidearm that projects from the dynactin Arp1 filament (Fig. 3, top; Schafer et al., 1994a). To explore this possibility further, platinum replicas of quick-frozen, deep-etched (Heuser, 1983) 9S complex were visualized by EM (Fig. 3, middle). The majority of molecules were elongated (\( >20 \) nm) and many contained a pair of globular heads similar in size and shape to those seen at the distal end of the dynactin sidearm. The structures were kinked in the middle to varying degrees, suggesting that, like the dynactin sidearm, this structure is flexible. Because the p150<sub>Glued</sub>/dynamitin/p24 assembly is biochemically and ultrastructurally similar to the projecting sidearm and shoulder domains of the intact dynactin molecule, we will refer to this protein complex as the dynactin shoulder/sidearm.

The remarkably similar ultrastructures of the projecting sidearm seen for intact dynactin and the isolated shoulder/sidearm subcomplex raises the question of where the excess dynamitin/p24 present in peak B might be accommodated within the dynactin structure. Mass arguments, plus the fact that free dynamitin can dissociate the p150<sub>Glued</sub> sidearm from the Arp1 filament, suggest that dynamitin is contained within the highly flexible, elastic shoulder that tethers the filamentous p150<sub>Glued</sub> sidearm to the Arp1 minifilament. Previous attempts to label intact dynactin with antibodies to dynamitin were unsuccessful (Schafer, D.A., J.E. Heuser, and T.A. Schroer, unpublished data) and appropriate p24 antibodies are not available, so we could not address this question by direct labeling methods. Instead, we imaged isolated dynamitin/
Table I. Hydrodynamic Properties, Composition, and Predicted Mass of Dynactin Subcomplexes

| Dynactin subcomplex          | Stokes' radius | Predicted dimensions | M₁, 1 | M₂, 2 | Subunit | Mᵣ | Stoichiometry* |
|------------------------------|----------------|----------------------|-------|-------|---------|-----|---------------|
|                              | S value        | nm                   | nm    | Da    | Da      |     |               |
| Shoulder/sidearm             | 9              | 10.7                 | 21.4 × 1.2 | 396,550 | 386,373 | p150 | 137,757       | 2               |
| Dynamitin                    | 4.7            | 9.4                  | 44,941 | 2     |         |     |               |
| p24                          | 1.9            | 135,570              | 140,108 |       | Arp11   |     |               |
| p27                          | 1.0            | 110,780              | 110,859 |       |         |     |               |
| p25                          | 1.2            | 396,550              | 386,373 |       | Dynamitin|     |               |
| p62                          | 4.4            | 61.0                 | 110,859 |       | p24     |     |               |
| p150                         | 4.7            | 9.4                  | 44,941 | 2     |         |     |               |
| Shoulder                     | 7              | 4.7                  | 9.4 × 1.9 | 135,570 | 140,108 | p27  | 46,230        | 1               |
| p27                          | 1.0            | 110,780              | 110,859 |       |         |     |               |
| Shoulder/sidearm             | 1.9            | 135,570              | 140,108 |       |         |     |               |
| p24                          | 1.0            | 110,780              | 110,859 |       |         |     |               |
| p25                          | 1.2            | 396,550              | 386,373 |       |         |     |               |
| p62                          | 4.4            | 61.0                 | 110,859 |       |         |     |               |
| p150                         | 4.7            | 9.4                  | 44,941 | 2     |         |     |               |

Subcomplex S values and diffusion coefficients were determined from multiple runs and compared with sucrose-gradient or gel-filtration standards run in parallel. Elution peaks were identified by A_{280} and confirmed by SDS-PAGE. Stokes radii were calculated from the diffusion coefficients. Subcomplex dimensions were predicted from the Perrin factor as described (Bloom et al., 1988). Molecular weights were calculated using the Svedberg equation (Mᵣ, left column) or from molecular weights predicted from cloned cDNAs (Mᵣ, right column). Mouse EST clones were used to predict the size of Arp11, p27, p25 (this study), and p24 (Pfister et al., 1998). The p62 mass was predicted from the cloned rat sequence (this study), p50 (human; Echeverri et al., 1996), and p150 (human; Tokito and Holzbaur, 1998) masses were predicted from the published sequences.

*Subunit molar ratios in each subcomplex, estimated by scanning densitometry of a dilution series of each purified subcomplex, compared with a BSA standard.

p24 heterotrimers as described above (Fig. 3, bottom). These molecules appeared as flexible, elongated structures that ranged in length from 11 to 30 nm and were occasionally seen to associate with each other end-to-end. The flexibility and apparent elasticity of these structures is consistent with a location within the dynactin shoulder. We will refer to the dynamitin/p24 heterotrimer as shoulder.

**p62 Is Present in a Complex with Three Other Dynactin Subunits**

K₁ disruption yielded two other protein peaks whose components behaved as protein complexes. Fig. 2 C, peak D, was most enriched in the capZ α/b heterodimer and was not pursued further. Peak C, in contrast, was enriched in p62, p27, and two previously unidentified dynactin subunits of 43 and 25 kD. This material eluted from the sizing column with an apparent Stokes’ radius of 4.7 nm (Table I). These behaviors predict a globular protein of Mᵣ 135,000 D. However, peak C also contained some capZ, so we were unsure as to whether the four predominant components (p62, p27, plus 43- and 25-kD species) comprised a single protein complex or they might be organized into multiple, independent protein assemblies. To explore this possibility further, the proteins in peak C were sedimented into a sucrose gradient (Fig. 4 A). CapZ sedimented at ≈5S, consistent with previous work (Cooper et al., 1984), while p62, p27, and the 43- and 25-kD proteins cosedimented at ≈7S. To further purify the 7S complex, it was subjected to ion exchange chromatography on a MonoS column (Fig. 4 B). p62 and the three associated polypeptides bound and coeluted in a broad peak, while other subunits of dynactin (capZ, dynamitin, and p24) flowed through the column. The approximate stoichiometry of the p62, p27, and 43- and 25-kD polypeptides was 1:1:1:1 (Table I), as estimated by densitometry of Coomassie blue-stained gels.

The sample was then subjected to ultrastructural analysis (Fig. 4 C). Round particles were observed with an approximate diameter of 8 nm, in agreement with our gel filtration size estimates (Table I). The particles appeared to have a depression in the center and were reminiscent of the Arp2/3 complex (Mullins et al., 1997, 1998), an important regulator of actin assembly that binds F-actin pointed ends (reviewed in Schafer and Schroer, 1999). Our previous ultrastructural analysis suggested that p62 is associated with the pointed end of the Arp1 filament (Schafer et al., 1994a). This implies that the p62, p27, and the 43- and 25-kD assemblies are associated with the Arp1 filament pointed end. We will refer to this heterotetrameric protein complex as pointed-end complex.

**Cloning of Pointed-End Complex Subunits**

Though several dynactin subunits have been characterized at the amino acid sequence level (p150<sup>Glul</sup>, dynamitin, Arp1, actin, capZ, and p24; for review, see Holleran et al., 1996), the four components of the pointed-end complex have not. To complete the molecular characterization of dynactin, we cloned all four remaining subunits. A full-length p62 clone (Fig. 5 A) was isolated from a rat brain expression library using mAb 62B (Schafer et al., 1994a). The predicted protein has an Mᵣ of 53,086 and pl of 7.4. In the sequence of the peak revealed a RING-finger–like motif (Saurin et al., 1996) and a series of predicted α helices with alternating acidic and basic character. Database searches indicate that p62 is homologous along its length with R0-2 (ropy-2), a Neurospora gene whose protein produce is also predicted to contain a metal binding motif (Vierula and Mais, 1997). A nalysis of ropy mutants reveals defects in dynactin-dependent nuclear movement into growing hyphae. Since other R0 genes encode subunits of dynactin or dynactin (Plamann et al., 1994; Tinsley et al., 1996), it seems likely that p62 is the vertebrate R0-2 homologue.

In addition to p62, the pointed-end complex contains subunits of ≈25 and ≈43 kD that had not been observed previously. However, close inspection revealed a minor polypeptide migrating just above p24 in undisrupted bovine brain dynactin (Fig. 2 A, left). Immunoblotting showed the ≈25-kD species to be immunologically distinct from p24 (data not shown). In intact dynactin, the novel
43-kD species is obscured by A rp1, the major component of dynactin (one third by mass). The 43-kD polypeptide in purified pointed-end complex was determined to be distinct from both A rp1 and actin by peptide fingerprinting (Cleveland, 1983) and immunoblotting (data not shown). The identification of the ~25- and 43-kD species as novel dynactin components was verified by peptide microsequencing and cDNA cloning (see below).

To obtain clones for the 25- and 43-kD polypeptides, for which antibodies were not available, we screened genomic databases with tryptic peptide sequences from each protein. This yielded multiple EST clones that were assembled, as needed, to yield full-length sequences (Figs. 5, B and C, and 6 A). Human p27 was previously identified by the Japanese genome sequencing project (Ichikawa et al., 1997) as a ubiquitously expressed gene. The mouse p27 gene encodes a protein of 20.6 kD with an isoelectric point of 6.0. p27 orthologs from fly and worm were identified using the predicted rat p27 amino acid sequence. Though the p27 gene appears to be evolutionarily conserved (Fig. 5 B), no novel features are revealed in an alignment of the sequences. The 25-kD subunit (p25; Fig. 5 C) is another
novel protein with a predicted Mr of 20.1 kD and pI of 7.9. Orthologs were identified in fly and worm; both had basic isoelectric points. Although we did not detect chick embryo brain dynactin p25 on two-dimensional (2-D) gels (Schafer et al., 1994a), presumably due to its alkaline pI, a spot corresponding to bovine p25 can be seen at a pI of \(<8.0\) by silver staining (data not shown). p25 is predicted to fold into a \(\beta\) structure.

**Pointed-End Complex Contains Arp11, a Novel Actin-related Protein**

The sequence of the 43-kD protein (Fig. 6 A) revealed it to be a novel Arp. A phylogenetic tree including representative actin and Arp sequences (Fig. 6 C) shows this new Arp to be less similar to actin than most other Arps. In keeping with existing nomenclature guidelines (Schroer et al., 1994; Poch and Winsor, 1997), we suggest the name Arp11. A alignment of the Arp11 sequence with actin reveals overall conservation of the “actin fold,” a primordial core structure that contains nucleotide and metal binding elements (Kabsch and Holmes, 1995). A s in other actin-related proteins, sequence differences, when they are observed, are largely limited to surface residues. In addition to scattered amino acid substitutions, the alignment reveals several changes in surface loops (Fig. 6 B) that play key roles in actin–actin and actin–protein interactions (Page et al., 1998). The Arp11 barbed-end face is generally well conserved and is not predicted to contain large insertions that would prevent interactions with the pointed end of an Arp1 (or actin) filament. The NH\(_2\) and COOH termini (found at the barbed-end face of subdomain 1) are only slightly longer (5–20 amino acids) than in actin, which should interfere only slightly, if at all, with subunit–subunit interactions. In contrast, the pointed-end face of Arp11 is predicted to be dramatically different from that of actin (Fig. 6 B). The most conspicuous difference is a large deletion (actin residues 38–57) that eliminates an entire surface loop (Fig. 6 B, red) from subdomain 2. Subdomains 3 and 4 are predicted to contain insertions, some of which (Fig. 6 B, green) map within two important actin–actin interfaces inferred from the F-actin x-ray crystal structure (Holmes et al., 1990; Kabsch et al., 1990). The predicted structure of Arp11 suggests it will not form filaments by itself and will only interact with filaments of Arp1, or conventional actin, at their pointed ends.

**Discussion**

The work presented here provides significant new insight into dynactin composition and subunit organization, and suggests novel mechanisms for Arp1 filament length regulation and dynactin-membrane binding. The availability of purified dynactin shoulder/sidearm, shoulder, and pointed-end complexes will facilitate biochemical and biophysical analysis of dynein–dynactin and dynactin–cargo
interactions. The four new dynactin subunit cDNAs we have isolated will be useful tools in studies of the assembly pathway of dynactin in vitro and should permit a comprehensive analysis of dynactin function in cells.

We are reasonably confident that the present effort represents the complete molecular characterization of dynactin subunits. 2-D gel analysis of isolated bovine brain dynactin reveals all of the proteins discovered thus far, including the novel subunits p25 and A rp11 (data not shown). Dynactin’s predicted mass, based on the stoichiometries of its 11 different gene products and their predicted molecular weights, is 1.03 MD. This value agrees well with values (1.10 and 1.11 MD) predicted by hydrodynamic measurements (this study) and previous STEM analysis (Schafer et al., 1994a).

Dynactin’s complicated, highly asymmetric structure suggests a multiplicity of functions. To serve its many roles in cells, dynactin must be able to interact with dynein, endomembranes, and other cytoplasmic particles, centrosomes, chromosomes, and the plasma membrane. While the present analysis does not provide any new information about these binding interactions specifically, it
The Shoulder/Sidearm and Shoulder Complexes

A large body of evidence indicates that dynein binds dynactin via the projecting p150Glued sidearm. p150Glued was first implicated as a dynein-binding subunit in blot overlay and affinity chromatography studies using recombinant proteins (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). We find that isolated dynactin shoulder/sidearm inhibits dynein-based microtubule aster formation in vitro, suggesting that this domain can act as a competitive inhibitor of the dynein–dynactin interaction. Moreover, overex-
pression of the dynactin-binding domain of p150Glued profoundly interferes with dynactin-based motility in cells (Quintyne et al., 1999), reinforcing the notion that this subunit interacts directly with dynactin in vivo. Recombinant or overexpressed p150Glued binds cellular microtubules via an NH2-terminal microtubule binding site (Waterman-Storer et al., 1995) that maps to the globular heads at the distal end of the dynactin sidearm. Native dynactin and isolated shoulder/sidearm subcomplex also bind microtubules in vitro, although the binding is transient. The dynamic binding of dynactin to microtubules allows dynactin to remain associated with the microtubule track throughout the dynactin ATPase cycle, which is believed to enhance motor processivity (King, S.J., and T.A. Schroer, manuscript submitted for publication).

Together, dynactin’s shoulder/sidearm and shoulder complexes form a flexible, elastic structure (Fig. 3, top) that can bind the Arp1 minifilament on one end, the microtubule substrate at the other, and dynein in between. The flexibility of the junction with the minifilament allows p150Glued to undergo a wide range of movement relative to the Arp1 backbone. This may facilitate passage of bulky cargoes through cytoplasm. The flexibility of this structure may also facilitate intercalation by exogenous dynamitin. Isolated shoulder/sidearm and shoulder complexes both exhibit a range of morphologies, much like the pleomorphic shoulder in intact dynactin. Two dynamitins in isolated shoulder complex are bound to one subunit of p24, suggesting that this may be a fundamental assembly unit of the dynactin molecule. However, dynamitin disruption leaves four dynamitin subunits on the Arp1 filament and four subunits on the p150Glued/p24 assembly, suggesting that dynamitin may be most stable in tetrameric form. That KI dissociates the dynamitin tetramer into two dimers suggests a “fault line” at the dimer–dimer interface.

Pointed-End Complex

Our disruption studies have also provided new insights into subunit–subunit interactions in the Arp1 minifilament. Dynamitin treatment appears to leave this structure intact. Cap27 remains associated, indicating that the barbed end is still capped. The pointed-end complex is also apparently retained. p62 is a component of the 19S complex (Cheverri et al., 1996; Fig. 1 B) and Arp11, p27, and p25 can be discerned by 2-D gel analysis (K.A. Melkonian, unpublished observations). The 19S structure also contains dynamitin, suggesting that this protein may contribute to Arp1 minifilament stability. KI disruption, in contrast, depolymerizes the Arp1 polymer, as previously seen for conventional F-actin (Szent-Gyorgi, 1951), but leaves the cap27 α/β heterodimer intact, as expected (Caldwell et al., 1989). Our KI disruption studies provide no new information about the location of the single actin monomer within the Arp1 minifilament, as actin elutes as monomer under these conditions (Bingham and Schroer, 1999). However, association with the conventional actin-capping protein, capZ is a likely possibility.

The predicted structure of Arp11, based on alignment with conventional actin and other Arps, suggests it has the capacity to bind Arp1 directly. Earlier attempts to demonstrate binding between p62 and Arp1 were unsuccessful (S.R. Gill, unpublished observations), as would be expected if this interaction were mediated by Arp1. The positions of insertions and deletions (relative to actin) within the Arp1 sequence predict it will associate with the Arp1 filament exclusively at its pointed end. Dynactin Arp1 minifilaments are uniform in length (37 nm; Schafer et al., 1994a) and they do not readily depolymerize, self-associate, or nucleate assembly of conventional actin (J.B. Bingham, unpublished observations), suggesting that they are capped at both ends. In contrast, the filaments formed by purified Arp1 in vitro are heterogeneous in length (mean length, 52 nm) and can anneal end-to-end (Bingham and Schroer, 1999). The conventional actin-capping protein α/β-heterodimer associated with dynamitin (Schafer et al., 1994a,b) is expected to prevent Arp1 growth and annealing at the Arp1 barbed end. Pointed-end complex may play a similar role at the opposite end of the filament and may also help template Arp1 assembly to ensure the filaments grow to a uniform size.

The association of p27 and p25 with p62 and Arp1 in the pointed-end complex appears to be somewhat labile. During KI disruption, p62 and Arp1 always copurify, but p27 and p25 sometimes copurify with monomeric Arp1 and actin (J.B. Bingham, Ph.D. thesis). When the resulting Arp1/actin/p27/p25 sample is further fractionated by MonoQ chromatography (Bingham and Schroer, 1999), p25 and p27 again copurify (J.B. Bingham, Ph.D. thesis), suggesting they are tightly associated with one another. Thus, within the pointed-end complex, p62 and Arp1 appear to be binding partners, as do p27 and p25.

The primary sequences of the remaining three pointed-end complex subunits provide fertile ground for speculation about the assembly and function of the Arp1 minifilament, dynactin’s proposed cargo-binding domain. The mechanism of dynactin-membrane binding remains obscure, though recent work suggests that Arp1 may interact with Golgi-associated spectrin (Holleran et al., 1996). However, the predicted biochemical properties of pointed-end complex subunits suggest that this structure may also participate in membrane binding. The conserved alkaline pls of p62, Arp11, and p25 suggest that any or all may interact electrostatically with negatively charged membrane lipids or other acidic cargoes such as lipid droplets or viral nucleocapsids. p25 is predicted to fold into a β structure that may contain a lipid-binding, plekstrin homology domain. Thioacetylation of p25 and/or p27 (both of which are cysteine rich) might further stabilize interactions between these subunits and membranes. Homologies of pointed-end complex subunits have been found in organisms as diverse as fly (all four subunits), worm (p62, p27, and p25), and Neurospora (p62 and Arp11), indicating that this structure is evolutionarily conserved. However, budding yeast, an organism that does not require cytoplasmic microtubules for endomembrane traffic, does not appear to contain pointed-end complex homologues. This suggests that yeast dynactin may not perform whatever function(s) this structure provides in other species and further supports a role in membrane interactions. But regardless of how dynactin binds membranes, it is important to keep in mind that dynein may have mechanisms for binding membranes that are independent of dynactin. Purified cytoplasmic dynein associates peripherally with purified synaptic

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vesicles and can bind purified phospholipid vesicles with similar affinity (Lacey and Haimo, 1992, 1994). The dynein light chain, Tctex, binds directly to the transmembrane protein rhodopsin (Tai et al., 1999). Thus the interaction of dynein and dynactin is likely to be complex and may involve multiple subunits of each protein.

Previous work indicates that all dynactin subunits except conventional actin and actin-capping protein are found in dynactin only (Paschal et al., 1993; Echeverri et al., 1996). We do not yet know if the same is true for A rp11, p27, or p25. It is intriguing that pointed-end complex ultrastructure so closely resembles the A rp 2/3 complex (Mul lins et al., 1997, 1998; J.E. H euser, unpublished observations), a structure that plays an important role in events at conventional actin filament pointed ends (reviewed in Schafer and Schroer, 1999). It will be interesting to determine whether pointed-end complex, or A rp11 alone, contributes to the dynamics of the conventional actin cyto skeleton. That Neurospora R O-2 mutants show defects in actin cytoskeletal organization (Vieruala and Mais, 1997) suggests that this may indeed be the case.

Further Directions

Dynactin is just one of a family of proteins proposed to mediate the interaction of membranes with microtubules. It shares a number of superficial similarities with CLIP-170, another protein in this class. CLIP-170 binds to and mediate the interaction of membranes with microtubules. Dynactin is just one of a family of proteins proposed to involve multiple subunits of each protein.

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