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

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Karen Kustedjo, Michael H. Bracey, and Benjamin F. Cravatt‡

From The Skaggs Institute for Chemical Biology and Department of Cell Biology, The Scripps Research Institute, La Jolla, California 92037

Early-onset torsion dystonia is an autosomal dominant hyperkinetic movement disorder that has recently been linked to a 3-base pair deletion in the *DYT1* gene. The *DYT1* 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 DYT1 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 resided 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.

Early-onset torsion dystonia represents the most severe and common form of hereditary dystonia in humans (1). This syndrome is inherited in an autosomal dominant fashion with approximately 30% penetrance (2) and manifests itself as sustained muscle contractions that induce twisting and repetitive movements and/or abnormal posture. Symptoms usually begin in the arms or legs at a mean age of 12 years, exhibited as twisting contractions that spread to other limbs within 5 years (3, 4). Torsion dystonia, also known as primary or idiopathic dystonia, is distinguished from other forms of dystonia by a lack of apparent additional neurological defects, with no loss of consciousness, intelligence, or perception being observed. No visible organic lesions can be ascribed as a causative agent for torsion dystonia, and there is an absence of any distinct neuropathology (5, 6). However, recent studies using positron emission tomography have identified *DYT1* dystonia-associated alterations in regional brain metabolic patterns, even in patients without clinical manifestations of the disease (7).

The gene responsible for early-onset torsion dystonia (*DYT1*) has recently been mapped to the human chromosome locus 9q34 and found by positional cloning to encode a putative ATP-binding protein, torsin A (8). The causative mutation in torsion dystonia has been identified as a 3-base pair deletion (GAG) in the coding sequence of *DYT1*, resulting in the deletion of one of a pair of glutamic acid residues near the C terminus of torsin A (glutamates 302 and 303; ΔE-torsin A). The torsion dystonia-associated GAG deletion in *DYT1* seems to have occurred several independent times throughout history, appearing in both Ashkenazi Jewish and multiple non-Jewish populations (9, 10). Homology searches with the torsin A protein sequence reveal that the protein possesses a putative ATP-binding domain and a potential N-terminal signal sequence. Torsin A also shares over 70% identity with a second human gene product termed torsin B, whose corresponding gene, *DYT2*, is found adjacent to *DYT1* on chromosome 9 (8). Transcripts encoding torsin A and B display broad tissue distributions (8), suggesting that this protein family is ubiquitously expressed.

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

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‡ To whom correspondence should be addressed: The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-784-8633; Fax: 858-784-2345; E-mail: cravatt@scripps.edu.

in a putative protein of unknown structure or function motivated us to investigate the biochemical and cell 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^{32}-P]CTP (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 *Bsp*EI site). The complete torsin A cDNA was subcloned into the pcDNA3 and pFLAG eukaryotic expression vectors, and both strands of the cDNA were sequenced completely. A cDNA encoding the ΔE-torsin A protein was generated by site-directed mutagenesis using the Quickchange procedure (Stratagene) (primer 1, 5′-GCAGAGTGGCTGAGATGACATTTTTCCC-3′; primer 2, 5′-GGGAAAAATGTCATCTCAGCCACTCTGC-3′). The ΔE-torsin A cDNA was sequenced completely and contained only the desired 3-base pair deletion.

Generation of Anti-torsin A Antibodies—The torsin A cDNA was subcloned into the TrcHisC 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-PAGE[1] and used for 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).

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 the torsin A or ΔE-torsin A cDNA in the eukaryotic expression vector pcDNA3 as described previously (19). Cells were harvested by trypsinization. After detachment, the cells were pelleted, washed twice with 1 ml of phosphate-buffered saline (PBS), and resuspended in buffer A (10 mM Hepes, pH 7.4, with 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 250 mM sucrose) containing a protease inhibitor mixture (Roche Molecular Biochemicals). Cells were lysed by passage through a 23-gauge needle 20 times to generate total cell extracts (further processed as described below).

Generation of Stably Transfected Torsin A and ΔE-Torsin A Cell Lines—Stably transfected cell lines expressing either torsin A or ΔE-torsin A were obtained from transient transfection of HEK-293 cells with the appropriate expression plasmid (pcDNA3 containing the *neo* antibiotic resistance gene and either the torsin A or ΔE-torsin A cDNA) and subsequent selection of colonies resistant to G418 (Geneticin, Life Technologies, Inc.). Cell lines were checked for torsin protein expression by immunofluorescence and by Western blotting.

Immunofluorescence Studies of Torsin A and ΔE-Torsin A—COS-7 and HEK-293 cells were grown on 11 × 22-mm coverslips to approximately 35% confluency and then transiently transfected as described previously (20). Coverslips used for HEK-293 cells were treated with 0.1 mM poly-D-lysine. Following transfection, the coverslips were washed with PBS and fixed in methanol for 5 min at −20 °C. After fixation, the coverslips were washed with PBS and blocked in 3% bovine serum albumin in PBS for 1 h. The blocking solution was removed, and the coverslips were then incubated with rabbit anti-torsin A antibodies and mouse anti-BiP antibodies (anti-KDEL antibodies, StressGen) in blocking solution for 1 h. After washing with PBS, the coverslips were then incubated with Alexa 488-labeled goat anti-rabbit antibody (Molecular Probes) and Alexa 568-labeled goat anti-mouse antibody (Molecular Probes) in blocking solution for 1 h. The coverslips were washed with PBS to remove excess goat antibody, and the coverslips were mounted on microscope slides with SloFade (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 supernatant and pellet from the second spin were considered the cytosolic and microsomal fractions, respectively. The 100,000 × *g* pellet was reconstituted in 200 μl of buffer A with protease inhibitors by passing through a 23-gauge needle 10 times. The protein concentration for each fraction was determined (D_c protein assay kit, Bio-Rad), and 50 μg of protein were analyzed by SDS-PAGE and Western blotting procedures. All SDS-PAGE protein samples were incubated with an equal volume of 2× loading buffer (125 mM Tris (pH 6.75), 20% glycerol, 4% SDS, 10% β-mercaptoethanol, and 0.005% bromphenol blue) and heated for 5 min at 90 °C prior to SDS-PAGE analysis.

Protease Protection Experiments with Stably Transfected HEK-293 Cell Lines—Confluent plates (250 mm) were harvested by trypsinization. After detachment, cells were pelleted, washed once with 1 ml of PBS, and resuspended in buffer A (800 μl per plate). Cells were lysed by passage through a 23-gauge needle 20 times. The cell extract was then spun at 1,000 × *g* for 5 min, and the supernatant removed and re-centrifuged at 15,000 × 10^4 × *g* for 10 min. The resulting pellet was gently resuspended in 260 μl of buffer C (10 mM Hepes, pH 7.4, with 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 250 mM sucrose, 0.1 M NaCl) and incubated with shaking at 4 °C for 15 min. The resuspended microsomes (67.5 μl) were treated with 7.5 μl of either buffer C, 10% digitonin (Wako Chemical) in buffer C, or 10% Triton X-100 in buffer C. These solutions were incubated at 4 °C for 1 h. Each 75-μl sample was then split in thirds, and one-third of the sample was treated with 1 μl of either buffer C, trypsin (Calbiochem, 0.8 μg; ~50 μg of trypsin per mg of microsomal protein) in buffer C, or α-chymotrypsin (Sigma, 1.6 μg; ~100 μg of chymotrypsin per mg of microsomal protein) in buffer C. The reactions were incubated at 30 °C for 15 min. The reactions were stopped by the addition of trypsin/chymotrypsin inhibitor (Sigma, 0.2 μg, Bowman-Birk inhibitor) and SDS-PAGE loading buffer. Fractions were analyzed by SDS-PAGE and Western blotting procedures.

Glycosylation Studies of Torsin A and ΔE-Torsin A—HEK-293 microsomes (50 μl) were centrifuged at 100,000 × *g,* and the pellet was resuspended in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol) with a 23-gauge needle. The fractions were denatured at 100 °C for 15 min, treated with PNGase F according to manufacturer's specifications (New England Biolabs), and then analyzed by SDS-PAGE and Western blotting techniques. cDNAs encoding the glycosylation mutants, N143Q and N158Q, were generated by site-directed mutagenesis using the Quickchange procedure (Stratagene).

Sucrose Gradient Ultracentrifugation of Torsin A and ΔE-Torsin A—HEK-293 microsomes derived from mock, torsin A, and ΔE-torsin A transiently transfected HEK-293 cells were treated with 1% Triton X-100 for 30 min and then centrifuged at 100,000 × *g* for 1 h. The supernatants (150 μl) were applied to 3–12% linear sucrose gradients (12 ml; 10 mM Hepes (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 0.1% Triton X-100). The sucrose gradients were then centrifuged at 39,000 rpm at 4 °C for 17 h in an SW40 Ti rotor (Beckman). Fractions were removed from the top of the gradient in 1-ml aliquots and precipitated with trichloroacetic acid using bovine serum albumin (50 μg) as an aid for precipitation. All fractions were analyzed by SDS-PAGE and Western blotting.

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 tran-

[1] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PNGase, peptide *N*-glycosidase; ER, endoplasmic reticulum.

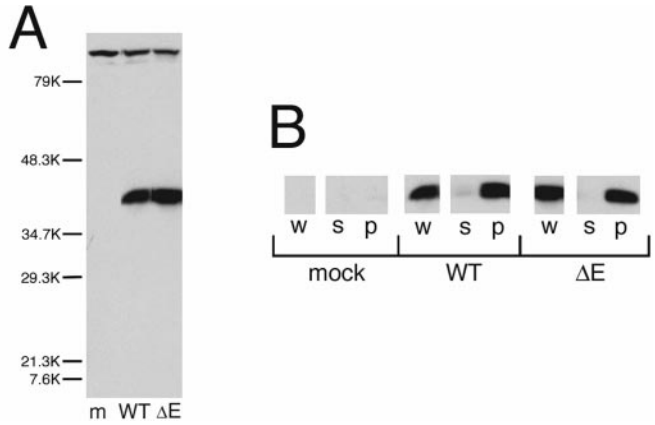

FIG. 1. **Evaluation of anti-torsin A antibodies and subcellular distribution of torsin proteins.** *A*, whole cell homogenates prepared from HEK-293 cells transiently transfected with empty pcDNA3 (*m*, mock), torsin A in pcDNA3 (*WT*), and ΔE-torsin A in pcDNA3 (Δ*E*) were analyzed by SDS-PAGE and Western blotting using rabbit polyclonal antibodies raised against a bacterially expressed His-tagged torsin A protein. Immunoreactive bands near the predicted molecular mass of torsin A were observed in both the torsin A and ΔE-torsin A-transfected cells but not in mock-transfected cells. *B*, both torsin A and ΔE-torsin A immunoreactivity associated with particulate fractions upon cellular fractionation; *w,* whole cell homogenate; *s*, supernatant from 100,000 × *g* spin; *p*, pellet from 100,000 × *g* spin.

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. 1*A*). 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. 1*B*). 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. 2*A*). 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. 2*B*). In order to evaluate whether the dramatically different subcellular localizations of torsin A and ΔE-

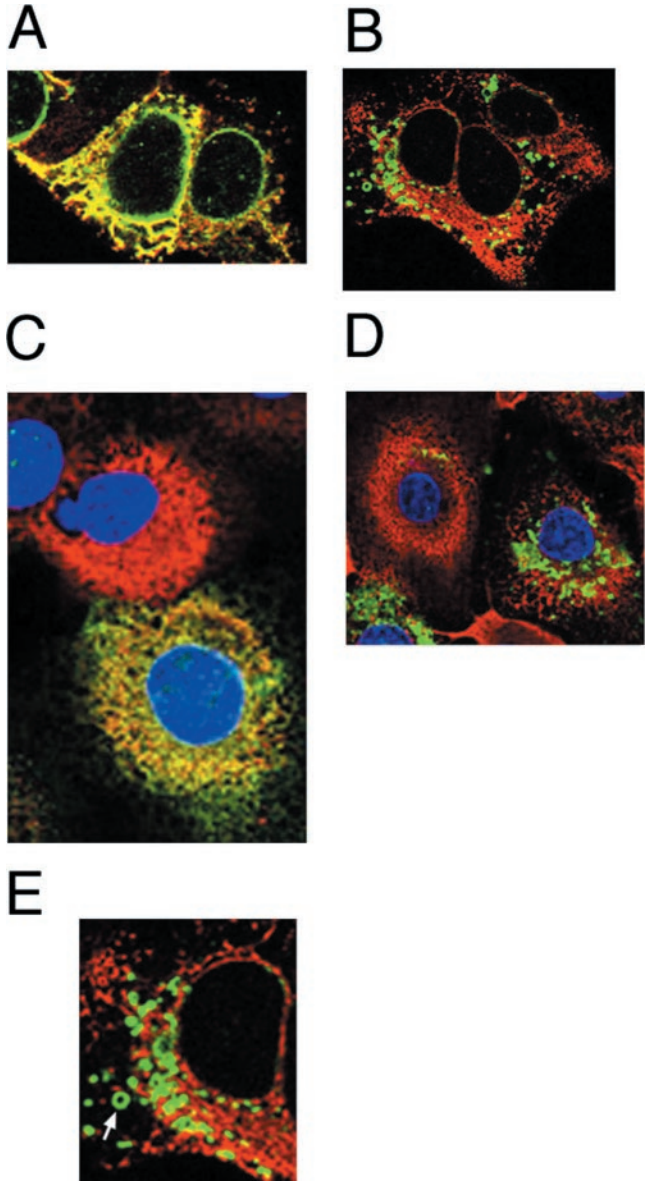

FIG. 2. **Immunofluorescence localization of transfected torsin A and ΔE-torsin A in mammalian cells.** *A* and *C*, immunolocalization of torsin A (*green*) and the ER-resident protein BiP (*red*) in HEK-293 (*A*) and COS-7 cells (*C*). Co-localization between torsin A and BiP is observed as a *yellow* signal. Weakly torsin A-transfected (*A*, *right cell*) or untransfected cells (*C*, *upper cell*) are also observed in these fields. *B* and *D*, immunolocalization of ΔE-torsin A (*green*) and BiP (*red*) in HEK-293 (*B*) and COS-7 cells (*D*). The lack of yellow signal indicates no detectable co-localization between ΔE-torsin A and BiP in these cells. *E*, *arrow* highlights a representative ΔE-torsin A-immunoreactive structure with an apparently hollow interior lacking ΔE-torsin A signal.

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. 2*C*). However, ΔE-torsin A was again found as a constituent of large intracellular structures that lacked evidence of BiP co-localization (Fig. 2*D*). Intriguingly, the ΔE-torsin A-immunoreactive structures sometimes appeared as hollow circles (Fig. 2*E*), 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

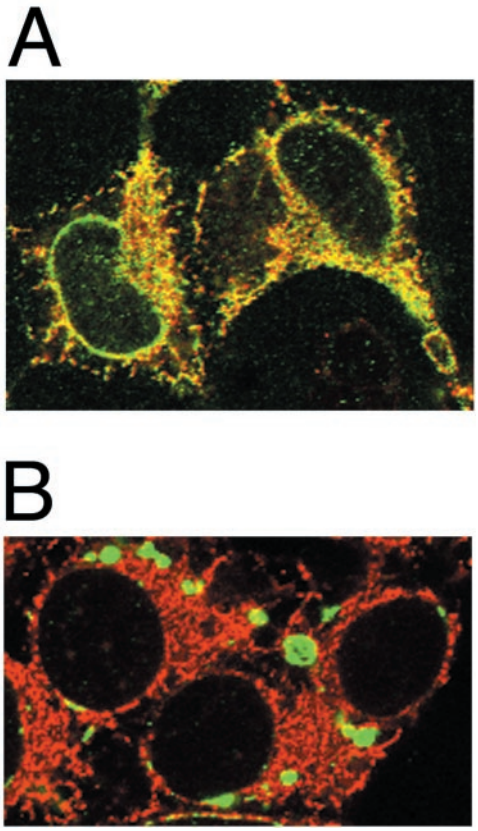

FIG. 3. **Immunofluorescence localization of stably transfected torsin A and ΔE-torsin A in HEK-293 cells.** *A,* immunolocalization of torsin A (*green*) and BiP (*red*). Co-localization of torsin A and BiP is observed as a *yellow* signal. *B,* immunolocalization of ΔE-torsin A (*green*) and BiP (*red*).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

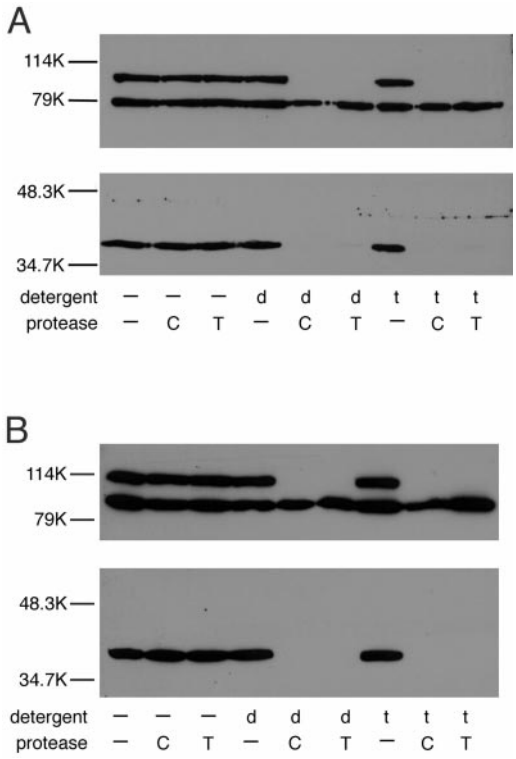

FIG. 4. **Protease protection experiments with stably transfected torsin A and ΔE-torsin A lines.** Microsomes derived from HEK-293 stable lines expressing torsin A (*A*) and ΔE-torsin A (*B*) were treated with protease (trypsin (*T*) or α-chymotrypsin (*C*)), in the presence or absence of detergent (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 lumenal microsomal orientation.

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. 4*A*) and ΔE-torsin A (Fig. 4*B*) 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 lumenal 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 lumenal 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. 5*A*), 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. 5*B*). Examination of the primary structure of torsin A identified two potential *N*-linked glycosylation sites (matching the consensus sequence N*X*(T/S)), 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.

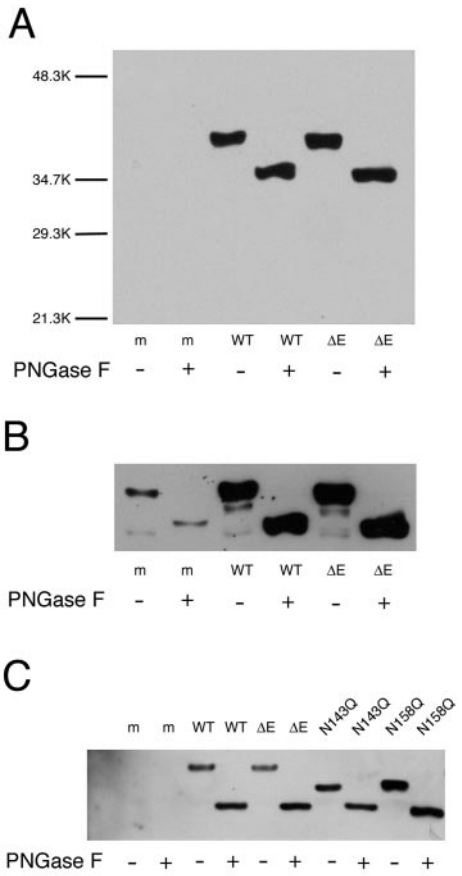

FIG. 5. **Glycosylation studies of torsin A and ΔE-torsin A.** *A*, microsomes derived from mock-, torsin A-, and ΔE-torsin A-transfected HEK-293 were treated with PNGase F and analyzed by SDS-PAGE and Western blotting with anti-torsin A antibodies. The shifts to a lower molecular mass upon treatment with PNGase F are indicative of *N*-linked glycosylation sites on both torsin A and ΔE-torsin A. *B*, longer exposures of the Western blot identified immunoreactive bands in the mock-transfected cells consistent with endogenous torsin A (−*PNGase F*) and its deglycosylated form (+*PNGase F*). *C*, mutation of either predicted *N*-linked glycosylation site in torsin A from asparagine to glutamine (N143Q and N158Q) resulted in a protein of molecular mass between that of the wild type (*WT*) torsin A and its PNGase F-treated form. Treatment of either 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*.

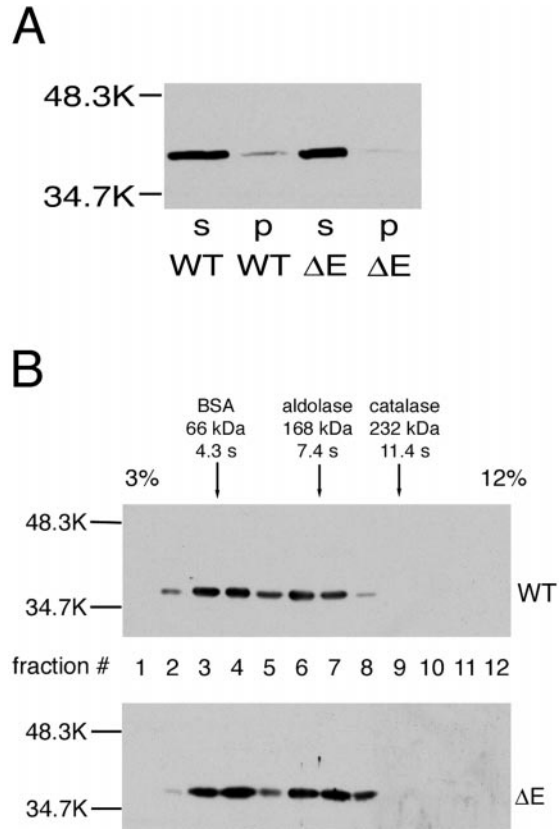

FIG. 6. **Solubilization and sucrose gradient sedimentation analysis of torsin A and ΔE-torsin A.** *A*, both torsin A and ΔE-torsin A were effectively solubilized from HEK-293 microsomes in the presence of 1% Triton X-100 (*s*, supernatant and *p*, pellet, from 100,000 × *g* spin) as judged by SDS-PAGE and Western blot analysis. *B*, application of solubilized torsin A and ΔE-torsin A to a linear 3–12% sucrose gradient revealed that both proteins are of similar solution sizes, migrating with *s* values ranging from 4.0 to 8.5 S, 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.

5*C*), indicating that both of the N*X*(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 reduced the protein to the molecular size of PNGase F-treated wild type torsin A (Fig. 5*C*). Collectively, these results support that both torsin A and ΔE-torsin A are lumenally oriented glycoproteins modified by carbohydrate on both Asn-143 and Asn-158.

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 the nonionic detergent Triton X-100 (Fig. 6*A*), suggesting that neither protein was grossly misfolded. Subsequent sucrose gradient sedimentation analyses revealed that torsin A and ΔE-torsin A were of similar oligomeric sizes in solution (Fig. 6*B*). Both torsin proteins displayed a relatively broad migration pattern on 3–12% sucrose gradients with apparently two peaks of immunoreactivity occurring at around 5 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 lumenally oriented glycoproteins. On this note, a comparison of the primary structures

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 lumenal 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 N*X*(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. 4*B*). 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 lumenally 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 torsins 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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