A Dynactin Subunit with a Highly Conserved Cysteine-rich Motif Interacts Directly with Arp1*

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Dynactin is a multisubunit complex and a required cofactor for most, or all, of the cellular processes powered by the microtubule-based motor cytoplasmic dynein. Using a dynein affinity column, the previously uncharacterized p62 subunit of dynactin was isolated and microsequenced. Two peptide sequences were used to clone human cDNAs encoding p62. Sequence analysis of the predicted human polypeptide of 53 kDa revealed a highly conserved pattern of eleven cysteine residues, eight of which fit the consensus sequence for a Zn\(^{2+}\)-binding RING domain. We have characterized p62 as an integral component of 20 S dynactin by biochemical and immunocytochemical methods. Affinity chromatography experiments demonstrate that p62 binds directly to the Arp1 subunit of dynactin. Immunocytochemistry with antibodies to p62 demonstrates that this polypeptide has a punctate cytoplasmic distribution as well as centrosomal distribution typical of dynactin. In transfected cells, overexpression of p62 did not disrupt microtubule organization or the integrity of the Golgi but did cause both cytosolic and nuclear distribution of the protein, suggesting that this polypeptide may be targeted to the nucleus at very high expression levels.

Dynactin is an oligomeric complex of subunits ranging in size from 22 to 150 kDa (see Ref. 1 for review). Dynactin binds to cytoplasmic dynein (2, 3); blocking this interaction has been shown to inhibit cytoplasmic dynein-mediated vesicular transport (4, 5) as well as spindle assembly and cell division (Refs. 6–9; reviewed in Refs. 1 and 10).

The morphology of dynactin has been investigated by immuno-electron microscopy (11). Dynactin is characterized by a 37-nm-long actin-like filament that forms the base of the complex and a 24-nm lateral projection from this filament. The filament is formed from the limited polymerization of 8–13 mol of Arp1 (actin-related protein 1), an actin-related protein, whereas the lateral projection is formed from a probable dimer of p150Glued (11, 12). p150Glued was found to bind to Arp1 via a central motif (2, 3), and to microtubules via a N-terminal CAP-Gly motif (13). Studies that have disrupted either the predicted amino acid sequence revealed that human p62 has significant homology to the predicted product of the \(\text{rop}-2\) gene of Neurospora crassa. Of particular interest is a highly conserved N-terminal cysteine-rich domain. We have characterized p62 as a bona fide subunit of dynactin by affinity chromatography, immunochemistry, and immunocytochemistry. We have used affinity chromatography to demonstrate that p62 binds directly to Arp1, suggesting that p62 may indeed serve as a pointed end capper for the Arp1 minifilament of dynactin and that this interaction may be mediated by the highly conserved RING domain of p62.

MATERIALS AND METHODS

Isolation of Dynactin—Dynactin was purified essentially as described in Karki and Holzbaur (2) by constructing an affinity column of bacterially expressed dynein intermediate chain. A 150,000 \(\times\) g cytosol prepared from 10 frozen rat brains (Pel-Freez) in PHEM buffer (50 mM Na-PIPES, 50 mM Na-HEPES, 1 mM EDTA, 2 mM MgCl\(_2\), pH 6.9) was loaded onto the column and washed with 50 column volumes of PHEM and 5 column volumes of PHEM containing 50 mM NaCl. Dynactin was eluted from the column with 1 mM NaCl in PHEM, dialyzed, and then concentrated using polyethylene glycol 20,000 (Fisher). The concentrated eluate was fractionated on a 5–20% sucrose gradient, and the peak fraction corresponding to 20 S was methanol precipitated.

Peptide Sequencing and cDNA Cloning—Purified dynactin was resolved by electrophoresis, transferred to Immobilon-P, and the polypeptide band corresponding to p62 was excised and subjected to in situ proteolysis and microsequencing. Two nonoverlapping peptide sequences were obtained: LIEYYQLAQK and LVAVNYIPEV. These two peptide sequences were used to search the National Center for Biotechnology Information (Bethesda, MD) data bases in a BLAST search (15) to identify overlapping human expressed sequence tags (ESTs) (16). One of these ESTs (AA223368) from NT2 neuronal precursor cells) was obtained, and the sequence was further extended by PCR using a

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One end of the short Arp1 filament is thought to be capped by the \(\alpha\) and \(\beta\) subunits of capping protein (CapZ), which are found in a 1:1 stoichiometry in dynactin (11). Capping protein has been shown to cap the barbed ends of actin filaments and by analogy is thought to cap the barbed end of the Arp1 filament. Pointed end capping of actin is less well understood, and within the dynactin complex no analog of a pointed end capper has been identified. However, immuno-electron microscopic studies (11) suggested that a polypeptide with an apparent molecular mass of 62 kDa, present in dynactin at a stoichiometry of one subunit/complex, localizes to the end of the Arp1 filament opposite to that bound to CapZ.

To address the role of p62 within the dynactin complex, we isolated this polypeptide using a dynein affinity column. Microsequencing of two tryptic peptides allowed us to isolate human cDNA clones encoding p62. The predicted product of these clones is a protein of 53 kDa that co-migrates through SDS-PAGE\(^1\) with the endogenous p62 polypeptide. Data base comparison of the predicted amino acid sequence revealed that human p62 homology to the predicted product of the \(\text{rop}-2\) gene of Neurospora crassa. Of particular interest is a highly conserved N-terminal cysteine-rich domain. We have characterized p62 as a bona fide subunit of dynactin by affinity chromatography, immunochemistry, and immunocytochemistry. We have used affinity chromatography to demonstrate that p62 binds directly to Arp1, suggesting that p62 may indeed serve as a pointed end capper for the Arp1 minifilament of dynactin and that this interaction may be mediated by the highly conserved RING domain of p62.

\(^1\) The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; ESTs, expressed sequence tags; PIPES, 1,4-piperazinediethanesulfonic acid; PCR, polymerase chain reaction.
retinoic acid-induced human NT2 cell cDNA library (Stratagene, La Jolla, CA). Sequence derived from PCR clones was verified through comparison of at least six independent clones. The longest clones obtained by PCR or by 5'-rapid amplification of cDNA ends appear to have short 5'-untranslated regions that lack stop codons upstream of the putative initiation codon. Extensive screening of the EST data base revealed no mammalian clones extending further 5' to our longest clone, suggesting difficult secondary structure in this region. High temperature reverse transcription-PCR or genomic library screening did not extend the cDNA sequence. All DNA sequencing was performed using the Sequenase T7 DNA Polymerase, version 2.0 (U. S. Biochemical Corp.).

Northern Blot Analysis—A 32P-labeled p62-specific probe was made from the putative full-length coding region of the cDNA clone with the Prime-It II Random Primer Kit (Stratagene). It was used to probe a human multiple tissue Northern blot using ExpressHyb hybridization solution (CLONTECH Laboratories, Palo Alto, CA) and manufacturer's protocol.

Affinity Production and Immunoprecipitation—cDNA clones corresponding to amino acids residues 1–149 (N-terminal fragment) and 151–460 (C-terminal fragment) were subcloned into pET15b expression vector (Novagen, Madison, WI), expressed in BL21(DE3) strain of Escherichia coli, and purified on a Ni2+ affinity column. The purified proteins were used as immunogens to inoculate rabbits, and p62-specific rabbit polyclonal antibodies were affinity-purified from serum on antigen columns.

Immunoprecipitation of rat brain cytosol in PHEM buffer was carried out using a monoclonal antibody to dynamitin (p50; Transduction Laboratories, Lexington, KY) according to the protocol previously described (17). Cytosol was preadsorbed with protein A-agarose beads for 30 min or 4 °C to eliminate any nonspecific adsorption. The preadsorbed cytosol was then incubated with antibody-bound beads for 3 h at 4 °C. As a control for nonspecific precipitation, beads without antibody were incubated with equal amounts of cytosol in parallel. Both antibody-loaded and control beads were washed extensively with 1× RIPA (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate), and then were boiled in SDS-PAGE sample buffer (18). The eluted samples were analyzed by SDS-PAGE and immunoblotting using antibodies to p62, Arp1, and p150Glued.

Affinity Chromatography—Two affinity columns of purified recombinant dynenine intermediate chain, each with a bed volume of 0.5 ml, were constructed as described previously (19). One column was preblocked with excess recombinant p150Glued (20), and was followed by the equivalent amount of bovine serum albumin (BSA). The columns were washed with 5 ml of PHEM, and rat brain cytosol was incubated with antibody-bound beads for 3 h at 4 °C. A control for nonspecific precipitation, beads without antibody were incubated with equal amounts of cytosol in parallel. Both antibody-loaded and control beads were washed extensively with 25 ml of PHEM buffer followed by 2 ml of HEM buffer (PHEM without PIPES) and then eluted in 1 ml of 1 M NaCl in HEM. The eluates were trichloroacetic acid precipitated and dissolved in 50 μl of 1× Laemmli sample buffer. The proteins were resolved by SDS-PAGE, transferred onto Immobilon-P, and probed with antibodies to p150Glued, Arp1, and p62 (20).

For Arp1-p62 binding analysis, an affinity column was made from recombinant p62 in the same manner as dynenine intermediate chain columns above but with a bed volume of 0.2 ml. As a control, a BSA column was also prepared. Human Arp1, previously subcloned into the pcDNA3 mammalian expression vector (Invitrogen, San Diego, CA), was translated in vitro using the TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI) in the presence of [35S]methionine. The translated product was diluted in HEM buffer, and equal amounts were loaded onto both the p62 and BSA columns. The columns were washed and eluted as described above, and the resulting precipitates were dissolved in 1× Laemmli sample buffer and analyzed by SDS-PAGE followed by autoradiography.

Cell Culture, Transient Transfections, and Immunocytochemistry—Mammalian cells (PK2 or COS-7) were maintained in minimum essential medium with Earle's salts or Dulbecco's modified Eagle's medium supplemented with 2 ml Glutamax, (Life Technologies, Inc.), 10% heat-treated fetal calf serum, 100 units/ml penicillin, and 100 mg/ml streptomycin and were split regularly immediately after reaching confluence using trypsin-EDTA. For overexpression studies, cells were transiently transfected by the calcium phosphate precipitation method (20) in 10 cm tissue culture dishes with p62 cDNA construct in pCDNA3 and 24-h transfection was followed by 24-h washout before fixation. Cells were fixed in –20 °C methanol with 1 ml EGTA for 10 min. The coverslips were rinsed in phosphate-buffered saline and blocked in 5% goat serum, 1% BSA, and 0.05% sodium azide in phosphate-buffered saline, pH 7.4. The fixed cells were assayed by immunofluorescence with primary antibodies as noted with incubation at 4 °C overnight. Fluorescein-labeled donkey anti-rabbit or anti-mouse and Texas Red-labeled donkey anti-mouse or anti-rat secondary antibodies were used for detection. Coverslips were mounted using ProLong Antifade Kit (Molecular Probes, Eugene, OR), viewed with an epifluorescence microscope (model DMRB; Leica, Deerfield, IL) fitted with a 100× objective (1.4 NA) and appropriate dichroic filters, and photographed using a Wild Leitz Photomicroscope system.

For size analysis of recombinant p62 encoded by the cloned cDNA, PK2 cells were transfected in a 100-mm dish with p62 construct fused to a FLAG tag, washed after 24 h, and grown in fresh medium for additional 24 h. Cells were then harvested and lysed in 100 μl of 1× SDS-PAGE loading buffer. Samples from in vitro translated recombinant p62, human brain, and rat brain microtubule extract were also prepared. Each of these samples was analyzed by SDS-PAGE and Western blot, probed with anti-p62 polyclonal antibody (UP1188).

**RESULTS**

Cloning and Sequencing of Human cDNAs Encoding p62—Dynactin was purified using a dynenine affinity column and SDS-PAGE, and a band corresponding to a molecular mass of 62 kDa was excised and subjected to tryptic digestion and microsequencing. Two tryptic peptides from the p62 polypeptide were obtained and used to identify EST clones in a BLAST search of the National Center for Biotechnology Information data base. An EST clone encoding the partial sequence of p62 was obtained, and the remainder of the coding sequence was determined from clones obtained from an NT2-N library using PCR with a p62-specific primer (Fig. 1a).

The open reading frame predicted by these human cDNAs encodes a protein of 460 amino acid residues with a predicted molecular weight of 52.3 kDa (Fig. 1a). Because this is somewhat smaller than the apparent size of the native polypeptide, we overexpressed this protein by transient transfection of PK2 cells and examined the migration of this protein through SDS-PAGE. As shown in Fig. 2, the product of the human cDNAs (lanes 1 and 3) we have isolated migrates more slowly than expected based on predicted size, co-migrating with endogenous p62 from human (lane 2) and rat brain (lane 5) and untransfected PK2 cells (lane 4). To examine the possibility that post-translational modification of the p62 polypeptide is responsible for the difference between the predicted and apparent size, we also expressed the p62 cDNA in vitro using a coupled transcription/translation system. However, the protein expressed in vitro (lanes 1 in panels a and b of Fig. 2), as well as protein expressed in E. coli (data not shown), both co-migrate with endogenous p62 from human brain, suggesting that the clones we have isolated encompass the open reading frame of this gene and that post-translational modifications are not likely to account for the difference between the predicted size and actual size of this polypeptide. Further, treatment of purified dynactin with a broad spectrum phosphatase did not result in any shift in the mobility of the p62 polypeptide (data not shown).

Data base searches using the predicted amino acid sequence for human p62 revealed significant sequence identity to the product of the *nopy-2* gene. This gene from *N. crassa* is required for proper nuclear migration and hyphal elongation and was suggested to encode a dynenine or dynactin subunit (21). Other *N. crassa* genes encoding subunits of either cytoplasmic dynein or dynactin have also been shown to produce similar phenotypes when disrupted (reviewed in Ref. 10). A schematic of the predicted human and *N. crassa* polypeptide sequences are shown in Fig. 1b. The *N. crassa* gene product is significantly larger, with a predicted molecular mass of 80 kDa. Although the N-terminal residues of these proteins show some homology (black bars in Fig. 1b), the *N. crassa* polypeptide has an insertion of about 200 residues within this homologous domain. This insert accounts for the larger size of the fungal homolog.

Data base searches also identified ESTs with predicted cod-
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FIG. 1. Molecular characterization and sequence analysis of the predicted primary sequence of human p62. a, primary sequence of p62 deduced from human cDNA clones. The sequences of the two tryptic peptides obtained from rat p62 are underlined. b, comparison of human p62 primary sequence with its N. crassa homolog, ro-2. The regions of high sequence homology are black bars. Following this region, the N. crassa polypeptide has an insertion of about 200 residues (boxed in). The regions of highest sequence homology are shaded in gray. Although the last eight cysteine residues fit well to a consensus Zn\(^{2+}\) binding RING domain, the first three cysteine residues may still be able to bind Zn\(^{2+}\) by making use of conserved histidine or serine residues. The mouse and Drosophila homologs are depicted in c. In contrast, the first three cysteine residues in this highly conserved cysteine-rich RING-like motif of p62 homologs from human, mouse, Drosophila, C. elegans, and Neurospora. There is a significant sequence conservation as shown between amino acid residues 30 and 114 (numbering corresponds to the human sequence); completely conserved residues are shaded in gray. Although the last eight cysteine residues fit the consensus Zn\(^{2+}\)-binding RING domain, the first three cysteine residues may still be able to bind Zn\(^{2+}\) by making use of conserved histidine or serine residues. The mouse and Drosophila sequences were assembled from ESTs in the database. The C. elegans sequence is a predicted open reading frame resulting from the genome project. The N. crassa sequence is from Vierula and Mais (21).

FIG. 2. Recombinant p62 co-migrates with endogenous p62 from human brain. Panel a shows the Coomassie-stained gel of samples containing recombinant and native p62, whereas the panel b is the Western of the same gel probed with anti-p62 antibody. Lanes 1, in vitro translated human p62; lane 2, human brain tissue; lane 3, FLAG-p62-transfected PtK2 cells; lane 4, control PtK2 cells; lane 5, ATP extract of microtubules from rat brain cytosol. Note the identical migration of both recombinant and native p62. Arrow indicates the position of p62 polypeptide.

FIG. 3. Northern analysis of the p62 expression in human tissues. A multiple tissue Northern blot was probed with a cDNA encoding human p62. Although the p62 mRNA is ubiquitously transcribed, note the relatively higher levels of expression in heart and skeletal muscles and to lesser extent in brain. Kb, kilobases.

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...ing sequences for p62 homologs from mouse and Drosophila, as well as a predicted protein sequence for a Caenorhabditis elegans homolog generated through the genome project (21). The predicted sequences for human, mouse, Drosophila, C. elegans, and N. crassa are aligned in Fig. 1c for the regions of highest homology. This alignment reveals a highly conserved cysteine-rich domain in the N-terminal domain of human p62 (amino acids 30–114). This domain is characterized by the highly conserved spacing of 11 cysteine residues. The last eight of these cysteine residues fit well to a consensus RING domain that is predicted to bind two zinc atoms in a characteristic cross brace structure (22). RING domains have been identified in a number of diverse proteins, many of which are members of multi-subunit complexes where the RING motif is thought to mediate protein-protein interactions. We speculate that this domain in p62 may be involved in interaction with other subunits of dynactin such as the binding to Arp1 described below. In contrast, the first three cysteine residues in this highly conserved motif do not fit into either a LIM or an additional RING motif and are unlikely to bind Zn\(^{2+}\) in the absence of a fourth ligand. Although there is an additional cysteine residue in this region in the sequences from N. crassa and C. elegans, this residue is not conserved in the sequences from human or mouse.

A multiple tissue Northern blot was probed with a cDNA encoding p62 to determine the tissue distribution of p62-encoding mRNA. As shown in Fig. 3, we observed a single band of ~4.2 kilobases in length. Although immunoblots of brain extract from human and rat brain suggest that two similarly sized isoforms may be expressed in these tissues (Fig. 2, lanes 2 and 5), the Northern blot did not resolve any alternatively spliced products of the p62 gene. Comparisons of p62 expression in various human tissues suggests that mRNAs encoding p62 are apparently ubiquitously transcribed but that transcription levels are highest in heart and skeletal muscle. Somewhat lower levels of p62 mRNA were detected in brain, although dynactin is thought to be enriched in neurons (17), and on immunoblots we see relatively high levels of p62 expression in this tissue (Fig. 2 and data not shown).

p62 Is a Dynactin Subunit—Although a previous comparison of the similarity of the rosy-2 phenotype to those resulting from mutations in dynactin or dynactin subunits led to the suggestion...
that ro-2 encoded a subunit of either protein complex (21), the absence of genetic interactions or biochemical data precluded a more definitive identification. Therefore, to characterize the 62-kDa vertebrate polypeptide as a dynactin subunit, we performed several biochemical and immunocytochemical assays. To test whether p62 exists exclusively as a 20 S component, like most dynactin subunits, or is also free in the cytosol, such as CapZ, a high speed cytosol was prepared from a rat brain homogenized in PHEM buffer. The cytosol was fractionated by sedimentation through a sucrose gradient, and the resulting fractions were analyzed by SDS-PAGE followed by Western blot using antibodies to p62 and Arp1. As shown in Fig. 4a, p62 is found exclusively in a single peak corresponding to 20 S, co-migrating with Arp1.

We also performed an immunoprecipitation experiment using a monoclonal anti-dynamitin (p50) antibody (Transduction Laboratories) to precipitate dynactin from rat brain cytosol. A panel of dynactin subunit antibodies (p150Glued, p50, and p22) was used in addition to anti-p62 antibodies to probe the immunoprecipitated proteins. As shown in Fig. 4b, p62 was co-precipitated by the anti-dynamitin antibody, along with other dynactin subunits.

We have previously demonstrated that dynactin is retained on an affinity column of dynein intermediate chain and that this binding can be specifically blocked with excess recombinant p150Glued (19). Two identical dynein intermediate chain affinity columns were constructed and preblocked either with BSA or with a fragment of recombinant p150Glued expressed and purified from E. coli. As shown in Fig. 4c, p62 bound to the dynein affinity column in the absence of excess p150Glued (BSA block), but this binding was significantly inhibited by excess p150Glued in parallel with the behavior of the dynactin subunit Arp1.

Using affinity-purified rabbit polyclonal antibodies to p62 (UP1188), we examined the distribution of this polypeptide in cultured PtK2 cells. Fig. 5 shows that p62 has a punctate perinuclear distribution along with a prominent localization to the centrosomes in interphase cells and is localized to the spindle poles and midbody of dividing cells. This distribution is indistinguishable from that previously demonstrated for other dynactin subunits such as p150Glued and p22 (reviewed in Ref. 10). Together, these biochemical and immunocytochemical results demonstrate that p62 is a bona fide subunit of dynactin complex.

p62 Binds Directly to Arp1—Using recombinant p62, we tested for a direct interaction between p62 and Arp1 using affinity chromatography. An affinity column of recombinant human p62 covalently bound to a Sepharose matrix was constructed and loaded with in vitro translated full-length human Arp1. A control BSA column was also loaded with in vitro translated Arp1. The columns were extensively washed and eluted with 1 M NaCl. Analysis of the resulting fractions, shown in Fig. 6, indicates that the p62 affinity column retained significantly more Arp1 (compare the flow-through fractions from the p62 and BSA affinity columns); the retained Arp1 was subsequently eluted from the p62 column with high salt buffer. This result is consistent with the previous immuno-electron microscopic data and establishes that p62 binds directly to the Arp1 subunit of dynactin. Direct interaction with p62 may either stabilize or cap the Arp1 filament, thereby contributing to the overall stability of dynactin in the cell.

Fig. 4. p62 is a dynactin subunit. a, a high speed supernatant of rat brain homogenate was loaded onto a 5–20% linear sucrose gradient, and resulting fractions were analyzed by SDS-PAGE followed by immunoblotting. The blot was probed with anti-p62 as well as anti-Arp1 antibodies. Result shows that p62, like Arp1, runs at the 20 S dynactin peak. b, immunoprecipitation was carried out using a monoclonal anti-p50 antibody (Transduction Laboratories), and the resulting precipitate was analyzed by Western blotting using a panel of dynactin subunit antibodies (p150Glued, p50, and p22). Like other dynactin subunits, p62 is present in the p50 immunoprecipitate, whereas it is absent in the control immunoprecipitate. c, two identical dynein columns were constructed and blocked with either BSA or excess recombinant p150Glued fragment before the cytosol was loaded. The eluted fractions were probed for p62 and Arp1. Although the BSA-blocked dynein column retains both p62 and Arp1, the p150Glued-blocked column does not. F.T., flow through.

Fig. 5. Cellular localization of p62. Epifluorescence micrographs of cultured PtK2 cells stained with p62 (green; a–c), anti-tubulin (red; b), and dynein (red; c). PtK2 cells were seeded on 18 x 18-mm glass coverslips and grown to 75% confluency. The cells were then rapidly fixed in –20 °C 100% methanol and 1 mM EGTA for 10 min and processed for immunocytochemistry. Micrographs show that p62, like other dynactin subunits, has a punctate cytoplasmic and centrosomal distribution (a). p62 is also found at the spindle poles (b) and the midbody (c) of dividing cells as has been shown for other dynactin subunits.
Transient Transfection Analysis—Overexpression of the dynactin subunit dynaminin (p50) has been shown to cause mitotic arrest, irregular spindle morphology, and disrupted morphology of the Golgi (6, 14), and overexpression of the Arp1 subunit has also been shown to disrupt the Golgi (23). In contrast, overexpression of other dynactin subunits such as p22 was observed to have no clear effects on either microtubule organization or Golgi morphology (24). To investigate the effects of p62 overexpression, a human cDNA encoding p62 was expressed in PtK2 and COS7 cells under the control of the cytomegalovirus promoter.

The most striking feature observed in transfected cells overexpressing p62 was the nuclear as well as cytoplasmic localization of the protein (Fig. 7, a and b). This nuclear localization phenotype was observed both in COS7 and PtK2 cell lines. Whether the nuclear localization of overexpressed p62 is an artifact because of very high levels of protein expression induced by transfection or whether it instead has some physiological significance remains to be determined.

When the cytoskeleton of transfected cells were compared with untransfected cells (Fig. 7a), we observed no clear effect on either the focusing or the radial distribution of the microtubules, similar to our observations on the overexpression of the p22 subunit of dynactin (24). No noticeable effects on the actin cytoskeleton because of p62 overexpression were observed (data not shown). When we compared the Golgi morphology of transfected and control cells, we failed to see any significant difference, implying that neither the microtubule organization nor the Golgi morphology was affected as a result of p62 overexpression, in contrast to observations made following the overexpression of either the Arp1 (23) or dynamin (6) subunits of dynactin.

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The functional interaction between cytoplasmic dynein and dynactin is critical to disperse cell processes such as vesicle transport into the Golgi, mitotic spindle assembly and orientation, and both fast and slow axonal transport. Although cytoplasmic dynein clearly provides motor function for these processes, the role of dynactin has been less clear. The identification of an independent microtubule-binding site on dynactin suggested that this complex might contribute to the processivity of the motor complex (13). Dynactin has also been proposed to be required to link the motor to its cargo, either to vesicles via an Arp1-spectrin interaction (23) or to kinetochores via an interaction between dynamin and ZW10 (25).

To further investigate the cellular role of dynactin, we cloned and characterized human cDNAs encoding the p62 subunit of dynactin. The 53-kDa predicted protein we identified showed substantial similarity to the predicted 80-kDa product of the roopy-2 gene from N. crassa (21). The phenotype of the ro-2 mutant, disrupted nuclear distribution along the fungal hyphae, led Vierula and Mais (21) to propose that this gene encoded a subunit of either dynein or dynactin. The strong sequence conservation we have detected between the human and fungal genes, and the biochemical data presented here clearly identify the human and fungal polypeptides as subunits of dynactin. These biochemical data, including immunoprecipitation, sucrose gradient fractionation, affinity chromatography, and immunocytotoxicity, also indicate that in vertebrate cells p62 is apparently found exclusively as a subunit of the 20 S dynactin complex. This observation is in contrast to the behavior of the capping protein CapZ, which is found both in dynactin and in association with the cellular actin cytoskeleton.

The known ability of capping protein to cap the barbed end of actin filaments and its localization within dynactin to a single end of the Arp1 minifilament have suggested that this dimer also caps the analogous barbed end of Arp1. p62 has been localized to the opposite end of the Arp1 filament and therefore has been proposed to bind to and potentially to cap the analogous pointed end of the Arp1 filament. Here we report data describing the direct binding of p62 and Arp1, supporting this model. The question still remains of how the polymerization of Arp1 is regulated to produce short filaments of defined length. Further studies investigating the in vitro assembly of filaments of Arp1, p62, and the α and β subunits of capping protein may shed light on this issue. However, the difficulty in expressing active recombinant Arp1 and the very low abundance of native Arp1 compared with actin significantly hinders this type of analysis.

The most striking feature of the p62 polypeptide is a very highly conserved pattern of cysteine residues. Comparisons of the sequences of p62 from human, mouse, Drosophila, C. el-
egans, and N. crassa indicate a pattern of 11 highly conserved cysteine residues. The last eight of these closely fit to the consensus RING motif, indicating that p62 is likely to include this core cross-brace structural element (22), in which two zinc atoms are ligated tetrahedrally by four cysteine residues. The strong conservation of the first three cysteine residues is more puzzling, because these residues are not sufficient to bind to another zinc atom. One possibility is that the serine located at position 38 in the human and murine sequences may serve as a fourth ligand; in the Drosophila, C. elegans, and N. crassa sequences there is a conserved cysteine residue at this position. Although histidine is the more common replacement for cysteine in Zn$^{2+}$ binding motifs, mutagenesis studies performed in another RING protein have demonstrated that a replacement of serine for cysteine does not abrogate function under some conditions (26). Alternatively, the mammalian forms may employ either of the conserved His residues found at positions 44 or 49 to complex a third zinc atom. In either model, the possible Zn$^{2+}$ binding motif formed in human p62 from three cysteine residues and either a serine or a histidine residue is not predicted to conform to a consensum LIM/RING motif but could still form a zinc binding finger, meaning that overall p62 would be predicted to bind up to three zinc atoms. It is not unusual to find additional zinc finger-like domains adjacent to RING motifs (22).

The RING finger proteins that have been characterized to date differ widely in function, although they can be grouped in general categories of function such as oncogenesis (e.g. BRCA1) and signal transduction (TRAF2) (22). The putative cellular roles of p62 and dynactin do not fit easily into these categories. The RING domain is thought to participate in or to facilitate protein-protein interactions, and therefore this motif may allow an effective interaction between p62 and Arp1. More interestingly, perhaps, is the observation that FYVE finger proteins have been shown to bind directly to membrane-associated phospholipids (27). Although there clearly exists a soluble pool of dynactin in the cell, at least half of the complex is associated with cellular membranes (17), and dynactin is clearly involved in dynein-mediated vesicle motility. It would be of particular interest if the zinc-mediated structure of p62 allowed this polypeptide to interact both with Arp1, as shown above, and directly with membranes. Although a direct association of p62 with phospholipids on the surface of vesicles may not in itself be sufficient to link both dynein and dynactin to its vesicular cargo, this type of interaction might contribute to the association. We are currently investigating this hypothesis.

One surprising observation we have made is the nuclear distribution of p62 in transfected cells, in contrast to the cytosolic and centrosomal distribution of p62 observed in control cells. None of the previous transfections with other dynactin subunits have given such a phenotype. Although we did not detect any obvious nuclear localization signal in the predicted primary sequence of p62, we speculate that because its homolog, ro-2, has a nuclear distribution function (possibly associating with the nuclei during dynein/dynactin-based transport), overexpression of human p62 may exaggerate its association with the nuclei. Further, with the cysteine-rich zinc finger-like motif it is not unlikely that p62 may associate with DNA at nonphysiological expression levels. Finally we note that unlike p50 or Arp1 overexpression, which yielded a clear phenotype of disrupted Golgi morphology, we do not observe any clear changes in the structure of the Golgi, nor in the underlying organization of cellular microtubules. These differences in effects of overexpression are more likely to reflect the results of biochemical differences in the polypeptides rather than significant distinctions in dynactin function within the cell.

In summary, the molecular characterization of p62 from a range of species from human to Drosophila as reported here and previously in fungi by Vierula and Mais (21) have provided insight into the biochemical properties of this polypeptide and its role within the dynactin complex. Specifically, we have demonstrated the direct binding of p62 to Arp1, supporting the hypothesis that this polypeptide may cap the pointed end of the Arp1 filament. The nature of the RING domain structure that is at the core of the human p62 polypeptide also suggests that this dynactin subunit may have further binding interactions, such as a direct interaction with membrane phospholipids, which will be the focus of further investigations.

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