Disease-Associated Mutations in the p150^{Glued} Subunit Destabilize the CAP-gly Domain[†]

Shubbir Ahmed,[‡] Shangjin Sun,[§] Amanda E. Siglin,[||] Tatyana Polenova,[§] and John C. Williams*,[‡]

[‡]*Department of Molecular Medicine, Beckman Research Institute at City of Hope, Duarte, California 91010,* [§]*Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716-1100, and* [||]*Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107*

Received February 15, 2010; Revised Manuscript Received June 1, 2010

ABSTRACT: Point mutations within the CAP-gly domain of the p150^{Glued} subunit of the dynactin complex have been identified in patients with distal spinal bulbar muscular atrophy (dSBMA) and Perry's syndrome. Herein, we show by CD and NMR experiments that each mutated CAP-gly domain is folded but less stable than the wild-type (WT) domain. We also demonstrate that the domains harboring these mutations bind to microtubules but fail to bind to EB1. These data indicate that these disease-associated, point mutations affect the stability of this domain and inhibit their interaction with EB1 but do not inhibit their interaction with microtubules.

Dynein and dynactin concomitantly drive the bulk of microtubule (MT)-based, retrograde transport of membranous organelles and play a critical role in highly polarized cells (*1*). This is especially true for neurons, in which axons can extend more than a meter in length and signaling between the synapse and cell body through vesicular transport is required for development and survival (*2*). Not surprisingly, mutations in dynein, dynactin, or other dynein-interacting partners are commonly associated with neurological diseases. Recently, a new set of point mutations in the p150^{Glued} subunit of dynactin have been identified in patients diagnosed with Perry's syndrome, a severe neurological disease that shows symptoms of parkinsonism and weight loss and is accompanied by depression and suicide attempts (*3*). These mutations, G71A, G71R, G71E, T72P, and Q74P, map to the N-terminal CAP-gly domain of dynactin's p150^{Glued} subunit (Figure 1). While this is the same domain that harbors the G59S mutation identified in patients with dSBMA (*4*), the characteristics of the diseases are distinct (*3*).

The p150^{Glued} CAP-gly domain weakly binds MTs and is proposed to anchor the dynactin complex to MTs to facilitate its interaction with dynein and increase the processivity of the dynein motor (*5, 6*). Recent studies, however, suggest the CAP-gly domain is necessary for MT organization, but not for dynein activity (*7*). In yeast, the CAP-gly domain was demonstrated to be essential for dynein-dependent spindle and nucleus positioning, suggesting that the CAP-gly domain is important for dynein-mediated processes under load (*8*).

The CAP-gly domain of p150^{Glued} also binds to EB1, a MT plus-end tracking protein (*9, 10*). Recent in vivo studies suggest that this interaction is required for anchoring the minus end of

MTs to the centrosome (*10*). In vitro data also suggest that p150^{Glued} recruits EB1 to the MT +TIP and modulates MT dynamics (*11*). While these studies mark the importance of this interaction, the pleiotropic effects of EB1 and p150^{Glued} overexpression or depletion [as well as the addition of purification tags (*12*)] have made it difficult to confidently assign a specific function to this interaction.

To determine whether point mutations in the CAP-gly domain found in Perry's syndrome affect the stability, MT binding, or EB1 interaction, each point mutation was generated by site-directed mutagenesis in a construct that spanned residues 19–107. The domains with point mutations were expressed in *Escherichia coli* and purified to homogeneity (see the Supporting Information). Each mutated domain required expression at 18 °C to produce a soluble protein. In addition, to test whether the point mutations associated with Perry's syndrome behave differently than the point mutation associated with dSBMA, we also generated the G59S point mutation. This mutation was not soluble despite attempts to express it at 15 °C, the induction of bacterial chaperones by cold shock (midlog phase cells were cooled to 10 °C for 20 min), and addition of ethanol to the medium [2% (v/v) added at midlog growth]. Because G59 is located in the hydrophobic core of the CAP-gly domain and simple modeling of the mutation (using Protein Data Bank entry 2HKQ) indicated that the hydroxyl group of the Ser mutation produces significant steric clashes irrespective of the rotamer chosen, we generated a domain with the G59A mutation to reduce (but not eliminate) the number of these clashes. This construct, G59A, was soluble.

Next, we collected CD and NMR spectra to characterize the effect of these mutations on the overall structure of the CAP-gly domain. Previously, we showed that the CD spectra of the WT CAP-gly domain exhibited a positive inflection at 223 nm, and we attributed this anomalous spectrum to the unusual packing of aromatics in the core (*13*). The CD spectra of the point mutations deviated from the WT spectrum, indicating that each mutation affects the overall fold (*13*) (see the Supporting Information).

We collected ^{1}H$-^{15}$N HSQC spectra to measure the extent of the perturbations due to the G71A, Q74P, and G59A mutations in the WT CAP-gly domain (Figure 2A and the Supporting Information). First, we noted that each domain with a point mutation displayed well-resolved spectra, indicating that each is folded. We also observed that a large number of peaks for the domains with point mutations were shifted relative to the WT spectrum and that spectra of the Perry's syndrome point mutations differed from the G59A mutant spectrum. These data suggest the disease-associated point mutations perturb the fold globally.

To address the extent of these perturbations, we characterized the thermal properties of each point mutation by CD (Figure 2B).
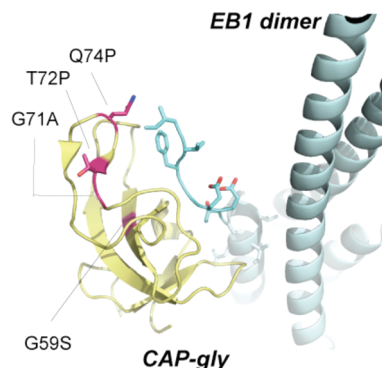
FIGURE 1: Ribbon diagram of the p150[Glued] CAP-gly domain (yellow) bound to EB1 (cyan). The point mutations identified in Perry's syndrome are shown as sticks colored magenta. These mutations are located in a long loop that connects strands $\beta 3$ and $\beta 4$ and part of the "GKNDG" binding motif. The location of G59 is also shown.
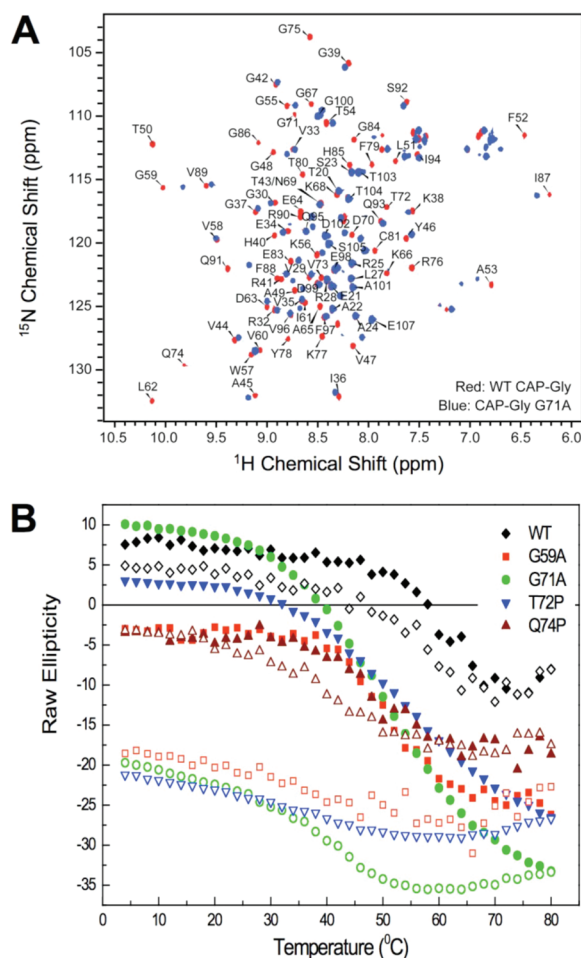

FIGURE 2: Characterization of the CAP-gly domain point mutations. (A) NMR spectrum of the G71A point mutation indicating the domain is folded but perturbed globally compared to the WT domain. (B) Thermal denaturation of each point mutation and the WT CAP-gly domain. The filled symbols represent the ellipticity of the sample at 223 nm upon heating (e.g., from 4 to 80 °C), and the empty symbols represent cooling (e.g., from 80 to 4 °C).

The WT CAP-gly domain exhibited a single and reversible transition at 60 °C, while the mutation, Q74P, also produced a single transition at 45 °C and was reversible. The remaining point mutations, G59A, G71A, and T72P, produced a broad, irreversible transition with a midpoint at 45 °C. These data further
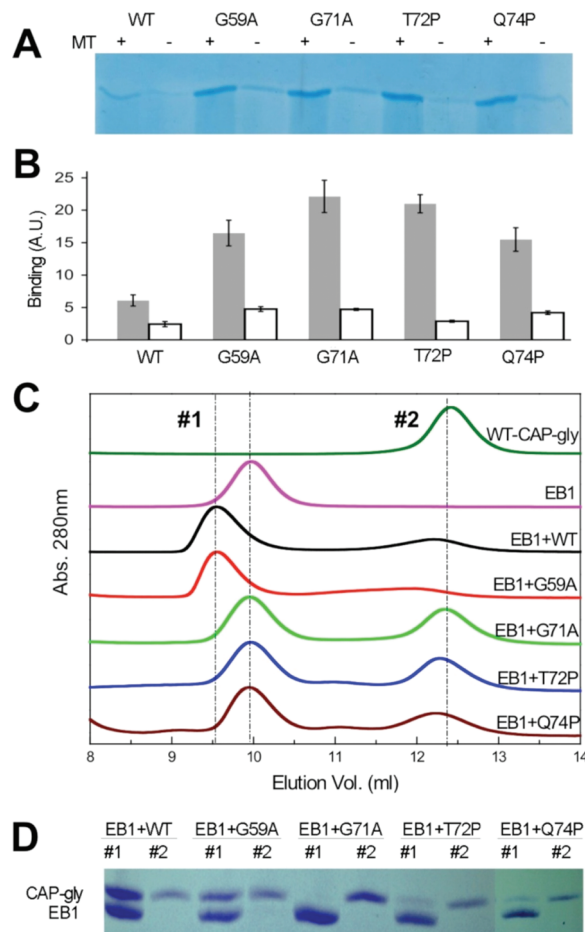

FIGURE 3: Characterization of CAP-gly domain binding interactions. (A) SDS−PAGE gel of the pelleted fraction of CAP-gly domain constructs in the presence and absence of MTs (see the Supporting Information). (B) Quantification of the intensities of precipitated fractions indicating that the mutated domains bind to paclitaxel-stabilized MTs better than the WT construct (gray bars; $n = 3$). The amount of protein precipitated in the absence of MTs is shown as white bars. (C) SEC analysis of the interaction of the CAP-gly domain with EB1. (D) SDS−PAGE analysis of fractions 1 (first peak) and 2 (second peak) shown in panel C.

suggest that the point mutations affect the CAP-gly domain globally.

Next, we asked whether these point mutations affect the MT binding properties of the CAP-gly domain. To do this, we used a standard MT precipitation assay; paclitaxel-stabilized MTs (5 μM) were incubated with each point mutation (75 μM) and precipitated by centrifugation at 200000g. SDS−PAGE analysis of the supernatant and the pelleted MTs revealed that each point mutant could be precipitated with MTs. We also observed minor precipitation of the domains with point mutations in the absence of MTs as compared to WT; however, each construct harboring a mutation bound MTs better than the WT construct (Figure 3A).

To test whether the point mutations affected the interaction of the CAP-gly domain with EB1, we generated, expressed, and purified an EB1 fragment (residues 194−268) that binds to the WT CAP-gly domain (*9, 10, 14*). We used SEC (size exclusion chromatography) to characterize their hydrodynamic properties and interaction. Each CAP-gly domain mutation and the WT CAP-gly domain eluted at 12.5 mL, while the EB1 construct eluted at 10.0 mL (Superdex G75 in PBS) (Figure 3C). We note that EB1 is an elongated coiled coil (2 × MW = 17.4 kDa) and therefore elutes earlier than the CAP-gly domain (MW = 9.5 kDa).

We observed a significant shift to an earlier elution volume for an admixture of the WT CAP-gly domain and EB1, as well as for the admixture of G59A and EB1. The elution profiles for admixtures of the remaining CAP-gly domain mutants and EB1 (where the CAP-gly domain was added at a 1.2-fold excess) appeared as a sum of the individual components. SDS−PAGE of both peaks (Figure 3D) confirmed that WT and G59A coeluted with EB1 while the other CAP-gly domain mutations did not (Figure 3D). Although we could not test the dSBMA point mutation, G59S, point mutations identified in patients with Perry's syndrome failed to bind to EB1.

Taken together, the results of this study show that each point mutation identified in patients with Perry's syndrome affects the EB1 interaction and the stability of the CAP-gly domain. These results also indicate that the mutations strengthen MT binding. Although we were unable to produce a soluble form of G59S to obtain a quantitative measure of its instability and to differentiate the dSBMA and Perry's syndrome mutations, we did show that the G59A mutation (which we propose is an intermediate step to G59S sterically) is less stable than the WT domain and could bind to EB1. We infer that the additional steric clashes stemming from the G59S mutation produce an unfolded protein under physiological conditions.

The role of these point mutations that lead to or compound the pathogenesis of these distinct diseases remains unclear. Previous studies showed that the G59S mutation leads to formation of inclusion bodies, suggesting that the loss of stability promotes aggregation (*15*). The point mutations found in Perry's syndrome also reduce the stability of the CAP-gly domain, but to a lesser degree. Relevant to the difference in stability, expression of these Perry's syndrome point mutations in HEK293T cells also produced inclusion bodies, but to a lesser extent than the G59S mutation (*3*).

Recent findings also indicate that the CAP-gly domain in p150[Glued] is necessary for processes requiring greater dynein force production (e.g., spindle positioning) and microtubule organization, but not dynein-mediated, retrograde transport (*7, 8, 16*). In fact, deletion of the EB1 homologue in yeast phenocopied the G59S mutation as well as the deletion of the CAP-gly domain, indicating that they belong to the same pathway (*8*). The p150[Glued] G59S mutation that was expressed in mammalian cells also failed to colocalize with EB1 at the MT +TIP (*15*). Taken together, these observations also suggest that the loss of the CAP-gly domain−EB1 interaction could play an important role in disease progression.

Finally, although we observed that the Perry's syndrome mutations strengthened MT binding, the role of the p150[Glued] CAP-gly domain in dynein-mediated cargo transport (e.g., processivity) appears to be minimal (*7, 8, 16*). Thus, this increase in the level of MT binding is unlikely to be responsible for compounding the pathology of these diseases.

In summary, we provide biochemical and spectroscopic evidence using homogeneous samples that these disease-related mutations reduce the stability of the CAP-gly domain and affect EB1 and MT binding. These studies also suggest future experiments need to differentiate the loss of EB1 binding, stability, and potentially MT binding to improve our understanding of the roles of these point mutations in disease progression.

SUPPORTING INFORMATION AVAILABLE

Materials, experimental procedures, and additional spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Vallee, R. B., Williams, J. C., Varma, D., and Barnhart, L. E. (2004) Dynein: An ancient motor protein involved in multiple modes of transport. *J. Neurobiol. 58*, 189–200.
2. Gunawardena, S., and Goldstein, L. S. (2004) Cargo-carrying motor vehicles on the neuronal highway: Transport pathways and neurodegenerative disease. *J. Neurobiol. 58*, 258–271.
3. Farrer, M. J., Hulihan, M. M., Kachergus, J. M., Dachsel, J. C., Stoessl, A. J., Grantier, L. L., Calne, S., Calne, D. B., Lechevalier, B., Chapon, F., Tsuboi, Y., Yamada, T., Gutmann, L., Elibol, B., Bhatia, K. P., Wider, C., Vilarino-Guell, C., Ross, O. A., Brown, L. A., Castanedes-Casey, M., Dickson, D. W., and Wszolek, Z. K. (2009) DCTN1 mutations in Perry syndrome. *Nat. Genet. 41*, 163–165.
4. Puls, I., Jonnakuty, C., LaMonte, B. H., Holzbaur, E. L., Tokito, M., Mann, E., Floeter, M. K., Bidus, K., Drayna, D., Oh, S. J., Brown, R. H., Jr., Ludlow, C. L., and Fischbeck, K. H. (2003) Mutant dynactin in motor neuron disease. *Nat. Genet. 33*, 455–456.
5. Schroer, T. A., and Sheetz, M. P. (1991) Two activators of microtubule-based vesicle transport. *J. Cell Biol. 115*, 1309–1318.
6. King, S. J., and Schroer, T. A. (2000) Dynactin increases the processivity of the cytoplasmic dynein motor. *Nat. Cell Biol. 2*, 20–24.
7. Kim, H., Ling, S. C., Rogers, G. C., Kural, C., Selvin, P. R., Rogers, S. L., and Gelfand, V. I. (2007) Microtubule binding by dynactin is required for microtubule organization but not cargo transport. *J. Cell Biol. 176*, 641–651.
8. Moore, J. K., Sept, D., and Cooper, J. A. (2009) Neurodegeneration mutations in dynactin impair dynein-dependent nuclear migration. *Proc. Natl. Acad. Sci. U.S.A. 106*, 5147–5152.
9. Honnappa, S., Okhrimenko, O., Jaussi, R., Jawhari, H., Jelesarov, I., Winkler, F. K., and Steinmetz, M. O. (2006) Key interaction modes of dynamic +TIP networks. *Mol. Cell 23*, 663–671.
10. Askham, J. M., Vaughan, K. T., Goodson, H. V., and Morrison, E. E. (2002) Evidence that an interaction between EB1 and p150[Glued] is required for the formation and maintenance of a radial microtubule array anchored at the centrosome. *Mol. Biol. Cell 13*, 3627–3645.
11. Manna, T., Honnappa, S., Steinmetz, M. O., and Wilson, L. (2008) Suppression of microtubule dynamic instability by the +TIP protein EB1 and its modulation by the CAP-Gly domain of p150glued. *Biochemistry 47*, 779–786.
12. Zhu, Z. C., Gupta, K. K., Slabbekoorn, A. R., Paulson, B. A., Folker, E. S., and Goodson, H. V. (2009) Interactions between EB1 and microtubules: Dramatic effect of affinity tags and evidence for cooperative behavior. *J. Biol. Chem. 284*, 32651–32661.
13. Sun, S., Siglin, A., Williams, J. C., and Polenova, T. (2009) Solid-state and solution NMR studies of the CAP-Gly domain of mammalian dynactin and its interaction with microtubules. *J. Am. Chem. Soc. 131*, 10113–10126.
14. Hayashi, I., Wilde, A., Mal, T. K., and Ikura, M. (2005) Structural basis for the activation of microtubule assembly by the EB1 and p150Glued complex. *Mol. Cell 19*, 449–460.
15. Levy, J. R., Sumner, C. J., Caviston, J. P., Tokito, M. K., Ranganathan, S., Ligon, L. A., Wallace, K. E., LaMonte, B. H., Harmison, G. G., Puls, I., Fischbeck, K. H., and Holzbaur, E. L. (2006) A motor neuron disease-associated mutation in p150Glued perturbs dynactin function and induces protein aggregation. *J. Cell Biol. 172*, 733–745.
16. Culver-Hanlon, T. L., Lex, S. A., Stephens, A. D., Quintyne, N. J., and King, S. J. (2006) A microtubule-binding domain in dynactin increases dynein processivity by skating along microtubules. *Nat. Cell Biol. 8*, 264–270.