A motor neuron disease–associated mutation in p150\textsuperscript{Glued} perturbs dynactin function and induces protein aggregation

Jennifer R. Levy,1 Charlotte J. Sumner,2 Juliane P. Caviston,1 Mariko K. Tokito,1 Srikanth Ranganathan,2 Lee A. Ligon,1 Karen E. Wallace,1 Bernadette H. LaMonte,1 George G. Harmison,2 Imke Puls,2 Kenneth H. Fischbeck,2 and Erika L.F. Holzbaur1

1Department of Physiology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104
2Neurogenetics Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892

The microtubule motor cytoplasmic dynein and its activator dynactin drive vesicular transport and mitotic spindle organization. Dynactin is ubiquitously expressed in eukaryotes, but a G59S mutation in the p150\textsuperscript{Glued} subunit of dynactin results in the specific degeneration of motor neurons. This mutation in the conserved cytoskeleton-associated protein, glycine-rich (CAP-Gly) domain lowers the affinity of p150\textsuperscript{Glued} for microtubules and EB1. Cell lines from patients are morphologically normal but show delayed recovery after nocodazole treatment, consistent with a subtle disruption of dynein/dynactin function. The G59S mutation disrupts the folding of the CAP-Gly domain, resulting in aggregation of the p150\textsuperscript{Glued} protein both in vitro and in vivo, which is accompanied by an increase in cell death in a motor neuron cell line. Overexpression of the chaperone Hsp70 inhibits aggregate formation and prevents cell death. These data support a model in which a point mutation in p150\textsuperscript{Glued} causes both loss of dynein/dynactin function and gain of toxic function, which together lead to motor neuron cell death.

Introduction

The microtubule motor cytoplasmic dynein and its activator dynactin, which mediate minus end–directed movement, have important roles in both interphase and dividing cells. In interphase cells, the dynein–dynactin complex is essential for vesicle and organelle transport, such as ER-to-Golgi vesicular trafficking (for review see Schroer, 2004). The dynein–dynactin motor complex also transports RNA particles (Carson et al., 2001), aggresomes (Johnston et al., 2002), and virus particles along microtubules (Dohner et al., 2002). During cell division, dynein and dynactin play a critical role in both nuclear envelope breakdown and spindle formation (for review see Schroer, 2004).

Consistent with these multiple cellular roles, dynein and dynactin function are required in higher eukaryotes. Loss of dynein or dynactin is lethal in \textit{Drosophila melanogaster} (Gepner et al., 1996), and mice homozygous for loss of cytoplasmic dynein heavy chain die early in embryogenesis (Harada et al., 1998). Cells cultured from dynein heavy chain–null embryos show fragmented Golgi and a dispersal of endosomes and lysosomes throughout the cytoplasm (Harada et al., 1998).

Neurons appear to be particularly susceptible to defects in dynein–dynactin complex function. The dominant-negative mutation in \textit{D. melanogaster} Glued, which encodes a truncated form of the p150\textsuperscript{Glued} subunit of dynactin, shows defects that are most profound in neurons (Harte and Kankel, 1983). Two \textit{N}-ethyl-\textit{N}-nitrosurea–induced point mutations in cytoplasmic dynein heavy chain cause slowly progressive motor neuron disease in mice (Hafezparast et al., 2003). \textit{Legs at odd angles (Loa)} and \textit{Cramping (Cra1)} mice each carry missense mutations in a highly conserved domain of cytoplasmic dynein that mediates subunit interactions. When homozygous, these mutations are lethal; heterozygous mice exhibit progressive loss of motor neurons, leading to muscle weakness and atrophy (Hafezparast et al., 2003). A similar phenotype is observed in transgenic mice with a targeted disruption of dynactin in motor neurons (LaMonte et al., 2002).
In humans, a G59S missense mutation has been identified in the gene encoding p150 Glued (DCTN1) in a kindred with slowly progressive motor neuron disease (Puls et al., 2003). Affected patients develop adult-onset vocal fold paralysis, facial weakness, and distal-limb muscle weakness and atrophy. Clinical, electrophysiological, and pathological investigations have confirmed the selective loss of motor neurons in this disorder (Puls et al., 2005). p150 Glued is the dynactin subunit responsible for binding to dynein and microtubules (Vaughan and Vallee, 1995; Waterman-Storer et al., 1995). The G59S substitution occurs in the highly conserved NH2-terminal cytoskeleton-associated protein, glycine-rich (CAP-Gly) domain, which interacts directly with microtubules (Waterman-Storer et al., 1995) and the microtubule plus-end protein EB1 (Ligon et al., 2003).

In this study, we examined the biochemical and cellular effects of the G59S substitution in p150 Glued. Our data suggest that the G59S mutation leads to both decreased microtubule binding and enhanced dynein and dynactin aggregation, suggesting that both loss of function and toxic gain of function contribute to the motor neuron degeneration observed in affected patients.

Results

The G59S mutation disrupts the binding of p150 Glued to microtubules and EB1

The G59S mutation is located within the highly conserved CAP-Gly domain of the p150 Glued polypeptide, a domain that mediates the binding of dynactin to microtubules. We compared the microtubule binding affinities of wild-type and G59S p150 Glued peptides (Fig. 1 A). The CAP-Gly domain of wild-type p150 Glued, which spans residues 1–107, bound weakly to microtubules (unpublished data). This 1–107 peptide lacks the serine-rich region of p150 Glued (111–191), which may be required for efficient microtubule binding by CAP-Gly proteins (Hoogenraad et al., 2000). In contrast, the binding of NH2-terminal residues 1–333 of the wild-type protein to microtubules was robust, with a $K_d$ of 1.1 ± 0.2 μM. The 1–333 fragment of p150 Glued carrying the G59S mutation bound to microtubules with a $K_d$ of 2.6 ± 0.5 μM, indicating a modest decrease in affinity. More striking, however, was the observation that even at saturating microtubule concentrations, only half of the mutant protein was able to bind to microtubules in this assay (Fig. 1 B). Similar results were observed in experiments with full-length wild-type and G59S p150 Glued (unpublished data).
We performed sequential microtubule binding experiments, in which the unbound fraction of G95S p150Glued (1–333) protein was incubated for a second time with a saturating concentration of microtubules (25 μM), and observed that ~60% of the protein pellet with microtubules (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200511068/DC1). These data suggest that there may be a rapid equilibrium between two populations of the mutant polypeptide, one that can bind and one that cannot. Mixing of wild-type and G95S p150Glued at a 1:1 ratio resulted in 60% of protein pelleting with 25 μM microtubules (Fig. S1 B). These data suggest that mutant protein does not significantly inhibit the binding of wild-type polypeptide to microtubules.

We next investigated the effects of the mutation on the binding of p150Glued to microtubules in cells. We used transient transfection assays to compare the distribution of wild-type and G95S p150Glued in COS7 cells as well as MN1 cells, motor neuron–like cells that extend neurites (Salazar-Grueso et al., 1998). Although endogenous dynactin generally has a punctate cellular localization, with decoration of dynamic microtubule plus ends, overexpression of p150Glued results in the decoration of the microtubule cytoskeleton (Waterman-Storer et al., 1995). As shown in Fig. 1 C, 24–48 h after transfection of GFP-tagged full-length constructs of wild-type p150Glued, there was decoration of microtubules, as assessed by colocalization with tubulin. In contrast, GFP-tagged full-length G95S p150Glued was distributed diffusely in the cytoplasm and showed no colocalization with tubulin (Fig. 1 C). Similar results were obtained using GFP-tagged NH2-terminal 1–333 constructs of wild-type and G95S p150Glued, as well as untagged full-length wild-type and G95S p150Glued constructs (unpublished data). We performed microtubule binding experiments using protein extract from COS7 cells that had been transfected with GFP-tagged, full-length p150Glued. Almost all of the exogenous polypeptide from wild-type and G95S p150Glued–transfected cells pelleted with taxol-stabilized microtubules. However, only approximately half of the protein from G95S p150Glued–transfected cells pelleted with microtubules (unpublished data). This observation confirms our in vitro data that only a portion of the G95S p150Glued protein population may be available for microtubule binding.

The NH2-terminal CAP-Gly domain of p150Glued binds to EB1 (Ligon et al., 2003). Crystallographic studies demonstrate that the COOH terminus of EB1 contacts p150Glued in a hydrophobic cleft of the CAP-Gly domain (Hayashi et al., 2005). We therefore examined the binding of G95S p150Glued to EB1 using affinity chromatography. The wild-type peptide bound to the EB1 column and was retained until elution with high ionic strength buffer, but the G95S peptide had decreased retention on the column, indicating reduced affinity for EB1 (Fig. 2 A).
Previous studies have shown that p150Glued tracks dynamically with growing microtubule ends together with EB1 (Vaughan et al., 1999). To investigate the effect of the G59S mutation on the localization of p150Glued to microtubule plus ends, we transfected COS7 cells with GFP-labeled wild-type or G59S p150Glued. We selected for cells with low levels of expression, as microtubule plus-end tracking behavior is not evident at higher expression levels because of the decoration and bundling of microtubules induced by high levels of exogenous p150Glued. Wild-type p150Glued tracked dynamically with growing microtubule ends (Fig. 2 B and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200511068/DC1), whereas the G59S construct showed no microtubule association, even at tips (Fig. 2 C and Video 2). In cells with higher levels of expression of the G59S construct, we noted apparent aggregates of misfolded protein, but these aggregates showed no directed movement (Video 2).

The G59S mutation does not alter the structural integrity of the dynactin complex

To study the cellular effects of the G59S mutation in the p150Glued subunit of dynactin, we established fibroblast and lymphoblast cell lines from two symptomatic individuals known to be heterozygous for the G59S missense allele. Control fibroblast cell lines were obtained from two age-matched control individuals, and a control lymphoblast cell line was derived from an age-matched subject.

In these lines, we examined whether the G59S mutation alters the expression of dynein and dynactin. In both lymphoblasts and fibroblasts, quantitative RT-PCR analysis of RNA levels showed no difference in p150Glued transcript levels between cell lines heterozygous for the G59S mutation and control cell lines (Fig. 3 A). Western blots of protein extract from patient cell lines showed up-regulation of levels p150Glued, but not of dynein or other dynactin subunits, compared with control cell lines (Fig. 3, B and D).

To determine whether the wild-type and mutant proteins are both expressed in cells cultured from patients heterozygous for the G59S mutation, we performed quantitative Western blotting using both a monoclonal antibody to the microtubule binding region of p150Glued, which binds the wild-type protein with a much higher affinity than the mutant protein, and a polyclonal antibody to p150Glued, which recognizes both forms equally well (Fig. 3 C). Analysis of patient cells indicated that the total level of p150Glued expression (as determined using the polyclonal antibody) is 147 ± 7% the level observed in control cells (Fig. 3 D). Western blots with the monoclonal antibody demonstrated that patient cells express 82 ± 4% of the wild-type p150Glued that control cells express (Fig. 3 D). Thus, we estimate that the mutant protein makes up ∼44% of the total p150Glued population in patient cells.

To examine the structural integrity of the dynactin complex, we fractionated cell extracts from the patient-derived and control fibroblast cell lines by sucrose density gradient centrifugation. Intact dynactin was observed to sediment at ∼19S in both the patient and control samples, consistent with the large size of the multimeric complex. No significant pool of unincorporated p150Glued subunits was observed in the lower S value fractions from either the patient or control cells (Fig. 3 E), suggesting that expression of the mutant polypeptide does not significantly disrupt dynactin structure and that the mutant polypeptide is incorporated into dynactin in these cells.
The heterozygous G59S mutation in p150Glued does not disrupt dynein/dynactin localization, Golgi morphology, microtubule organization, or spindle assembly

Incorporation of the mutant polypeptide into dynactin might be expected to disrupt dynactin localization in patient-derived cells; however, we observed no change in the cellular localization of dynactin in fibroblasts derived from patients compared with control fibroblasts (Fig. 4 A and Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200511068/DC1). Dynactin was present diffusely in the cytoplasm in a fine, punctate pattern, with no visible dynactin aggregates. We also noted no change in the cellular localization of cytoplasmic dynein, which was also found in a punctate cellular distribution, partially overlapping with dynactin staining in both patient and control cells (Fig. 4 B), or EB1, which was localized specifically to microtubule tips (Fig. 4 C).

We examined the effects of the G59S mutation on the integrity of the Golgi and the assembly of the mitotic spindle in the patient-derived fibroblasts. Disruption of dynactin by dynamin overexpression has been shown to disrupt the Golgi in interphase cells (Burkhardt et al., 1997) and the mitotic spindle in dividing cells (Echeverri et al., 1996). However, no gross morphological defects in the organization of the Golgi or the mitotic spindle were evident in patient-derived heterozygous cells under normal growth conditions (Fig. 4 D and Fig. S2 B). In addition, no consistent defects in the growth rate were observed in the patient fibroblasts (unpublished data).

The G59S mutation in p150Glued impairs dynactin function

To test the patient fibroblasts for dynactin function, we looked at several dynactin-dependent processes. Dynactin, as well as dynein and the dynein-interacting protein LIS1, are necessary for directed fibroblast migration (Dujardin et al., 2003). However, wounded monolayers of patient cells recovered at the same rate as control cells (unpublished data). Aggresome formation has also been shown to be dynein dependent (Johnston et al., 2002). To test the effect of the mutation on aggresome formation, an androgen receptor containing an expanded polyglutamine repeat that induces inclusion formation (Merry et al., 1998) was expressed in patient fibroblasts. These fibroblasts formed inclusions at a rate indistinguishable from control cells (unpublished data).

Although a single wild-type copy of the gene for p150Glued may be sufficient to mediate dynein-dependent processes under normal conditions, conditions of cellular stress may reveal latent effects of the G59S mutation. Nocodazole, a microtubule-depolymerizing drug, causes dispersal of the Golgi. During recovery from nocodazole treatment, microtubules reassemble and the Golgi fragments coalesce near the centrosome in a dynein/dynactin-dependent manner (Corthesy-Theulaz et al., 1992). Hafezparast et al. (2003) have shown a slowing in the recovery of the Golgi after nocodazole treatment in fibroblasts cultured from homozygous Loa mice. Therefore, we assayed the cytoskeletal and organelle recovery rates in heterozygous G59S and control fibroblasts after nocodazole washout. Microtubules were depolymerized and the Golgi body dispersed after 1 h of nocodazole treatment. 1 h after drug washout, microtubules had reassembled in both control and patient-derived cells; however, Golgi complex morphology was significantly different in patient cells. In control cells, 75 ± 2% of cells had an intact Golgi complex, 22 ± 3% of cells had a partially disrupted Golgi complex, and 3 ± 1% of cells had completely disrupted Golgi complex (Fig. 5, A and B). In contrast, in patient-derived cells only 46 ± 8% of cells had intact Golgi complexes, whereas 44 ± 5% of cells showed partial disruption and 11 ± 6% of cells showed complete disruption of the Golgi. Golgi reassembly after 24 h was essentially normal in patient-derived fibroblasts (unpublished data), indicating that expression of mutant dynactin slows but does not block the minus end–directed transport of Golgi elements toward the microtubule organizing center.

We also observed that the localization of EB1 to microtubule plus-end tips was altered in patient cells during nocodazole recovery. After microtubule depolymerization with nocodazole, EB1 demonstrated diffuse cytoplasmic staining. After 30 min of recovery in conditioned growth media, EB1 was localized specifically to the plus ends of microtubules in control cells, forming comet tails that were 1.20 ± 0.06 μm long (Fig. 5 C). In patient-derived cells, EB1 was not limited to microtubule tips but was also observed to localize along microtubules (Fig. 5 C). EB1 tail length increased significantly in patient-derived cells, often to >5 μm, although overlap of adjacent microtubules prevented exact measurements of the elongated EB1 tails. These data suggest a defect in the specific localization of EB1 to microtubule plus ends.

To compare these data to a loss of function of dynactin, we used RNA interference to knockdown p150Glued expression levels in HeLa cells by 70–90% (Fig. 5 E). This knockdown caused dispersal of the Golgi throughout the cell body (Fig. 5 D). In addition, we observed an increase in the length of EB1 comet tails from 1.08 ± 0.05 μm in mock-transfected cells to 1.28 ± 0.07 μm in cells transfected with small interfering RNA (Fig. 5 F). The lengthening of EB1 comet tails is similar to what was observed in patient fibroblasts recovering from nocodazole treatment and correlates with a loss of dynactin function.

The G59S mutation leads to aberrant aggregation of p150Glued

In the microtubule binding assays described in Fig. 1, we observed the binding of only half of the mutant p150Glued polypeptide to microtubules, suggesting that some portion of the mutant protein population is unavailable for binding to microtubules. To investigate this further, we expressed differentially tagged (T7 and His) truncated forms of wild-type and G59S p150Glued in vitro and performed immunoprecipitation with an antibody to the T7 tag. Although our constructs, which include amino acids 1–333, span part of the first coiled-coil domain of p150Glued hypothesized to mediate dimerization (Schroer, 2004), we observed no association of the T7- and His-tagged wild-type polypeptides in vitro and performed immunoprecipitation with an antibody to the T7 tag. Although our constructs, which include amino acids 1–333, span part of the first coiled-coil domain of p150Glued hypothesized to mediate dimerization (Schroer, 2004), we observed no association of the T7- and His-tagged wild-type polypeptides in vitro and performed immunoprecipitation with an antibody to the T7 tag. Although our constructs, which include amino acids 1–333, span part of the first coiled-coil domain of p150Glued hypothesized to mediate dimerization (Schroer, 2004), we observed no association of the T7- and His-tagged wild-type polypeptides in vitro and performed immunoprecipitation with an antibody to the T7 tag. Although our constructs, which include amino acids 1–333, span part of the first coiled-coil domain of p150Glued hypothesized to mediate dimerization (Schroer, 2004), we observed no association of the T7- and His-tagged wild-type polypeptides in vitro and performed immunoprecipitation with an antibody to the T7 tag.
has a tendency to self-associate. There was no coimmunoprecipitation after incubation of differentially tagged wild-type and G59S p150Glued (unpublished data), indicating that the wild-type and G59S proteins do not interact under these conditions.

We next investigated whether aberrant biochemical species of the G59S p150Glued protein could be isolated from protein extracts of cells overexpressing this protein. COS7 cells were transfected with full-length wild-type or G59S GFP-tagged p150Glued. 24 h after transfection, the extract from these cells was fractionated over a sucrose gradient and analyzed by SDSPAGE gel electrophoresis and Western blot. In cells transfected with wild-type p150Glued, the peak concentration of dynamitin and endogenous p150Glued was at 19S (Fig. 6B). The exogenous p150Glued protein (as determined by the increase in molecular weight that is due to the GFP tag) was present at 19S, as well as at less dense fractions. This indicates that some exogenous

Figure 5. Cells heterozygous for the G59S mutation in p150Glued have delayed recovery after microtubule depolymerization. Nocodazole washout experiments were performed on patient and control fibroblasts. Cells were treated with nocodazole for 1 h, washed twice with PBS, and returned to normal growth media. (A) After 1 h of recovery, cells were fixed and stained for the cis-Golgi marker GM130 (red) and microtubules (green). Control cells have compact and perinuclear Golgi, but patient cells have partially disrupted Golgi at the same time point after drug washout. (B) Quantification of Golgi morphology after 1 h of recovery, ± SD (*, P < 0.05; **, P < 0.01). n = 3. (C) After 30 min of recovery, cells were fixed and stained for EB1 (red) and microtubules (green). Enlargements of merged images are shown at the bottom. Control cells show distinct tip localization of EB1, but patient cells show subtle mislocalization of EB1 along microtubules. (D) HeLa-M cells, either mock-transfected or transfected with small interfering RNA against p150Glued, stained with antibodies for EB1 or trans-Golgi marker 46. (E) Knockdown of p150Glued, compared with cells transfected with a fluorescein-labeled, nontargeting oligo or mock-transfected cells. (F) Quantification of the length of EB1 tails, ± SD (*, P < 0.05). Bars, 10 μm.
protein is incorporated into the dynactin complex but some remains unincorporated in lower molecular weight fractions, most likely because its expression is in excess of the other subunits of dynactin. In contrast, extracts from cells transfected with GFP-tagged G59S p150\textsuperscript{Gluated} demonstrated higher molecular weight species in fractions 2–4. This suggests the presence of aggregated forms of G59S p150\textsuperscript{Gluated} with a molecular weight well above that of endogenous dynactin (Fig. 6 B). Endogenous p150\textsuperscript{Gluated} and dynamitin are not present in these fractions, indicating that they do not copurify with the aggregated protein. The aggregated protein remains soluble, as we did not observe the formation of detergent-insoluble aggregates (unpublished data).
As shown in Fig. 1C, G59S p150Glued was cytoplasmically dispersed in COS7 cells 24–48 h after transfection, whereas wild-type p150Glued decorated microtubules. At longer time points, however, we noted a centripetal localization of the proteins. Wild-type p150Glued became preferentially localized along microtubules in the perinuclear region (Fig. 6C). In contrast, G59S p150Glued localized to inclusions surrounding the nucleus, which may correspond to the aggresomes of misfolded protein described by Johnston et al. (2002). These structures were also observed in very highly expressing cells at earlier time points, but their frequency increased with time after transfection (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200511068/DC1). In some MN1 cells transfected with GFP-tagged wild-type (D) or G59S (E) p150Glued and fixed and stained using antibodies for p150Glued and Hsp60. Bar, 10 μm. (F) Quantification of area of cells containing mitochondria in arbitrary units, ± SEM (*, P < 0.05). Wild type, n = 8; G59S, n = 12.

As shown in Fig. 1C, G59S p150Glued was cytoplasmically dispersed in COS7 cells 24–48 h after transfection, whereas wild-type p150Glued decorated microtubules. At longer time points, however, we noted a centripetal localization of the proteins. Wild-type p150Glued became preferentially localized along microtubules in the perinuclear region (Fig. 6C). In contrast, G59S p150Glued localized to inclusions surrounding the nucleus, which may correspond to the aggresomes of misfolded protein described by Johnston et al. (2002). These structures were also observed in very highly expressing cells at earlier time points, but their frequency increased with time after transfection (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200511068/DC1). In some MN1 cells transfected with GFP-tagged G59S p150Glued, single or multiple inclusions were evident most often in the cell body (Fig. 6D) and rarely in neurites. They were similar in appearance to those observed in motor neurons from the brainstem of an affected patient (Puls et al., 2005), and their frequency increased with time after transfection (Fig. S3). Inclusions stained positive for dynein intermediate chain (DIC), the Golgi marker GM130, and the 20S proteasome but not kinesin heavy chain, microtubules, neurofilaments, vimentin, microtubule-associated protein 2, Cu/Zn superoxide dismutase (SOD1), and survival of motor neurons, (unpublished data). Thus, in both neuronal and nonneuronal cells, mutation of the glycine 59 appears to decrease microtubule binding by the p150Glued CAP-Gly domain and leads to aggregation and inclusion formation by the mutant protein.

Inclusions of mutant protein are granular and associated with mitochondria

To look at the ultrastructure of the inclusions, transfected COS7 cells and MN1 cells were observed by EM. MN1 cells transfected with GFP-tagged full-length G59S p150Glued and labeled with immunogold showed granular, nonfibrillar inclusions of mutant protein (Fig. 7, A and B). Analysis of nonimmunogold-labeled, glutaraldehyde-fixed COS7 cells demonstrated that the inclusions were not membrane bound (Fig. 7C). These micrographs show inclusions that look remarkably like the dynein- and dynactin-containing inclusions seen in patient neurons by immunohistochemistry (Puls et al., 2005).

In these ultrastructural studies, mitochondria frequently surrounded or were contained within the G59S p150Glued inclusions (Fig. 7C). To examine the possibility that mitochondria localization was altered by the inclusions, COS7 cells were transfected with wild-type or G59S p150Glued and stained with an antibody to mitochondrial chaperone Hsp60. Mitochondria
were partially relocalized in the area of the aggregates (Fig. 7, D and E). Quantification of the cross-sectional area of the cells that contained mitochondria demonstrated that mitochondria in cells transfected with G59S p150Glued were less widely distributed than in cells transfected with wild-type protein (Fig. 7 F). It may be that mitochondria cannot be transported to the cell periphery because of aberrant interaction with the aggregated G59S p150Glued. Alternatively, it is possible that loss of dynein/dynactin transport causes mitochondrial mislocalization, as expression of dynamin has also been shown to cause an inward collapse of the mitochondrial array (Varadi et al., 2004).

Expression of G59S p150Glued induces death in neuronal cells

The expression of the G59S polypeptide led to an increase in cell death in MN1 cells, as determined by propidium iodide (PI) exclusion. Cells were transfected with GFP-tagged wild-type p150Glued, G59S p150Glued, or GFP alone. The MN1 cells transfected with G59S p150Glued demonstrated a significantly higher percentage of cell death than cells transfected with wild-type p150Glued or GFP alone (Fig. 8 A). Furthermore, the percentage of cell death increased with time after transfection, corresponding to an increase in the percentage of cells containing inclusions visible by immunofluorescence (Fig. S3). Embryonic rat motor neurons expressing G59S p150Glued also demonstrated an increase in cell death compared with motor neurons expressing exogenous wild-type p150Glued in a time-dependent manner (Kalb, R.G., personal communication). Neuronal cells may be uniquely sensitive to the G59S polypeptide, as the expression of G59S p150Glued does not increase cell death in COS7 cells (Fig. 8 A).

Overexpression of Hsp70 inhibits formation of G59S p150Glued aggregates and prevents cell death

Overexpression of the chaperone Hsp70 has been reported to suppress protein aggregate formation and prevent cell death in several protein misfolding disease models (Barral et al., 2004). 56 ± 6% of COS7 cells expressing the G59S p150Glued protein for 2 d contained visible inclusions (Fig. 8, B and E). However, cells expressing both Hsp70 and G59S p150Glued exhibited a disperse localization of both exogenous proteins and only 17 ± 4% of transfected cells contained visible inclusions (Fig. 8, C and E). Hsp70 containing the T13G mutation cannot undergo the conformational change necessary for chaperone activity (Sousa and McKay, 1998). In cells cotransfected with G59S p150Glued and T13G Hsp70, the proportion of cells containing inclusions was not significantly different from that of cells transfected with G59S p150Glued alone (Fig. 8, D and E). A Western blot of the cell protein lysates showed that levels of G59S p150Glued were decreased when wild-type, but not T13G, Hsp70 was co-expressed (Fig. 8 F). The chaperone function of Hsp70 may aid proper folding of G59S p150Glued, thereby avoiding the formation of inclusions and allowing effective degradation of the mutant protein by the ubiquitin–proteasome pathway.

Transfection of G59S p150Glued into MN1 cells led to an increase in cell death compared with cells transfected with wild-type p150Glued (Fig. 8 A). However, coexpression of G59S p150Glued and wild-type Hsp70 reduced the percentage of MN1 cell death to levels similar to those of cells transfected with wild-type p150Glued (Fig. 8 G). This protection was not observed when MN1 cells were cotransfected with G59S p150Glued and either empty vector or T13G Hsp70 (Fig. 8 G). These data demonstrate that expression of active Hsp70 reduces the amount of...
Discussion

A key question in the analysis of many neurodegenerative diseases is the cell-type specificity observed: why would a mutation in a ubiquitously expressed protein preferentially affect a single cell type? This question is particularly critical in the study of motor neuron diseases, such as Amyotrophic Lateral Sclerosis, in which multiple mutations in a ubiquitously expressed protein, SOD1, result in motor neuron–specific degeneration and cell death. Several mechanisms have been proposed, including neuron-specific aggregation and defects in axonal transport (for review see Bruijn et al., 2004).

We focus on the cellular effects of a point mutation in the p150Glued subunit of dynactin. Dynactin is ubiquitously expressed protein, SOD1, result in motor neuron–specific degeneration and cell death. Several mechanisms have been proposed, including neuron-specific aggregation and defects in axonal transport (for review see Bruijn et al., 2004).

We focus on the cellular effects of a point mutation in the p150Glued subunit of dynactin. Dynactin is ubiquitously expressed in vertebrates, interacting with cytoplasmic dynein to serve as the major motor for microtubule minus end–directed transport in the cell. The dynein–dynactin complex is required for a range of cellular functions, including mitotic spindle assembly, ER-to-Golgi trafficking, and endosome and lysosome motility. Although complete loss of dynactin function is therefore likely to affect all cell types, patients expressing the G59S mutation in the p150Glued subunit of dynactin develop an autosomal-dominant, slowly progressive degeneration specific to motor neurons (Puls et al., 2003).

The G59S missense mutation results in a subtle impairment of dynactin function. A subtle loss of function in a protein required for retrograde axonal transport may be sufficient to induce a slow degeneration of motor neurons. Mice with a targeted disruption in dynactin function or with point mutations in cytoplasmic dynein heavy chain exhibit a slowly progressive loss of motor neurons, resulting in muscle atrophy (LaMonte et al., 2002; Hafezparast et al., 2003).

The G59S mutation also results in a toxic gain of function, as the G59S polypeptide is prone to aggregate. Parrini et al. (2005) have shown that evolutionarily conserved glycines inhibit aggregation because of their low propensity to form β structure. The G59S substitution alters a highly conserved glycine residue within the NH2-terminal CAP-Gly domain of the protein and is predicted to result in steric crowding and misfolding of this domain (Puls et al., 2003).

Comparisons of aggregate formation in both neuronal and nonneuronal cells overexpressing the G59S mutation suggest that motor neurons are uniquely vulnerable to aggregate formation, leading to enhanced cell death. One explanation for this observation is that motor neurons may not express adequate levels of chaperones to cope with the high levels of misfolded protein. Recent studies have shown that motor neurons are not able to up-regulate Hsp70 in response to cellular stress and that they are particularly vulnerable to depletion of Hsp70 (Robinson et al., 2005). Consistent with this mechanism, overexpression of Hsp70 led to decreased aggregations of the G59S polypeptide and decreased cell death.

The mechanism by which aggregate formation leads to cell death remains to be determined. However, EM analysis of the aggregates demonstrates the presence of trapped organelles, including mitochondria. The aggregates may either actively trap organelles or passively disrupt microtubule-based transport via “organelle jams” (for review see Holzbaur, 2004). The sequestration of cytoplasmic dynein in these aggregates, as observed in both transfected cells and patient motor neurons (Puls et al., 2005), would further disrupt axonal transport. This disruption in transport is likely to be the most deleterious to motor neurons because of their overall size and extended axons.

Based on our observations, we propose the following model to explain the motor neuron–specific phenotype observed in patients expressing the G59S mutation in dynactin (Fig. 9). The mutation leads to a decreased efficiency in minus end-directed transport. This subtle loss of function does not significantly perturb nonneuronal cells but may be sufficient to affect the overall efficiency of retrograde axonal transport in neurons. However, the mutation also results in a gain of function, as the G59S polypeptide has an enhanced propensity to misfold. Aggregation of the misfolded protein is concentration dependent, and the p150Glued polypeptide is highly expressed in motor neurons (Melloni et al., 1995; unpublished data). Further, motor neurons may be specifically vulnerable to misfolding and aggregation of the G59S polypeptide because of insufficient expression of molecular chaperones. Finally, both the sequestration of active motors and the trapping of organelles by the p150Glued aggregates will further exacerbate the inhibition of axonal transport.

Together, our data provide the foundation for a testable model for the cellular mechanisms leading to the motor neuron–specific degeneration observed in patients expressing the G59S mutation, involving both loss of function and toxic gain of function. We anticipate that these studies will provide further insight into the mechanisms by which a mutation in an essential cellular protein can result in specific degeneration of motor neurons in vivo.
Materials and methods

Microtubule binding
Wild-type and G59S p150\(^{\text{Glu19}}\) were expressed and labeled with \(^{35}\)S-methionine using the TNT T7 Quick System (Promega), followed by centrifugation at 39,000 g for 30 min, incubated for 30 min at 20°C with increasing concentrations of microtubules polymerized from purified tubulin (Cytoskeleton, Inc.), and stabilized with paclitaxel (Cytoskeleton, Inc.). Microtubule bound and unbound proteins were separated by centrifugation at 39,000 g for 20 min and analyzed by SDS-PAGE and fluorography. Results were quantitated by densitometry using NIH Image. Prism Software (GraphPad) was used to fit the binding data to the one-site ligand binding equation \( y = \frac{B_{\text{max}}}{K_d + x} \).

Affinity chromatography and Western blot
Affinity-purified recombinant EB1 was cross-linked to activated CH Sepharose 4B (GE Healthcare) beads at 4 mg/ml ligand. In vitro–expressed p150\(^{\text{Glu19}}\) was incubated with the EB1 beads for 30 min at room temperature. These mixtures were loaded onto a column and washed extensively with 50 mM Tris and 25 mM KCl, pH 7.4, with 0.1% Triton X-100. Retained proteins were eluted with 2 M NaCl. Fractions were analyzed by SDS-PAGE and Western blot.

Cell culture, transfections, immunocytochemistry, and nocodazole recovery assay
Fibroblast cell lines were derived from forearm punch skin biopsies from two symptomatic patients with the G59S mutation and from an age-matched, unaffected control sibling (Priest, 1997). An additional age-matched control fibroblast line (AG02222) was obtained from the Coriell Institute for Medical Research Laboratories. Deconvoluted images were acquired with a Silicon Graphics workstation.

Immunocytochemistry
Immunostaining was visualized with Alexa 350–, 488–, and 594–conjugated secondary antibodies (Invitrogen). Images were acquired by a laser scanning confocal microscope (DMIRBE; Leica) with a 63× PlanApo objective using OpenLab software (Improvision) and a charged-coupled device camera (Orca ER; Hamamatsu). Cells were transfected with plasmids encoding either wild-type or G59S full-length human p150\(^{\text{Glu19}}\) or truncated constructs of the wild-type and G59S polypeptide spanning residues 1–333, both fused to GFP and untagged. Hemagglutinin-tagged His70 constructs were a gift from Y. Argon (University of Pennsylvania, Philadelphia, PA).

Cell death assays
A FACS-based survival assay was used to measure cell death (Taylor et al., 2003). MN1 or COS7 cells were transfected with pEGFP wild-type and G59S p150\(^{\text{Glu19}}\) constructs in 6-well plates. Each transfection condition was performed in triplicate. After 24, 48, or 72 h, cells were harvested with trypsin, gently pelleted with centrifugation, and resuspended in 1 ml of PBS. Cells were stained with 2 μg/ml PI (Sigma-Aldrich) and gently vortexed. For each sample, 50,000 ungated events were acquired using a FACSCalibur instrument (BD Biosciences) and Cell Quest software (Becton Dickinson). GFP fluorescence was collected in the FL-1 channel, and PI fluorescence was collected in the FL-3 channel in dot and density blot formats. Results were expressed as a percentage of PI-positive cells (cell death) divided by the total number of GFP-positive cells (transfected cells).

Microtubule plus-end dynamics assay
Live cell time-lapse recordings were performed on transiently transfected COS7 cells expressing either full-length wild-type or G59S p150\(^{\text{Glu19}}\) or NH\(_2\)-terminal residues 1–333 of wild-type or G59S p150\(^{\text{Glu19}}\), all fused to GFP. Cells on glass coverslips were imaged in an imaging chamber (ICS2; Biophotix) and maintained at 37°C in culture media. Sequential time-lapse fluorescent images were acquired at 12-s intervals.

RNA interference
HeLa-M cells were transfected with Oligofectamine (Invitrogen) with 100 nM of a mixture of four RNA duplexes targeting different regions of human DCTN1 (SMARTpool siRNA reagent [Dharmacon]; available from GenBank/EMBL/DDB) under accession no. NM_004082). 5′-gaagagcucugacuagca-3′, 5′-cgagagcucugacuagca-3′, 5′-caagagcucugacuagca-3′, and 5′-gagagcucugacuagca-3′. Cells were maintained for 72 h and processed for immunocytochemistry or resuspended in denaturing sample buffer and processed for Western blot analysis.

RNA extraction and quantitative RT-PCR
RNA was extracted from cells using TRIzol (Invitrogen) and purified with the RNAeasy clean-up kit according to the manufacturer's protocol (QIAGEN), and cDNAs were generated using the High Capacity cDNA Achieve kit (Applied Biosystems). Quantitative PCR reactions were run in triplicate using the ABI Prism 7900 sequence detection system (Applied Biosystems). A forward primer (5′-gaagagcucugacuagca-3′), a reverse primer (5′-gaagagcucugacuagca-3′), and a fluorescent probe (5′-FAM-cagctcagatcct-BHQ1-3′) were designed to amplify p150\(^{\text{Glu19}}\) transcripts. Endogenous controls were simultaneously amplified using commercially available primers (Applied Biosystems). The reactions were performed in triplicate and averaged, and p150\(^{\text{Glu19}}\) Ct values (cycle number when signal reaches a threshold above background) were corrected for endogenous control values using the ΔΔCt method per the Applied Biosystems User Bulletin 2.

Sucrose density gradient centrifugation
Cells from 3–6 flasks of patient fibroblasts and 3–6 flasks of control fibroblasts were washed in PBS, harvested, and homogenized in 20 mM Tris-HCl, pH 7.4, 2 mM EGTA, 1 mM EDTA with protease inhibitors (leupeptin, pepstatin A, Np-tosyl-Argine methyl ester, and PMSF). Triton X-100 was added to 0.4%, and the homogenate was clarified by low-speed centrifugation. The resulting supernatant fraction was layered over a 5–25% linear sucrose density gradient and centrifuged at 126,000 g for 16 h. The gradients were eluted in 0.5-ml fractions, which were resolved by SDS-PAGE, and analyzed by Western blot.

Aggregation assays
For aggregation assays, His- and T7-tagged constructs of wild-type and G59S p150\(^{\text{Glu19}}\) were coexpressed in vitro and incubated for 2 h at 30°C. The reactions were then incubated sequentially with protein A beads to precipitate extracts, followed by protein A beads with bound monoclonal antibody to T7 (Novagen). After a 1-h incubation with antibody bound beads, the beads were isolated by centrifugation; washed four times with 50 mM Tris, pH 7.3, 50 mM NaCl, and 0.1% Triton X-100; and eluted by boiling in denaturing gel sample buffer. The immunoprecipitates were analyzed by Western blots probed with antibodies to the His and T7 tags.

EM
MN1 and COS7 cells were plated on permanox chambered slides (Lab-Tek; Nunc) and transfected with pEGFP wild-type and G59S p150 constructs or wild-type and G59S p150\(^{\text{Glu19}}\) constructs. 48 h after transfection, one set of cells (for immunogold labeling) was fixed with 4% paraformaldehyde in PBS for 1 h and another set was fixed in...
4% glutaraldehyde in cocomal buffer. Paraformaldehyde-fixed cells were washed three times with PBS and then blocked and permeabilized with 0.1% saponin for 1 h. The p150<sup>Glued</sup>-GFP-transfected cells were then incubated with mouse polyclonal GFP antibody (Invitrogen), and p150<sup>Glued</sup>-transfected cells were incubated in dynactin polyclonal antibody (UP235) followed by anti-mouse or anti-rabbit Nanogold (Nanoprobes). Slides were subjected to staining and silver enhancement as described previously [Tannen et al., 1996]. After dehydration, embedding, and sectioning, samples were examined with an electron microscope [1200EX; JEOL].

Online supplemental material

Fig. S1 shows sequential and mixed microtubule binding of G59S p150<sup>Glued</sup>. Fig. S2 shows that the localization of p150<sup>Glued</sup> and the formation of spindles are normal in cells heterozygous for the G59S mutation in p150<sup>Glued</sup>. Fig. S3 shows that the percentage of cells containing G59S p150<sup>Glued</sup> inclusions increases with time after transfection. Video 1 shows microtubule plus-end tracking of GFP-labeled wild-type p150<sup>Glued</sup> in transfected COS7 cells. Video 2 demonstrates a loss of microtubule tip localization and plus-end tracking of GFP-labeled G59S p150<sup>Glued</sup> in transfected COS7 cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200511068/DC1.

We gratefully acknowledge the members of the affected family for their willing participation. We thank Susan Cheng and Virginia Crocker in the National Institute of Neurological Disorders and Stroke EM Core Facility, Barbara Crain in the Johns Hopkins Department of Pathology, and Anish Patel and Maura Straunam for technical contributions.

This work was supported by National Institutes of Health grant GM48661 (to E.L. Holzbaur), National Institutes of Health/National Institute on Aging training grant T32 AG00255 (to J.R. Levy), National Institute of Neurological Disorders and Stroke Career Transition award K22NS004819-01 (to C.J. Sumner), funds from the Intramural Research Program of the National Institutes of Health (to C.J. Sumner, S. Ranganathan, G.G. Harmison, I. Puls, and K.H. Fischbeck), and a grant from the Amyotrophic Lateral Sclerosis Institutes of Health (to C.J. Sumner, S. Ranganathan, G.G. Harmison, I. Puls, and K.H. Fischbeck), and a grant from the Amyotrophic Lateral Sclerosis Institutes of Health (to C.J. Sumner, S. Ranganathan, G.G. Harmison, I. Puls, and K.H. Fischbeck).

Submitted: 17 November 2005
Accepted: 31 January 2006

References

Barral, J.M., S.A. Brodley, G. Schaffar, and F.U. Hartl. 2004. Roles of molecular chaperones in protein misfolding diseases. Semin. Cell Dev. Biol. 15:17–29.

Brooks, B.P., D.E. Merry, H.L. Paulson, A.P. Lieberman, D.L. Kolos, and K.H. Fischbeck. 1998. A cell culture model for amyotrophic lateral effects in motor neurons. J. Neurochem. 70:1054–1060.

Bruijn, L.I., T.M. Miller, and D.W. Cleveland. 2004. Unraveling the mechanisms involved in motor neuron degeneration in ALS. Annu. Rev. Neurosci. 27:723–749.

Burkhardt, J.K., C.J. Echeverri, T. Nilsson, and R.B. Vallee. 1997. Overexpression of the dynamin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. J. Cell Biol. 139:469–484.

Carson, J.H., H. Cui, and E. Barabrese. 2001. The balance of power in RNA trafficking.Curr. Opin. Neurobiol. 11:558–563.

Courtney-Theulaz, I., A. Pauloin, and S. Rfeffer. 1992. Cytoplasmic dynein participation in the centrosomal localization of the Golgi complex. J. Cell Biol. 118:1333–1345.

Dohner, K., A. Wolfstein, U. Prank, C. Echeverri, D. Dujardin, R. Vallee, and B. Sodeik. 2002. Function of dynein and dynactin in herpes simplex virus capsid transport. Mol. Biol. Cell. 13:2795–2809.

Dujardin, D.L., E.G. Barnhart, S.A. Stelman, E.R. Gomes, G.G. Gundersen, and R.B. Vallee. 2003. A role for cytoplasmic dynein and LIS1 in directed cell movement. J. Cell Biol. 163:1205–1211.

Echeverri, C.J., B.M. Paschal, K.T. Vaughan, and R.B. Vallee. 1996. Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. J. Cell Biol. 132:617–633.

Gepner, J., M. Li, S. Ludmann, C. Kortas, K. Boylan, S.J. Iyadurai, M. McGrail, and T.S. Hays. 1996. Cytoplasmic dynein function is essential in transfected COS7 cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200511068/DC1.
of mitochondria by controlling the recruitment of the fission factor dynamin-related protein-1. J. Cell Sci. 117:4389–4400.

Vaughan, K.T., and R.B. Vallee. 1995. Cytoplasmic dynein binds dynactin through a direct interaction between the intermediate chains and p150Glu1. J. Cell Biol. 131:1507–1516.

Vaughan, K.T., S.H. Tynan, N.E. Faulkner, C.J. Echeverri, and R.B. Vallee. 1999. Colocalization of cytoplasmic dynein with dynactin and CLIP-170 at microtubule distal ends. J. Cell Sci. 112:1437–1447.

Waterman-Storer, C.M., S. Karki, and E.L. Holzbaur. 1995. The p150Glu1 component of the dynactin complex binds to both microtubules and the actin-related protein centractin (Arp-1). Proc. Natl. Acad. Sci. USA. 92:1634–1638.