

Effects of genetic variations in the dystonia protein torsinA: identification of polymorphism at residue 216 as protein modifier

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Four naturally occurring sequence variations have been found in the coding region of the *DYT1* gene encoding torsinA. One of these, a 3 bp (Δ GAG) deletion, underlies dominantly inherited cases of early-onset torsion dystonia. Others, including a single nucleotide polymorphism that replaces aspartic acid (D) at residue 216 with histidine (H) in 12% of normal alleles and two other rare deletions, have not been clearly associated with disease. To gain insight into how these sequence variations affect torsinA, we used the structure of the related protein ClpB to provide a model of torsinA's AAA+ domain. Motifs important for ATP hydrolysis—sensor 1 and sensor 2—were identified, mutagenized and used to validate predictions of this model. Inspection revealed that the Δ GAG deletion associated with dystonia removes one residue from an α -helix in the C-terminal portion of the AAA+ domain. The resulting distortion in torsinA structure may underlie this mutant's known tendency to produce ER-derived inclusions as well as its proposed loss of function. The D/H polymorphism at residue 216 falls in the N-terminal portion of the AAA+ domain near the sensor 1 motif. Surprisingly, cells expressing torsinA with the polymorphic histidine developed inclusions similar to those associated with Δ GAG-torsinA, indicating that this change may also affect torsinA structure. Introducing H216 into Δ GAG-torsinA reduced its tendency to form inclusions, suggesting that the two changes offset each other. Our findings point to a structural basis for the defects associated with the disease-linked Δ GAG deletion in torsinA. They also suggest possible connections between the allelic polymorphism at residue 216 and the penetrance of *DYT1* dystonia, as well as a possible role for this polymorphism in related disease states.

INTRODUCTION

Early-onset generalized torsion dystonia is a dominantly inherited movement disorder in humans. Positional cloning of the defective gene, *DYT1*, led to the discovery of the protein it encodes, torsinA, and a novel family of related proteins (1,2). Most cases (~80%) of early-onset torsion dystonia are caused by a single mutation in *DYT1*: an in-frame deletion of three nucleotides (Δ GAG) in exon 5, resulting in the loss of

a glutamic acid residue at position 302 or 303 in the 332 amino acid protein. Remarkably, this same mutation has arisen independently in different families. Still unclear are how the Δ GAG deletion causes early-onset torsion dystonia and why this mutation acts in a dominant manner.

Three other variations have been found that change the amino acid sequence of torsinA in humans. First, a polymorphism in the coding sequence for residue 216 encodes

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aspartic acid in 88% and histidine in 12% of alleles in control populations (1,3,4). Secondly, an 18 bp deletion ($\Delta 18$ bp) that causes loss of residues 323–328 was found in three members of a single family with varying degrees of dystonia, confounded in two of these individuals by myoclonus due to a concomitant mutation in the ϵ -sarcoglycan gene (3). Whether the torsinA deletion contributed to disease in this family has not been clearly established (5,6). Finally, a 4 bp deletion ($\Delta 4$ bp) that causes a frameshift and truncation starting at residue 312 was found in a single control blood donor who was not examined neurologically (7).

On the basis of sequence similarity, torsinA has been classified as a member of the superfamily of ATPases associated with a variety of cellular activities (AAA+) (1,8,9). AAA+ proteins typically form six-membered homo-oligomeric rings that control assembly, disassembly and function of many types of protein complexes involved in protein processing, membrane trafficking, cytoskeletal dynamics and organelle biogenesis (10–12). The AAA+ domain of torsinA is most similar to the second AAA+ domain of the bacterial heat-shock protein ClpB (9). ClpB and its ortholog in *Saccharomyces cerevisiae*, Hsp104, help resolubilize aggregated proteins and participate in conformational modification of prions (13). The crystal structure of *Thermus thermophilus* ClpB has been described (14) and is what we used in the present study to generate a structural framework for analysis of the torsin family of AAA+ proteins.

Although its function is not known, torsinA and its immediate family members have been implicated in a diverse set of cellular functions, including protein folding and degradation (15–17), response to viral infection (18), toxic conditions (19) and oxidative stress (20,21), rotation of the nucleus during early embryogenesis in *C. elegans* (22), processing of polytopic membrane proteins (23) and regulation of the interaction between outer and inner membranes of the nuclear envelope (24–26). Binding partners identified so far for torsinA include the nuclear envelope protein LAP1 and the related endoplasmic reticulum (ER) protein LULL1/NET9 (26) and kinesin light chain 1 (27). These interactions point to roles for torsinA in regulating some aspect of nuclear envelope organization and/or in regulating microtubule-based movement of membrane compartments within cells. In addition, torsinA and torsinB have been found to interact with each other, suggesting that torsin family members may assemble and possibly function as hetero-oligomeric complexes (28,29).

Although torsinA is expressed in most tissues in the body (1,30), the effects of the Δ GAG *DYT1* gene are thought to be exclusively neurologic, suggesting a critical function for torsinA in the brain (31). TorsinA message and protein are preferentially expressed in certain neuronal populations within the adult brain (32–34). Pathologic examination of the brains of Δ GAG *DYT1* carriers affected with dystonia reveals no evidence of neuronal loss (35). The dopaminergic cells in the substantia nigra, which express among the highest levels of torsinA message in the brain (32), appear to have enlarged cell bodies but no other obvious abnormalities such as protein aggregates or inclusions (33). Perinuclear inclusions that are immunoreactive for torsinA, ubiquitin and lamin A/C have recently been described in the brainstem of Δ GAG *DYT1* patients (36).

It is unclear how the disease-causing Δ GAG mutation perturbs cellular and especially neuronal function. What is known is that when Δ GAG-torsinA is overexpressed in cultured cells, it concentrates in the nuclear envelope and, at higher levels of expression, in large, whorled membrane inclusions derived from the ER (25,37–42). Whether the recently described brainstem inclusions in Δ GAG *DYT1* patients represent the same phenomenon remains to be established (36), but the lack of membranous inclusions in at least most cells derived from patients as opposed to in those transfected with Δ GAG-torsinA in culture presumably reflects the higher levels of expression in the latter (40). A recent study of torsinA knock-out mice indicates that the Δ GAG mutant cannot rescue the lethality associated with the lack of torsinA, pointing to loss of normal function as one effect of the Δ GAG mutation (43).

The present study was undertaken to gain insight into how the disease-causing Δ GAG deletion as well as other naturally occurring sequence variations affect torsinA. By aligning torsinA with the homologous protein ClpB, we delineated N- and C-terminal subdomains within its AAA+ domain and tested the effect of mutating predicted AAA+ motifs on the ability of wild-type and Δ GAG-torsinA to concentrate in the nuclear envelope and form inclusions in transfected cells. Changes in both subdomains of the AAA+ domain affected the tendency of overexpressed torsinA to form inclusions. The H216 polymorphism promoted formation of torsinA-positive inclusions similar to those induced by Δ GAG-torsinA. Surprisingly, introducing H216 into Δ GAG-torsinA reduced the tendency of this mutant to form inclusions, indicating that the regions of torsinA affected by these two changes interact in some manner. Our findings suggest a structural basis for the defects associated with the Δ GAG deletion and indicate that a search for possible connections between the allelic polymorphism at residue 216 and the penetrance of disease caused by the Δ GAG deletion, as well as for associations of these changes with related disease states, is likely to be worthwhile.

RESULTS

Structural features of torsinA predicted by sequence alignment with ClpB

The overall domain organization of torsinA is shown in Figure 1A. Structure- and sequence-based analyses of AAA+ proteins place torsinA in a family together with the C-terminal AAA+ domains of ClpA and ClpB (ClpAB-C/torsin family) (9). This family in turn belongs to a clade of AAA+ proteins, which also includes HslU, ClpX, Lon and RuvB (9). Among these, torsinA and its close relatives are most similar to the C-terminal AAA+ domain of ClpB. Alignment of torsinA with this domain from *T. thermophilus* ClpB for which a crystal structure has been solved (14) is shown in Figure 1B and is based on multiple sequence alignment of all torsin family members and representative ClpB proteins (Supplementary Material, Fig. S1). Conserved structural elements of the ClpB AAA+ domain are numbered according to the general scheme for AAA+ domains proposed by Iyer *et al.* (9,12) and can be divided into N-terminal α/β ($\alpha 0$ – $\alpha 4$, $\beta 1$ – $\beta 5$) and C-terminal predominantly α -helical

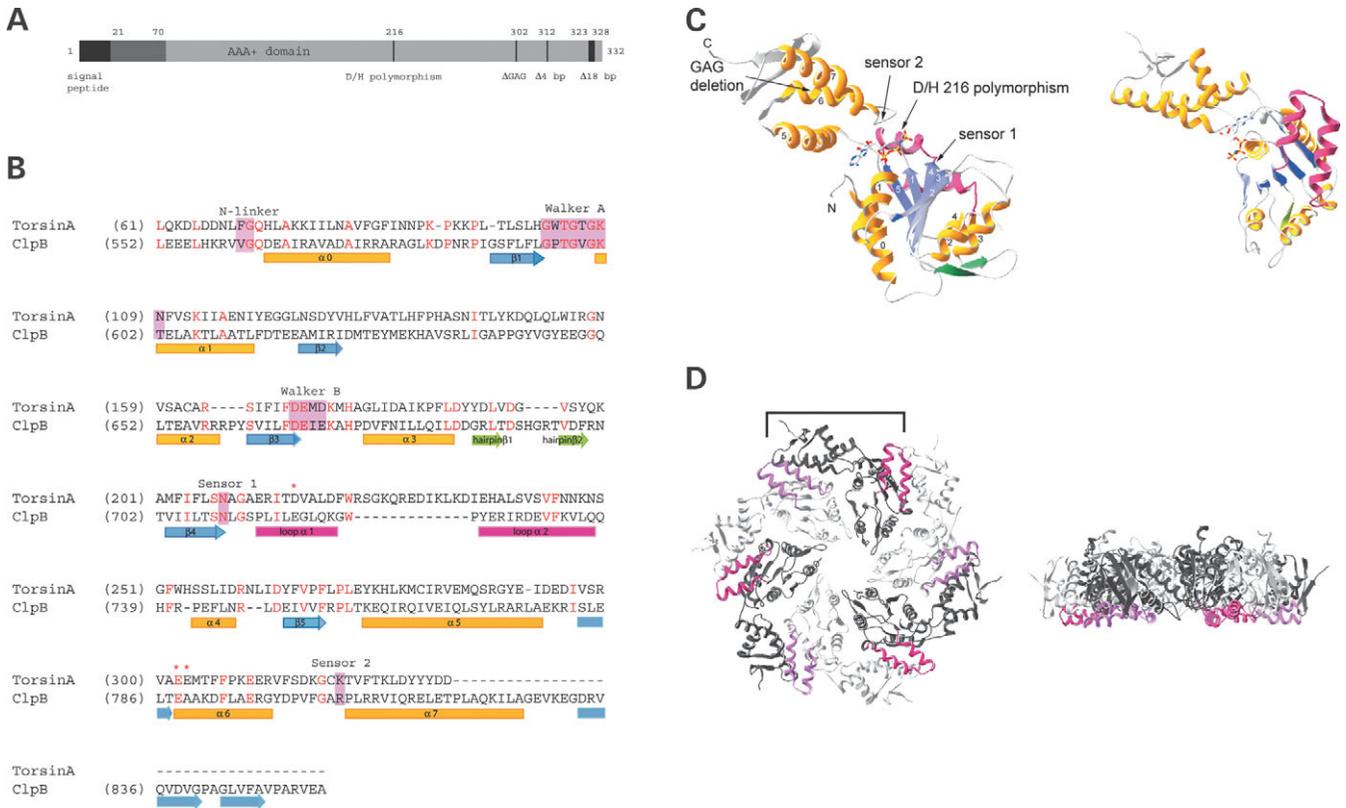


Figure 1. Predicted structural features of torsinA. **(A)** Overall organization and naturally occurring sequence variations in torsinA. **(B)** Structure-based alignment of torsinA with the C-terminal AAA+ domain of *T. thermophilus* ClpB (14). Helices and strands from ClpB are numbered according to the Iyer *et al.* (9). Conserved motifs are shaded. Mutations are starred. Red letters indicate identity in the sequence of the two proteins. Overall identity between the two is 21% and similarity is 40%. **(C)** Three-dimensional view of the C-terminal AAA+ domain of ClpB (PDB 1QVR). Coloring is the same as in (B). Predicted positions of ATP-interacting motifs as well as the Δ GAG (E302/303) deletion and D216H polymorphism in torsinA are indicated, on the basis of the alignment in (B). (Left) *En face* view; (right) $\sim 90^\circ$ rotated view with C-terminal end nearest the reader. **(D)** Model of hexamer formed by ClpB C-terminal AAA+ domains (14) shown from the C-terminal end (left) or the side (right), with alternating subunits shaded in dark and light colors. Bracket encloses the subunit shown from the same vantage point as right view of (C). The $\beta 4$ - $\alpha 4$ insertion (loop helix 1 and loop helix 2) is shown in magenta, again with dark and light colors on alternating subunits. Note the proximity between this insertion in one subunit and the C-terminal subdomain of an adjacent subunit.

($\alpha 5$ - $\alpha 7$) subdomains. In torsinA, the N-terminal subdomain of the AAA+ fold extends from residues 70-271 and the C-terminal subdomain from residues 272-332. Additional features of the ClpAB-C/torsin subfamily are numbered separately and include a presensor 1 β -hairpin (hairpin- $\beta 1$ and hairpin- $\beta 2$ between $\alpha 3$ and $\beta 4$) and an insertion of varying length and structure between $\beta 4$ and $\alpha 4$ (loop- $\alpha 1$ and loop- $\alpha 2$, located in the same position as the second region of homology in classical AAA proteins) (9). There is also an extended loop between $\beta 2$ and $\alpha 2$ that contains a conserved hydrophobic pore motif in several family members. The few breaks in the alignment between torsinA and ClpB are within predicted loops in the N-terminal subdomain. Similarity among the proteins is lower in the C-terminal subdomain, but a conserved sensor 2 motif (GCK in torsins, GAR in ClpB and ClpA) is present. The torsinA C-terminal subdomain is shorter than that of ClpB, ending 12 residues after the predicted sensor 2 motif and before the two β -strands that cap the C-terminus of ClpB.

The close structural similarity among the many AAA+ proteins crystallized to date (11) suggests that the alignment of torsinA with related proteins should enable initial prediction

of its structure. The three-dimensional structure of the second AAA+ domain of *T. thermophilus* ClpB is shown in Figure 1C (14), with its strands and helices numbered as in Figure 1B. In the left panel, the N-terminal α/β subdomain is shown below the C-terminal α -helical subdomain, with the predicted positions of conserved motifs and sequence variations in torsinA indicated. In the right panel, the AAA+ domain is rotated by $\sim 90^\circ$. Most naturally occurring changes in torsinA (Δ GAG, $\Delta 4$ bp and $\Delta 18$ bp deletions) fall within the AAA+ domain's C-terminal subdomain: the Δ GAG deletion is near the beginning of helix $\alpha 6$, which interacts extensively with other helices in the C-terminal subdomain and precedes the sensor 2 motif; the frameshift truncation resulting from the $\Delta 4$ bp deletion eliminates the entire sensor 2 motif; and the $\Delta 18$ bp deletion eliminates much of helix $\alpha 7$. The D/H polymorphism at residue 216 is in the N-terminal subdomain on the helix that follows the sensor 1 motif (magenta in Fig. 1C, between $\beta 4$ and $\alpha 4$).

Studies with purified torsinA have not yet defined its functional oligomeric state, but other AAA+ proteins including ClpB typically operate as hexameric rings. A model of how the ClpB C-terminal AAA+ domain—and thus the torsinA

AAA+ domain—might oligomerize to form a hexamer is shown in Figure 1D (14). Alternating subunits are shown in light and dark colors, and the two helices inserted between β_4 and α_4 are highlighted in magenta. The hexamer is shown from below (left panel, with top subunit in the same orientation as right panel of Fig. 1C) or from the side (right panel). The extensive interface between subdomains of adjacent subunits implies that mutations such as the Δ GAG deletion may affect not only torsinA's interactions with ATP but also its oligomeric state. The helices inserted between β_4 and α_4 lie on the surface of the predicted oligomer and are near the subunit–subunit interface. They may therefore play roles both in oligomer assembly and interaction with other proteins.

Effect of ATPase mutations on localization of torsinA: validating the AAA+ model

To explore predictions derived from this model, we introduced mutations into the key residues of two AAA+ motifs involved in ATP hydrolysis, the sensor 1 and sensor 2 motifs (12). All mutants were expressed at approximately equivalent levels in transiently transfected U2OS human osteosarcoma cells (Fig. 2A). Both N208A (sensor 1) and K320M (sensor 2) mutations led to a striking concentration of torsinA in the nuclear envelope of transfected U2OS cells (Fig. 2B), similar to the previously studied Walker B (E171Q) mutant (25,41). Enrichment of these mutants in the nuclear envelope was also seen in CAD and PC12 cells and with myc and GFP-tagged proteins (data not shown). None of these three mutants (E171Q, N208A or K320M) formed inclusions (Fig. 2C). The common effect of the three mutations is consistent with them all impairing ATP hydrolysis and suggests that the sensor 1 and sensor 2 motifs are correctly identified by our alignment.

Effect of mutations on Δ GAG-torsinA induced inclusions: role for C-terminal AAA+ subdomain in inclusion formation

Δ GAG-torsinA induces formation of membranous ER-derived inclusions in transfected cells (37,38). Interfering with its ability to bind ATP (K108A mutation in the Walker A motif) (23,25) or to undergo glycosylation (treatment with tunicamycin or mutation of N158) (44) prevents inclusions from developing. As nucleotide binding and glycosylation are often prerequisites for protein folding among ATPases and glycoproteins, respectively, these observations suggest that inclusions only develop when Δ GAG-torsinA is correctly folded. To understand further which features of torsinA contribute to the formation of inclusions, we combined Walker B, sensor 1 and sensor 2 mutations with the Δ GAG-deletion and examined the distribution of double mutants in transfected U2OS cells (Fig. 2). The mutant proteins were expressed at similar levels. E171Q/ Δ GAG- and N208A/ Δ GAG-torsinA-expressing cells developed inclusions, whereas cells transfected with K108A/ Δ GAG torsinA did not, as previously shown (25). Interestingly, K320M/ Δ GAG-expressing cells also did not form inclusions. We conclude that the inability to hydrolyze

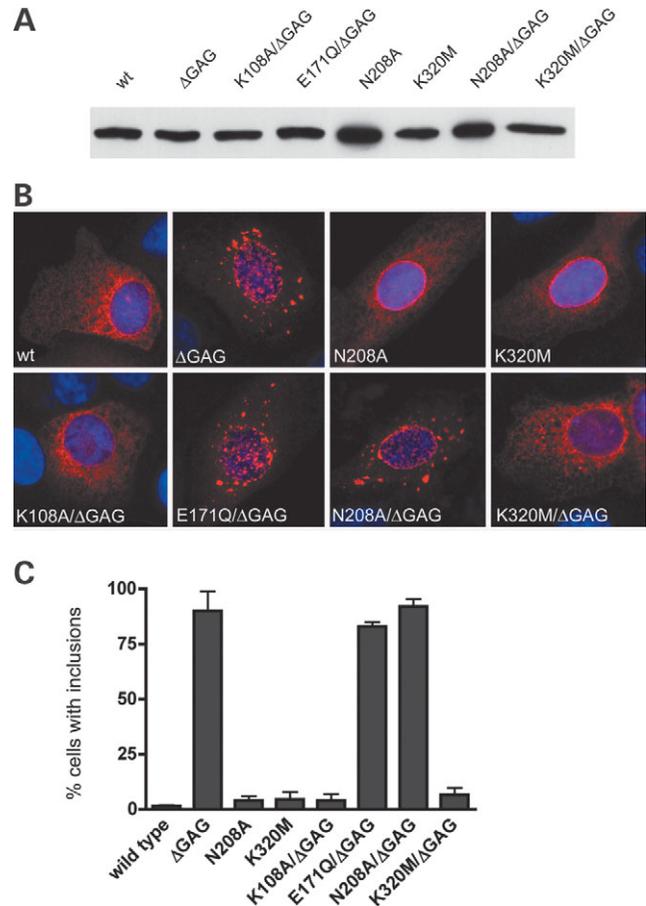


Figure 2. Effects of mutations in conserved ATPase motifs on localization of torsinA. (A) Western blot showing expression of indicated torsinA mutants in transfected U2OS cells. (B) Representative images of untagged torsinA mutants expressed by transient transfection of U2OS human osteosarcoma cells and detected with torsinA-specific antibody. Red shows torsinA and blue shows DAPI in the nucleus. (C) Quantitation of proportion of transfected cells containing torsinA-positive inclusions. Mean and standard error of the mean from three experiments (each >200 cells) are shown.

ATP does not by itself induce inclusions, but at the same time does not impede their formation in the context of the Δ GAG-torsinA deletion. The lack of Δ GAG-induced inclusions when sensor 2 is mutated (K320M) points to a special role for the C-terminal subdomain in this phenomenon.

In addition to the glutamic acid deletion caused by the Δ GAG mutation, other single amino acid deletions between residues 302 and 309 have been found to promote the development of torsinA-positive inclusions (41). These residues are all predicted to lie on helix α_6 preceding sensor 2 (Fig. 1), and deleting them one at a time may cause a common distortion in the organization of this helix and in the overall structure of the protein. To better understand the role of torsinA's C-terminal subdomain in inducing inclusions, we made a series of deletions in wild-type and Δ GAG-torsinA. These deletions were designed to eliminate some or all of the predicted helices surrounding the Δ GAG deletion. After transfection into CAD cells, all expressed protein at similar levels (Fig. 3A). TorsinA

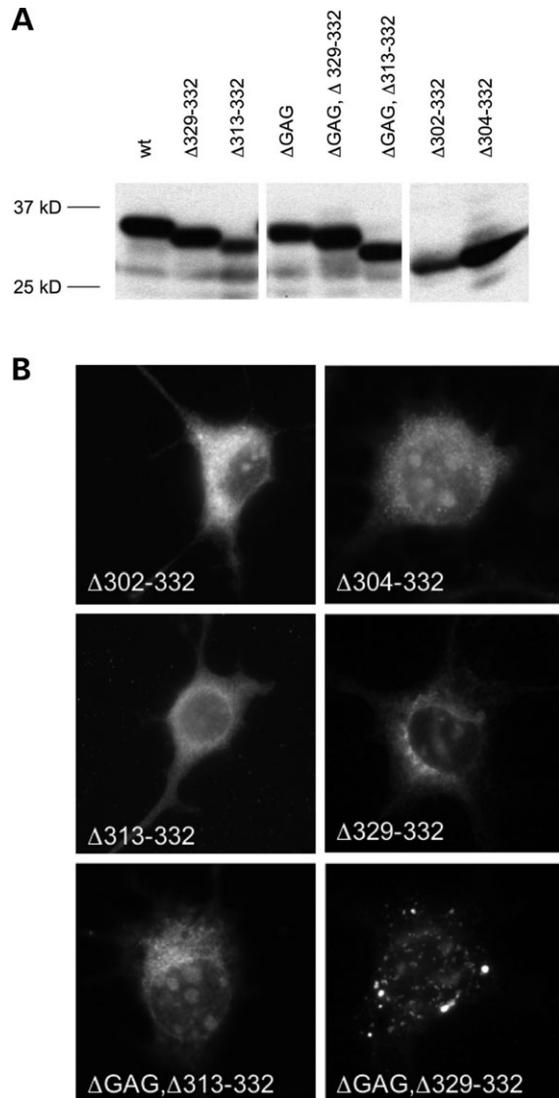


Figure 3. Effects of C-terminal deletions on torsinA localization. (A) Western blot showing expression of indicated deletion mutants in CAD cells transfected with amplicon vectors encoding the indicated protein. (B) Representative images of CAD mouse neuronal-like cells transfected with amplicon vectors encoding the indicated deletion mutants. Transfected cells were identified by co-expressed soluble GFP encoded in the amplicon backbone (data not shown).

was visualized using torsinA-specific antibodies in transfected cells, which were identified by the presence of co-expressed GFP (Fig. 3B). None of these larger deletions caused wild-type torsinA to form inclusions. On the background of the ΔGAG deletion, removal of residues 313–332, but not 329–332, blocked development of inclusions. Similar results were also seen in other cell lines (data not shown). These findings suggest that inclusions are not the default outcome of expressing truncated or non-functional torsinA. Instead, they form only in response to expression of particular mutants (single amino acid deletions between 302 and 309) (37,38,41) and require specific C-terminal elements including the sensor 2 K320 residue.

Effects of naturally occurring sequence variations: polymorphism at residue 216 changes behavior of wild-type and ΔGAG -torsinA

cDNA sequences for known naturally occurring torsinA coding sequence variants [ΔGAG , $\Delta 18$ bp, $\Delta 4$ bp (all with D216), H216 and H216/ ΔGAG] were cloned in an expression cassette and initially transfected into 293T cells. Cell lysates were resolved by SDS-PAGE and immunoblotted for torsinA. Wild-type, ΔGAG , H216 and H216/ ΔGAG immunoreactive proteins migrated with a molecular weight (MW) of 37 kDa, whereas the $\Delta 18$ and $\Delta 4$ bp forms had slightly lower MW as expected (Fig. 4A). Expression levels were roughly equivalent in both 293T (Fig. 4A) as well as CAD cells (data not shown). All forms of torsinA could be deglycosylated with endonuclease H, confirming that they were normally targeted to and retained in the ER.

Localization of overexpressed torsinA was evaluated in neuron-like CAD cells transfected with these expression cassettes. As earlier, torsinA was visualized using torsinA-specific antibodies in transfected cells, which were identified by the presence of co-expressed GFP (Fig. 4B). The behavior of the different mutant forms was quantified by counting the number of transfected cells with torsinA-positive inclusions (Fig. 4C). Inclusions were most prevalent in D216/ ΔGAG -torsinA-expressing cells (inclusions present in $\sim 80\%$ of transfected cells). Surprisingly, wild-type torsinA containing the H216 polymorphism also had a tendency to form inclusions ($\sim 25\%$ of cells), whereas a combined H216/ ΔGAG torsinA formed inclusions at a lower frequency ($\sim 60\%$ of cells) than did D216/ ΔGAG torsinA. This suggested the interesting possibility that H216 might distort torsinA's structure into a form that is somehow complementary to that of ΔGAG -torsinA, thereby achieving a more normal structure and reducing its tendency to form inclusions. Alternatively, the combined presence of the ΔGAG and H216 changes could have the same effect as inhibiting ATP binding or glycosylation. Other naturally occurring variations ($\Delta 4$ and $\Delta 18$ bp deletions) did not generate inclusions, consistent with a previous report of consequences of the $\Delta 18$ bp deletion (45).

The paradoxical finding that the H216 polymorphism promotes the formation of inclusions in the context of wild-type torsinA but inhibits induction of inclusions by ΔGAG -torsinA in CAD cells prompted us to examine the effects of these mutations on torsinA in a second cell line, human U2OS osteosarcoma cells. U2OS cells are larger than CAD cells and have a more extensive and clearly resolved ER and are thus advantageous for localization studies. To selectively visualize transfected protein, we used torsinA constructs containing a C-terminal myc epitope tag. All constructs expressed equivalent levels of protein (Fig. 5A). Once again, we found that cells-expressing D216 wild-type torsinA had no torsinA-reactive inclusions, whereas $\sim 85\%$ of those expressing D216/ ΔGAG torsinA did (Fig. 5B and C). Expression of wild-type torsinA containing H216 generated inclusions in $\sim 30\%$ of the cells and expressing H216/ ΔGAG led to inclusions in $\sim 60\%$ of the cells. Replacing D216 with alanine instead of histidine (A216) produced only few cells with inclusions, indicating that the inclusions are associated with the introduction of histidine and not the loss of aspartic

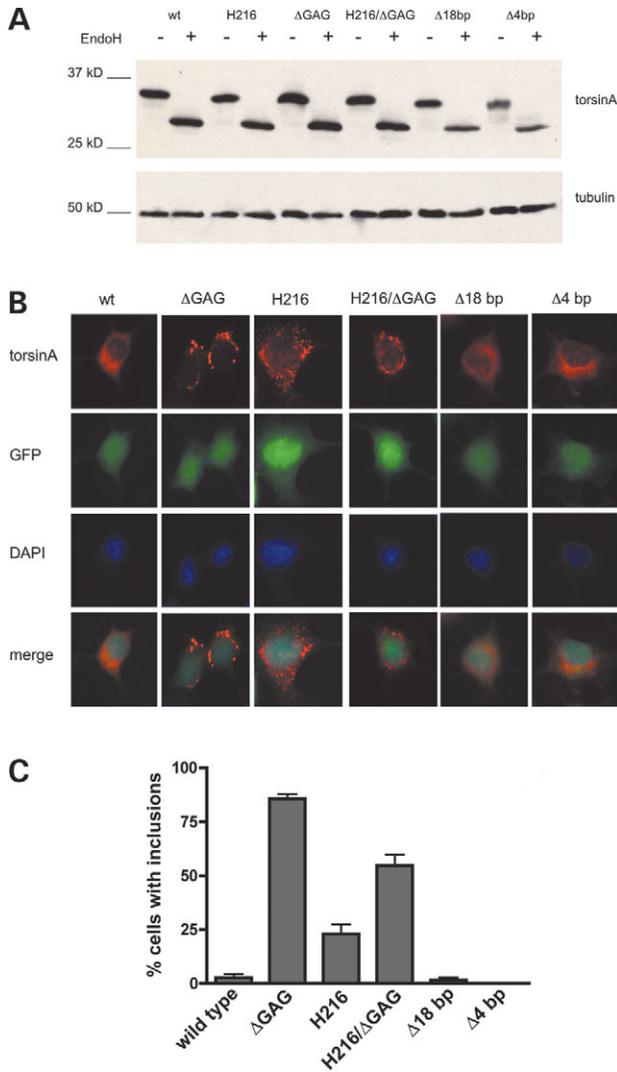


Figure 4. Localization of naturally occurring torsinA sequence variants in transfected cells. (A) Western blot showing expression of torsinA variants in HEK293T cells transfected with amplicons encoding the variant proteins. (B) Representative images of torsinA distribution in CAD cells transiently transfected with the indicated amplicon. Co-expressed GFP was used to distinguish transfected from untransfected cells. Blue shows DAPI in the nucleus. (C) Quantitation of proportion of transfected cells with torsinA-positive inclusions. Mean and standard error of the mean from three experiments are shown.

acid *per se*. Similar observations for all of these mutants were made using untagged and GFP-tagged torsinA constructs (data not shown). These observations parallel and support the results seen in CAD cells and indicate that the striking effects of these changes are general phenomena associated with expressing these different variants of torsinA.

DISCUSSION

Since identification of the *DYT1* gene in 1997 (1), the function of its encoded protein, torsinA, has remained elusive. Similarly, how mutations in torsinA compromise its structure and function to induce disease is unclear. Clues to both of these

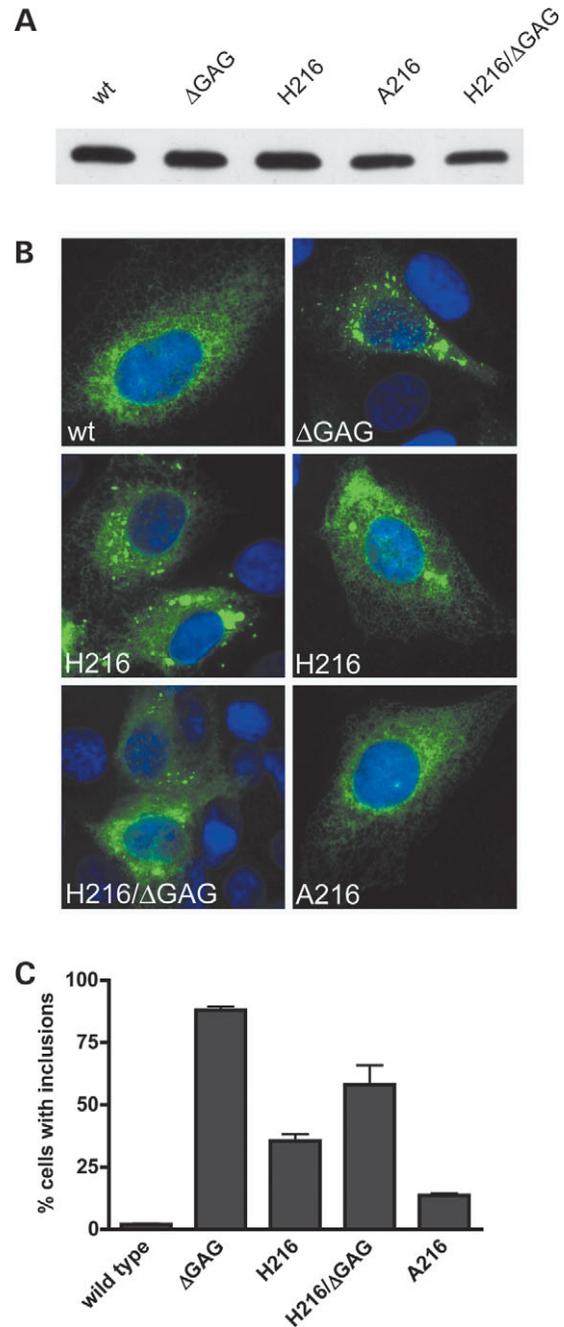


Figure 5. Effects of variations at residue 216 on localization of torsinA in U2OS cells. (A) Western blot showing expression of indicated torsinA-His₆myc mutants in transfected U2OS cells. (B) Representative images of U2OS cells transiently transfected with plasmids, encoding torsinA-His₆myc containing the indicated mutation(s). Green shows torsinA and blue shows DAPI. (C) Quantitation of proportion of transfected U2OS cells with torsinA-positive inclusions. Mean and standard error of the mean from three or more experiments (each >200 cells) are shown.

issues have come from studying the behavior of torsinA mutants in transfected cells and, more recently, in genetically modified animals (43,46–48). Whereas wild-type torsinA distributes throughout the ER proper, an engineered mutant torsinA predicted to function as a ‘substrate trap’ because it

is unable to hydrolyze ATP accumulates in the contiguous nuclear envelope (25,41). This suggests that torsinA functions in part within the nuclear envelope, and the recent finding that knock-out animals lacking torsinA have ultrastructural defects in the nuclear envelopes of their neurons supports this hypothesis (43). Dystonia-associated Δ GAG-torsinA accumulates both within the nuclear envelope and in ER-derived membrane-whorls or inclusions when overexpressed in cultured cells (37,38) and is unable to rescue the lethality of the torsinA knock-out mouse (43). This, together with the fact that DYT1 dystonia is a dominantly transmitted disease, suggests that the Δ GAG mutation may cause more than a simple loss of function in torsinA. Here, we use sequence-based alignment of torsinA with other AAA+ proteins to provide a working model of torsinA's structure and how it is modified by the disease-linked Δ GAG mutation as well as other naturally occurring changes. Importantly, we find that the relatively common polymorphic replacement of D216 with H216 affects the properties of both wild-type and Δ GAG-torsinA. These findings indicate that this polymorphism needs to be examined both for association with the low (<30%) penetrance of dystonia in carriers of the Δ GAG mutation and as a predisposing factor to other disease states.

TorsinA D/H216 polymorphism: modulator of protein structure and function

One of the major goals of this study was to explore the effects of naturally occurring variations in the torsinA protein sequence on its structure and function. In the absence of a defined and measurable activity for torsinA, we took advantage of the striking variations seen in the distribution of different mutant forms within the ER of transfected cells to assess protein behavior. As is well established in the literature, Δ GAG-torsinA readily forms ER-derived inclusions when overexpressed in cultured cells (37,38). The Δ 4 and Δ 18 bp deletions have no obvious effects on torsinA localization (Fig. 4) (45) and may or may not be associated with disease (5–7). Two surprising findings emerged regarding the effects of the D/H polymorphism at residue 216 (Figs 4 and 5). On the one hand, simple replacement of D216 with H216 changed the behavior of torsinA in a manner reminiscent of the Δ GAG deletion, inducing formation of torsinA-positive inclusions in a significant, although smaller, fraction of transfected cells. On the other hand, when combined with the Δ GAG deletion (H216/ Δ GAG torsinA), the change at residue 216 reduced the proportion of torsinA-expressing cells that developed inclusions.

How might the D/H polymorphism at residue 216 affect torsinA? Some insight comes from looking at the structure of the related AAA+ protein ClpB (Fig. 1). Residue 216 is part of a helix–loop–helix insertion in the core of the AAA+ domain, which is a characteristic feature of members of the ClpAB-C/torsin subfamily of AAA+ proteins (9). This insertion falls between conserved β 4 and α 4 elements and is predicted to be exposed on the surface of assembled hexamers (Fig. 1D). Residues in this inserted loop are known to mediate interaction between the AAA+ proteins ClpA and ClpX and their cognate protease ClpP (49) and have recently been engineered into ClpB to give it the new

ability to interact with and deliver proteins to ClpP (50). In torsin proteins, the β 4– α 4 insertion is longer than that in ClpB, highly charged, conserved and could be involved both in subunit–subunit interactions and in interactions with other proteins (Fig. 1) (Supplementary Material, Fig. S1). Any changes in the disposition of this insertion (such as might be induced by replacing an aspartic acid with histidine) could affect these interactions. As aspartic acid at position 216 is not conserved among torsin-related proteins (Supplementary Material, Fig. S1), the specific interactions affected by changing this residue to histidine will be unique to torsinA. The fact that histidine at this position reduces the tendency of Δ GAG-torsinA to form inclusions (Figs 4 and 5) suggests that the β 4– α 4 insertion might interact with the C-terminal (Δ GAG-containing) subdomain from an adjacent subunit to achieve a close-to-normal structure (Fig. 1D, note proximity between dark β 4– α 4 insertions in one subunit and adjacent light colored α -helical domains from another subunit). Alternatively, the combined presence of two destabilizing mutations (H216 and Δ GAG deletion) could interfere with torsinA structure, resulting in a decrease in inclusion formation similar to that seen when glycosylation or ATP binding is prevented (23,25,44).

TorsinA-positive inclusions: indicators of more than a loss of torsinA function

A major question in the study of DYT1 dystonia is whether the Δ GAG deletion in torsinA simply decreases normal torsinA function or instead has additional toxic effects on the cells in which it is expressed. The tendency of overexpressed Δ GAG-torsinA to promote formation of ER-derived inclusions might point to the latter, but the fact that inclusions form most efficiently when Δ GAG-torsinA is expressed at high levels in cultured cells (40) and do not appear to be present in neurons in most regions of the brain in affected patients (33–35) has led to lingering questions about what this phenomenon tells us about torsinA function and dysfunction. The recent description of torsinA containing inclusions in the brainstem of Δ GAG DYT1 patients raises the possibility that inclusions could be directly associated with disease (36). However, even if macroscopic inclusions are not typically present in affected patient tissue, their presence in transfected cells, such as we have studied here, appears likely to reflect the abnormality, structural or functional, in torsinA that leads to disease.

In support of this hypothesis, we note that inclusions containing torsinA form when expressing only a small set of mutants, including the Δ GAG deletion (37,38), the H216 polymorphic form (Figs 4 and 5) and a few single amino acid deletions in the predicted helix α 6, which also contains the Δ GAG deletion (41). Other mutations in ATP-interacting motifs as well as in sequences near the C-terminus of the protein do not promote development of inclusions (Figs 2–5). In addition, some mutations in ATP-interacting motifs prevent Δ GAG-torsinA from forming inclusions (K108A Walker A and K320M sensor 2), whereas others do not (E171Q Walker B and N208A sensor 1) (Fig. 2). Deletions within the C-terminal domain adjacent to the Δ GAG-deletion also prevent inclusions from developing (Fig. 3). Taken

together, these observations suggest that Δ GAG-torsinA forms inclusions in a nucleotide-bound conformation, but only when its C-terminal subdomain remains largely intact.

Inclusions thus represent more than a simple loss of function in torsinA and instead appear to be ER responses to an abnormal form or forms of torsinA. Several observations indicate that torsinA may be present in abnormally stabilized oligomers or complexes in the inclusions. First, Δ GAG-torsinA in transfected cells is more readily cross-linked to itself than is wild-type torsinA (23). Secondly, other ER-derived inclusions (often referred to as 'organized smooth ER' or OSER) form as the result of oligomerization of overexpressed ER proteins (51). The possibility that torsinA interacts with itself within the lumen of the ER to induce inclusions is consistent with the tight apposition seen between the ER membranes in thin sections of torsinA inclusions (25,37,42). Finally, adding GFP (with its tendency to dimerize) (51) but not a mutant form of GFP that does not dimerize (51) to the C-terminus of torsinA causes even wild-type torsinA to form inclusions at high levels of expression (Teresa V. Naismith and Phyllis I. Hanson, unpublished data). Whether these observations can be extended to suggest that Δ GAG-torsinA and other similarly defective mutants form abnormal oligomers, but not full-blown inclusions, even at lower levels of expression remains to be determined.

On the basis of the marked differences in behavior of D216-torsinA versus H216-torsinA with respect to the development of inclusions by both wild-type and Δ GAG-torsinA, it will now be important to evaluate the frequency of this allele in manifesting versus non-manifesting Δ GAG *DYT1* carriers, as well as in patients with other forms of dystonia and related movement disorders. It seems possible that the identity of the residue at this polymorphic site could modify penetrance or development of disease.

MATERIALS AND METHODS

TorsinA/ClpB alignment

Torsin, ClpB and ClpA protein sequences were aligned using ClustalX and Megalign software. Three-dimensional views of the ClpB D2 domain in monomeric (PDB 1QVR) and hexameric (14) forms were rendered using DeepView v. 3.7. Coordinates of the ClpB hexamer model were kindly provided by F. Tsai (Baylor).

Expression constructs and mutagenesis

cDNAs encoding wild-type and mutant forms of torsinA were cloned into the following vectors: (a) amplicon pHGCX which contains an independent expression cassette for GFP (52), as described (37,40); (b) plasmid vector pcDNA3 for untagged torsinA (37); and (c) plasmid vector pcDNA4/TO His₆myc-C for proteins with C-terminal His₆myc tags. All constructs contain D216 unless specifically noted otherwise. Mutations were generated either by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA, USA) or by overlap-extension PCR (53) using wild-type or Δ GAG-torsinA as template. Sequences of mutagenic primers are available upon request. Truncation mutations were created by PCR

using a single mutagenic reverse primer including the stop codon and a *NotI* restriction site and subcloned into pHGCX. The complete torsinA sequence was verified in all constructs.

Cell culture

Human embryonic kidney fibroblast line 293T (54) was maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Rockville, MD, USA), mouse neural cell line, CAD (Dr J. Wang, Tufts University) (55) in DMEM-F12 (GIBCO BRL) and human osteosarcoma cell line (U2OS, Invitrogen) in DMEM. Media were supplemented with 10% fetal bovine serum and for 293T and CAD cells with 50 U/ml penicillin and 50 μ g/ml streptomycin.

Transfection

293T and CAD cells were transiently transfected at 80% confluency in P100 dishes (for protein analysis) or on 12 mm glass cover slips in 24-well plates (for immunocytochemistry) with pHGCX constructs as indicated. Transfections were carried out using either calcium chloride (56) or Lipofectamine according to the manufacturer instructions (GIBCO BRL). Medium was replaced 7 h after transfection and cells were evaluated by immunocytochemistry or immunoblotting 48 h after transfection or lysed for further protein analyses. U2OS cells on glass cover slips (for immunocytochemistry) or in P60 dishes (for protein analysis) were transiently transfected with plasmid constructs as indicated using Lipofectamine 2000 and fixed for immunostaining 24–72 h after transfection.

Immunoblotting

Total cell lysates were prepared by washing cells twice with phosphate-buffered saline (pH 7.4) and resuspending them in lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40 and complete protease inhibitorsTM (Boehringer Mannheim, Indianapolis, IN, USA). Lysates were resolved by electrophoresis in 12.5% polyacrylamide gels, transferred to nitrocellulose and immunoblotted for torsinA or tubulin. TorsinA was detected using mouse monoclonal DM-2A8 (28) and tubulin using mouse monoclonal DM1A (Sigma, St Louis, MO, USA). Myc-tagged torsinA was detected with mouse monoclonal 9E10 (Developmental Studies Hybridoma Bank, University of Iowa). Immunoreactive proteins were revealed with horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Pharmacia, Uppsala, Sweden) and Supersignal reagent (Pierce). Cell lysates were treated with endoglycosidase H according to the manufacturer instructions (New England Biolabs, Beverly, MA, USA).

Immunocytochemistry

CAD cells were fixed 24–48 h after transfection with 4% paraformaldehyde and processed for immunostaining, as described (37). TorsinA was visualized with 1:200 monoclonal antibody D-M2A8 (28) and 1:1500 Alexa 594 goat anti-mouse antibody (Molecular Probes, Portland, OR, USA) in 1%

BSA. Cells were also stained with 1.0 µg/ml DAPI before mounting onto glass slides. Cells were examined using a 100× objective on an Olympus BX60 microscope with a Sony CCD color camera and analyzed with Image-Pro Plus Software. U2OS cells were processed similarly, except that fixation was with 3% paraformaldehyde containing 4% sucrose. Myc-tagged torsinA was detected using mouse monoclonal 9E10 (Developmental Studies Hybridoma Bank, University of Iowa) and Alexa 555 goat anti-mouse antibody (Molecular Probes, Portland, OR, USA). U2OS cells were examined on a Leica Diaplan microscope with a Zeiss Axiocam color camera, as described (57).

Quantitation of inclusions

For quantitation, cover slips were evaluated in a blinded fashion for the presence of torsinA-positive inclusions by scoring absence versus presence of inclusions in >100 (CAD) or >200 (U2OS) transfected cells on each of three cover slips. Transfected CAD cells were distinguished from non-transfected cells by the additional presence of amplicon-encoded GFP in the transfected cells. Transfected torsinA in U2OS cells was recognized by taking advantage of the C-terminal myc epitope tag.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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