Torsin A and Its Torsion Dystonia-associated Mutant Forms Are Lumenal Glycoproteins That Exhibit Distinct Subcellular Localizations*

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Early-onset torsion dystonia is an autosomal dominant hyperkinetic movement disorder that has recently been linked to a 3-base pair deletion in the DYTI gene. The DYTI gene encodes a 332-amino acid protein, torsin A, that bears low but significant homology to the Hsp100/Clp family of ATPase chaperones. The deletion in DYTI associated with torsion dystonia results in the loss of one of a pair of glutamic acid residues residing near the C terminus of torsin A (ΔE-torsin A). At present, little is known about the expression, subcellular distribution, and/or function of either the torsin A or ΔE-torsin A protein. When transfected into mammalian cells, both torsin A and ΔE-torsin A were found to behave as lumenally oriented glycoproteins. Immunofluorescence studies revealed that torsin A localized to a diffuse network of intracellular membranes displaying significant co-immunoreactivity for the endoplasmic reticulum resident protein BiP, whereas ΔE-torsin A reside in large spheroid intracellular structures exclusive of BiP immunoreactivity. These results initially suggested that ΔE-torsin A might exist as insoluble aggregates. However, both torsin A and ΔE-torsin A were readily solubilized by nonionic detergents, were similarly accessible to proteases, and displayed equivalent migration patterns on sucrose gradients. Collectively, these data support that both the wild type and torsion dystonia-associated forms of torsin A are properly folded, lumenal proteins of similar oligomeric states. The potential relationship between the altered subcellular distribution of ΔE-torsin A and the disease-inducing phenotype of the protein is discussed.

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† The human torsins have clear homologues in rat, mouse, and Caenorhabditis elegans (four torsin-related gene products are predicted to exist in C. elegans), whereas Saccharomyces cerevisiae lacks torsin-like proteins. Additionally, the torsins show low but significant homology to the HSP100/Clp family of ATP-dependent chaperones (11–13). These distant relatives to the torsin family are cytosolic proteins noted for their ability to disassemble higher order protein structures and aggregates (14), confer increased tolerance to high temperature (15), and promote specific proteolysis (16). The HSP100/Clp proteins bind ATP and/or have ATPase activity, often functioning as nucleotide-stabilized oligomeric complexes with other companion proteins (17, 18). Torsin A is 25–30% identical to several HSP100/Clp family members over a 140-amino acid domain that contains the putative ATP-binding cassette of the protein. Additionally, multiple key residues in the IV and SN domains of the HSP100/Clp proteins are also conserved in torsin A (11). Because torsins contain only one predicted ATP-binding domain, these proteins would be classified as Class 2-type HSP100/Clp subfamily members (11).

The intriguing observation that a complex and debilitating neurobiological disease is linked to a single amino acid deletion...
in a putative protein of unknown structure or function motivated us to investigate the biochemical and cellular biological features of both torsin A and its disease-inducing mutant form, ΔE-torsin A. These studies were in turn anticipated to provide a molecular framework upon which models could be constructed to describe the endogenous function(s) of torsin A, as well as the mode of pathological action of ΔE-torsin A.

EXPERIMENTAL PROCEDURES

Generation of Torsin Expression Constructs—The cDNA for human torsin A was obtained as follows. ESTs covering portions of the torsin A cDNA were identified by BLAST searches and purchased (Research Genetics). These partial clones were then internally radiolabeled with [α-32P]dCTP (Multiprime DNA Labeling Kit, Amersham Pharmacia Biotech) and used as probes to screen human brain and liver 5′-Stretch Plus cDNA libraries (CLONTECH); screens and phage DNA isolation were conducted according to manufacturer’s guidelines. A single human liver clone was isolated that contained the complete torsin A cDNA sequence as well as an additional internal intronic sequence. Polymerase chain reaction was used to generate a contiguous torsin A construct by amplification of the two surrounding exonic regions and ligation of these two halves to create a complete torsin A cDNA possessing a single silent mutation (generating an internal BspE1 site). The complete torsin A cDNA was subcloned into the pcDNA3 and pFLAG eukaryotic expression vectors. The complete torsin A cDNA was sequenced completely and contained only the de

Generation of Anti-torsin A Antibodies—The torsin A cDNA was subcloned into the TexasRed vector (Invitrogen) for generation of a torsin A His-tagged fusion protein (residues 61–332 of torsin A), which was expressed exclusively as inclusion bodies in Escherichia coli. The His-tagged torsin A was purified from inclusion bodies by SDS-PAGE1 and used as antigen. Rabbit polyclonal antibodies generated against the His-tagged torsin A protein were affinity purified by binding to Immobilon P transfer membrane (Millipore)-bound His-tagged torsin A. The membrane was washed three times with TBS/Tween (200 mM NaCl, 50 mM Tris, and 0.1% Tween 20) and once with water. Specific antibodies were eluted with elution buffer (100 mM glycine, 50 mM Tris, pH 2.5), and the elution fractions were immediately neutralized with 12% volume of neutralizing buffer (50 mM Tris, pH 9).

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Transient Transfection of COS-7 and HEK-293 Cells with Torsin A and ΔE-Torsin A—COS-7 and HEK-293 cells (100-mm plates) were transiently transfected at approximately 80% confluency with either torsin A or ΔE-torsin A cDNA encoding the wild-type or disease-inducing mutant form of torsin A, respectively. The cells were fractionated to provide cell microsomes as follows. Experiments with COS-7 cells were performed with 1,000 × g, 5 min, and the supernatant was removed and re-centrifuged at 100,000 × g for 1 h. The coverslips were washed with PBS to remove excess goat antibody, and the coverslips were mounted on microscope slides with SliOFade (Molecular Probes). The slides were viewed on a Zeiss Axiovert S100TV/Bio-Rad MRC1024 confocal microscopy system.

Subcellular Fractionation of Torsin A and ΔE-Torsin A—Total cell extracts were fractionated to provide cell microsomes as follows. Extracts were centrifuged at 1,000 × g for 5 min, and the supernatant was removed and re-centrifuged at 100,000 × g for 1 h. The coverslips were washed with PBS to remove excess goat antibody, and the coverslips were mounted on microscope slides with SliO Fade (Molecular Probes). The slides were viewed on a Zeiss Axiovert S100TV/Bio-Rad MRC1024 confocal microscopy system.

RESULTS

Expression and Localization of Torsin A and ΔE-Torsin A in Mammalian Cells—In order to generate reagents to detect the expression of torsin A and ΔE-torsin A in mammalian cells, rabbit anti-torsin polyclonal antibodies were raised against a fragment of the torsin A protein recombinantly expressed in E. coli (amino acids 61–332). The cDNAs for torsin A and ΔE-torsin A were subcloned into the pcDNA3 vector and trans
siently transfected into HEK-293 cells. Transfected cell extracts were probed with anti-torsin antibodies, and both torsin A and ΔE-torsin A-transfected cells, but not mock-transfected cells (transfected with the pcDNA3 vector alone), showed a strong immunoreactive band near the predicted molecular size of the torsins (38 kDa) (Fig. 1A). Torsin A and ΔE-torsin A were expressed to similar levels in these transfected cell populations. An immunoreactive band of equivalent molecular size to torsin A was also observed in mock-transfected cells but only upon much longer exposures (see below), indicating that HEK-293 cells express low levels of endogenous torsin A. A single cross-reactive protein of approximately 100 kDa was observed in both mock- and torsin-transfected cells, possibly representing a protein of the HSP100 family of heat shock proteins, which display low level homology to torsins (8). Complementary efforts to generate epitope-tagged forms of torsin A revealed that addition of either an N- or C-terminal tag dramatically altered the stability of torsin A, with an N-terminal FLAG-torsin A appearing as several bands by Western analysis and a C-terminal Myc-torsin A failing to express to detectable levels (data not shown).

Both torsin A and ΔE-torsin A segregated exclusively with particulate fractions of HEK-293 cells, appearing in the 100,000 × g cell pellet but not in the 100,000 × g supernatant (Fig. 1B). In order to evaluate in more detail the subcellular localization of the torsins, immunofluorescence light microscopy studies were conducted on mammalian cells transfected with the torsin cDNAs. In HEK-293 cells, torsin A was found to reside on an intracellular membrane network that displayed significant overlapping immunoreactivity with the ER resident protein BiP (21) (Fig. 2A). Torsin A immunoreactivity was also observed in the nuclear envelope, a region void of BiP signals. A low level of additional immunoreactivity was sometimes observed in the nucleus, but this signal was found in mock-transfected cells as well and thus not likely attributable to the expressed torsin A protein. In sharp contrast to the subcellular distribution of torsin A, ΔE-torsin A localized to large, spheroid intracellular structures that were exclusive of BiP immunoreactivity (Fig. 2B). In order to evaluate whether the dramatically different subcellular localizations of torsin A and ΔE-

torsin A represented a cell type-specific phenomenon, the distributions of these proteins were examined in transfected COS-7 cells. As was observed in the HEK-293 cells, COS-expressed torsin A was found on a diffuse intracellular network of membranes that also displayed significant BiP immunoreactivity (Fig. 2C). However, ΔE-torsin A was again found as a constituent of large intracellular structures that lacked evidence of BiP co-localization (Fig. 2D). Intriguingly, the ΔE-torsin A-immunoreactive structures sometimes appeared as hollow circles (Fig. 2E), perhaps suggesting that this intracellular structure represented a tubulovesicular membrane compartment (see “Discussion”). Collectively, these results indicate that torsin A and ΔE-torsin A display distinct distributions in mammalian cells, with the former protein exhibiting properties
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consistent with those of a resident of the ER, and the latter protein localizing to a distinct intracellular membrane structure.

Stable Transfection of HEK-293 Cells with Torsin A and ΔE-Torsin A cDNAs—In order to confirm that the cellular and biochemical phenotypes observed for the torsin proteins were not due to artifacts associated with transient transfection, we generated lines of stably transfected HEK-293 cells expressing either form of the torsin protein. Immunofluorescence analysis of multiple stably transfected clones identified numerous torsin A and ΔE-torsin A-transfected lines that expressed significant levels of torsin protein (depending on the clone, 50–100% of the cell population expressed torsin protein). Notably, the different subcellular localizations of the wild type and ΔE-torsin A proteins observed originally in transiently transfected cells were preserved in the stably transfected HEK lines (Fig. 3). In these stable lines, the ΔE-torsin A protein was again found to reside in a compartment distinct and apparently nonoverlapping with the torsin A/BiP compartment.

Protease Protection Experiments with Stably Transfected HEK-293 Cells—Although the aforementioned data from subcellular fractionation and immunofluorescence studies supported that both torsin A and ΔE-torsin A were localized to intracellular membrane compartments, further biochemical evidence was sought to address whether these proteins were cytosolically or lumenally oriented in their respective topologies. Toward this end, we evaluated the protease sensitivities of the two proteins in the absence and presence of nonionic detergents. Two different proteases (trypsin and α-chymotrypsin) and detergents (Triton X-100 and digitonin) were utilized in their various permutations for this study, as previous reports have called attention to the potential artifacts associated with single detergent-protease analyses (e.g. the unveiling of a cryptic protease-sensitive site in a cytosolic protein by a particular detergent; Refs. 22 and 23). Both torsin A (Fig. 4A) and ΔE-torsin A (Fig. 4B) were sensitive to either trypsin or α-chymotrypsin digestion in the presence of either Triton X-100 or digitonin (Fig. 4, A and B, lower panels) but not in the absence of detergents. The selective protease sensitivity of the ER luminal protein grp94 in the presence of these detergents confirmed the integrity of the microsomal preparations under study (Fig. 4, A and B, upper panels) (24). These data support a lumenal orientation for both torsin A and ΔE-torsin A.

Analysis of the Glycosylation States of the Torsin A and ΔE-Torsin A Proteins—To achieve further support for the luminal orientation of the torsin proteins, their respective glycosylation states were investigated. After incubation in the presence PNGase F, both torsin A and ΔE-torsin A showed increased mobilities on SDS-PAGE (Fig. 5A), consistent with these proteins possessing N-linked carbohydrate modifications. Overexposure of the anti-torsin A Western blot identified that the endogenous torsin A protein in the HEK cells was similarly glycosylated (Fig. 5B). Examination of the primary structure of torsin A identified two potential N-linked glycosylation sites (matching the consensus sequence NX(S/T)), Asn-143 and Asn-158. In order to evaluate whether Asn-143 and/or Asn-158 were glycosylated in torsin A, these residues were individually replaced with glutamine by site-directed mutagenesis. Both Asn to Gln mutants migrated as proteins of intermediate molecular size relative to torsin A and its PNGase F-treated form (Fig.

**FIG. 3.** Immunofluorescence localization of stably transfected torsin A and ΔE-torsin A in HEK-293 cells. A, immunolocalization of torsin A (green) and BiP (red). Co-localization of torsin A and BiP is observed as a yellow signal. B, immunolocalization of ΔE-torsin A (green) and BiP (red). The lack of yellow signal indicates no detectable co-localization between ΔE-torsin A and BiP.

**FIG. 4.** Protease protection experiments with stably transfected torsin A and ΔE-torsin A lines. Microsomes derived from HEK-293 stable lines expressing torsin A (A) and ΔE-torsin A (B) were treated with protease (trypsin (T) or α-chymotrypsin (C)), in the presence or absence of detergent (digitonin (d) or Triton X-100 (t)). All fractions were subjected to SDS-PAGE and Western blotting analysis using either anti-torsin A antibodies (bottom panels) or anti-KDEL antibodies (top panels). Both torsin A and ΔE-torsin A exhibited protease sensitivity exclusively in the presence of detergent, supporting their luminal microsomal orientation.
indicating that both of the NX(S/T) sites of torsin A are glycosylated in vivo. In further support of this notion, subsequent treatment of each Asn to Gln mutant with PNGase F further reduced these proteins to the molecular mass of PNGase F-treated wild type torsin A, supporting that both Asn-143 and Asn-158 are glycosylated in vivo.

Biochemical Characterization of Solubilized Torsin A and ΔE-Torsin A—The identification of ΔE-torsin A as a constituent of large, spheroid intracellular structures initially raised suspicions that the protein might be misfolded and by default accumulating as intracellular aggregates. However, both torsin A and ΔE-torsin A were solubilized to near completion in the presence of 1% Triton X-100 (s, supernatant and p, pellet, from 100,000 x g spin) as judged by SDS-PAGE and Western blot analysis. Both proteins appear to migrate as two oligomeric forms, with peaks of immunoreactivity observed at 5.0 and 7.5 S, respectively. Although these s values are consistent with dimeric and tetrameric forms of the torsins, further biophysical studies are required to assess with confidence the absolute oligomeric states of these proteins. Nonetheless, the respective migration patterns of torsin A and ΔE-torsin A clearly support that these proteins are similar at the level of quaternary structure.

DISCUSSION

A major breakthrough in our understanding of the molecular basis for early-onset torsion dystonia was recently achieved with the linkage of this dominantly inherited disease to a 3-base pair deletion (GAG) in the DYT1 gene (8). Several additional genetic studies have since greatly strengthened the association of this mutation with torsion dystonia (9, 10). The DYT1 gene product, torsin A, is a predicted 338-amino acid protein that displays distant homology to the HSP100/Clp family of ATP-dependent chaperones. The torsion dystonia-associated mutation in DYT1 results in the deletion of a single glutamic acid residue residing near the C terminus of torsin A (ΔE-torsin A).

To characterize comparatively torsin A and ΔE-torsin A, we have examined these proteins in transfected mammalian cells using a variety of biochemical and cell biological techniques. Both torsin A and ΔE-torsin A were found to partition with cell membrane fractions and behave as lumennally oriented glycoproteins. On this note, a comparison of the primary structures
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of the six known torsin family members (two mammalian and four C. elegans proteins) reveals that these proteins share exactly six conserved cysteine residues. In contrast, these cysteines are not present in other Hsp100/Clp proteins, suggesting that these residues may form disulfide bonds important for the tertiary structure of the torsin proteins. To our knowledge, torsin A represents the first identified luminal member of the Hsp100/Clp family, indicating that this class of ATP-binding proteins has evolved to function in both oxidizing and reducing cellular environments. Further biochemical studies of torsin A and ΔE-torsin A determined that both proteins possess N-linked carbohydrate appendages, with each of two consensus NX(T/S) glycosylation sites being modified (Asn-143 and Asn-158). Interestingly, only one of these glycosylation sites is present in the torsin B protein, indicating that at least one difference between the highly homologous torsin A and B proteins (greater than 70% sequence identity) is their respective patterns of glycosylation.

Immunofluorescence studies revealed a major difference in the respective subcellular localizations of torsin A and ΔE-torsin A. In either HEK-293 or COS-7 cells, torsin A displayed a diffuse staining pattern indicative of distribution on intracellular membranes of the endoplasmic reticulum and nuclear envelope. Consistent with this notion, significant overlap was found between torsin A signals and those of the resident ER protein BiP. In contrast, ΔE-torsin A was localized to large, spheroid intracellular structures that lacked BiP immunoreactivity. We were initially concerned that the distribution of ΔE-torsin A might reflect aggregates of misfolded protein. However, the ΔE-torsin A protein was readily solubilized by the nonionic detergent Triton X-100 and displayed a migration pattern on sucrose gradients indistinguishable from that of the wild type torsin A protein. Both torsin A and ΔE-torsin A migrated as diffuse proteins with s values ranging between 4 and 8.5. These molecular sizes indicate that the torsin proteins exist as multiple oligomeric species in solution, with peaks of torsin immunoreactivity occurring at 5 and 7.5 S, respectively. Although it remains to be determined whether these torsin species represent homo- or hetero-oligomers, a dynamic nature for torsin self-assembly would be consistent with the properties displayed by other Hsp100/Clp family members, which often show dramatic ATP-induced shifts in both their homo- and heterotypic interactions in vitro (17, 18).

Considering further the altered subcellular distribution observed for ΔE-torsin A, one potential concern is our reliance on transfected cells in which the torsin proteins have been significantly overexpressed. Whether ΔE-torsin A would display this distinct localization in cells in which the protein was expressed at endogenous levels remains uncertain. At present, our antibody reagents lack sufficient sensitivity to detect by immunofluorescence microscopy the low quantities of endogenous torsin A found in the HEK-293 cells (although we were able to visualize endogenous torsin A by Western analysis; see Fig. 4B). However, the localization of ΔE-torsin A to a distinct subcellular compartment was clearly not cell type-specific, as the protein displayed indistinguishable localizations in both HEK-293 and COS-7 cells. Additionally, the subcellular distributions of both N-linked glycosylation mutants of torsin A were examined, and both proteins displayed localizations equivalent to that of wild type torsin A (data not shown), indicating that the altered distribution of ΔE-torsin A was a special feature of this mutant protein. Finally, considering that the biochemical properties of ΔE-torsin A were indistinguishable from those of the wild type protein (including their respective topologies, glycosylation states, protease sensitivities, detergent solubilities, and oligomerization states), our data clearly argue against ΔE-torsin A being a grossly misfolded protein product. Instead, we hypothesize that the single amino acid deletion found in ΔE-torsin A may subtly alter the conformation of this protein in a manner that drives its relocalization to a distinct subcellular compartment. The precise identity of this ΔE-torsin A-containing cellular structure is presently unknown, and future studies will seek to identify additional proteins that co-localize with ΔE-torsin A.

In summary, the comparative characterization of torsin A and ΔE-torsin A described in this study provides the first insights into the biochemical and cell biological properties of these proteins. When evaluated in the context of the homology of torsin A to members of the Hsp100/Clp family of chaperone proteins, models emerge to describe the potential endogenous function of this protein. For example, the identification of torsin A as a luminaly associated glycoprotein localized to intracellular membranes of the ER and nuclear envelope indicates that this protein may serve as a molecular chaperone assisting in the proper folding of secreted and/or membrane proteins. As for how ΔE-torsin A acts as a dominant disease-inducing protein, several molecular mechanisms seem possible. ΔE-torsin A may 1) sequester torsin A in inactive complexes, thus acting as a dominant negative; 2) behave as a constitutively active torsin A, thus acting as a dominant positive; and/or 3) possess novel functionality distinct from the normal biological roles of torsin A. In any of these models, ΔE-torsin A would likely need to exist as a folded and at least partially functional protein product, and our work to date supports this. Additionally, the mislocalization of ΔE-torsin A to a distinct subcellular compartment could support either a gain of function or dominant negative mode of action, the latter possibility likely depending on the ability of this protein to form hetero-oligomers with wild type torsin A. On this note, initial attempts to evaluate the interactions between ΔE-torsin A and torsin A have been hampered by an inability to N- or C-terminally epitope tag these proteins. Future efforts will focus on epitope tagging torssins internally on the C-terminal side of their predicted signal sequences.

Finally, it remains unclear why a mutation in a protein product of apparently broad tissue distribution would produce a neurobiological disease with relatively low penetrance. Considering that DYT1-associated dystonia lacks the visible neuropathologies associated with other neurodegenerative diseases, one possibility is that a more general cell biological defect underlies torsion dystonia. In this regard, a potential chaperone function for torsin A might be compatible with mutations in this protein inducing a low penetrance, tissue-restricted disease, as defects in other ubiquitously expressed chaperones have been found to produce a variety of specific disease states depending on the genetic and environmental backgrounds in which they occur (25). In such a model, a GAG deletion in the DYT1 gene would increase an individual's susceptibility to a "second hit" brought on by either genetic or environmental variables (26). Perhaps in the presence of this second hit, ΔE-torsin A expression becomes most debilitating in the context of the neuron, a cell type whose special cell biological properties may lend it enhanced susceptibility to an aberrant function of torsin A.

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