Recombinant Expression, Purification, and Comparative Characterization of TorsinA and Its Torsion Dystonia-Associated Variant ΔE-TorsinA†

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ABSTRACT: Early-onset torsion dystonia is an autosomal dominant movement disorder that has been linked to the deletion of one of a pair of glutamic acid residues in the protein torsinA ($E_{302/303}$; ΔE-torsinA). In transfected cells, ΔE-torsinA exhibits similar biochemical properties to wild type (WT)-torsinA, but displays a distinct subcellular localization. Primary structural analysis of torsinA suggests that this protein is a membrane-associated member of the AAA family of ATP-binding proteins. However, to date, neither WT- nor ΔE-torsinA has been obtained in sufficient quantity and purity to permit detailed biochemical and biophysical characterization. Here, we report a baculovirus expression system that provides milligram quantities of purified torsin proteins. Recombinant WT- and ΔE-torsinA were found to be membrane-associated glycoproteins that required detergents for solubilization and purification. Analysis of the biophysical properties of WT- and ΔE-torsinA indicated that both proteins were folded monomers in solution that exhibited equivalent denaturation behaviors under thermal and chaotropic (guanidinium chloride) stress. Additionally, both forms of torsinA were found to display ATPase activity with similar k_{cat} and K_m values. Collectively, these data reveal that torsinA is a membrane-associated ATPase and indicate that the $\Delta E_{302/303}$ dystonia-associated mutation in this protein does not cause gross changes in its catalytic or structural properties. These findings are consistent with a disease mechanism in which ΔE-torsinA promotes dystonia through a gain rather than loss of function. The recombinant expression system for torsinA proteins described herein should facilitate further biochemical and structural investigations to test this hypothesis.

Torsion dystonia is a class of movement disorders characterized by abnormal muscle tone induced by cramping, tremors, twisting, and repetitive movements that may lead to permanently distorted posture (*1*). The early-onset form of torsion dystonia is the most severe and frequent form of hereditary dystonias. It is inherited in an autosomal dominant fashion with approximately 30% penetrance (*2*) and is distinguished by a lack of apparent additional neurological defects, with no loss of consciousness, intelligence, or perception observed. Additionally, no visible organic lesions can be ascribed as a causative agent for torsion dystonia, and there is an absence of any distinct neuropathology (*3*, *4*). In 1997, Ozelius and colleagues identified a mutation in the *DYT1* gene on human chromosome locus 9q34 as responsible for early-onset torsion dystonia (*5*). *DYT1* codes for torsinA, a protein with a putative Mg-dependent ATP-binding region and an *N*-terminal 40-aa stretch of hydrophobic residues that may represent a cleavable signal sequence and/or a transmembrane domain (*6*). The torsion dystonia disease is associated with a 3-bp deletion in *DYT1* that results in the deletion of one of a pair of glutamic acid residues near the C-terminus of torsinA ($Glu_{302/303}$; ΔE-torsinA).

Human torsin proteins have clear homologues in rat, mouse, and *Caenorhabditis elegans*, while *Saccharomyces cerevisiae* lacks torsin-like proteins (*5*). Torsin proteins are 25−30% identical to several members of the HSP/Clp subclass of AAA ATPases over a 140-amino acid stretch that contains the protein's putative ATP-binding cassette. Because torsin proteins contain only one predicted ATP-binding domain, they represent Class 2-type HSP100/Clp subfamily members (*7*). HSP100/Clp proteins are typically soluble, cytosolic proteins that bind ATP and/or have ATPase activity (*8*, *9*), often functioning as nucleotide-stabilized homo/hetero-oligomeric complexes that disassemble higher order protein structures and aggregates (*10*), confer increased tolerance to high temperature (*11*), and promote specific proteolysis (*12*). Consistent with a role for torsinA as a chaperone, this protein has been shown to suppress protein aggregation events in *C. elegans* (*13*) and in mammalian cells (*14*). Interestingly, in the latter case, ΔE-torsinA was not functional in suppressing protein aggregation. Nonetheless, how torsinA proteins carry out such cell biological functions at a molecular level remains unknown.

Previous studies have shown that wild type (WT) and ΔE-torsinA display distinct subcellular distributions in transfected cells (*6*, *15*), with WT-torsinA localizing to the endoplasmic reticulum (ER)[1] and the ΔE-torsinA distributing to membrane-

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bound inclusions distinct from the ER. However, whether these differences in cellular distribution are casually related to the torsion dystonia phenotype remains unknown. Indeed, recent studies with a second disease-promoting mutant form of torsinA, which contains a six-amino acid C-terminal deletion (*16*), indicated that this mutant protein does not display an altered subcellular distribution (*17*), suggesting that other biochemical and/or cell biological differences between WT- and mutant torsinA proteins may underlie torsion dystonia. To date, biochemical studies on torsinA proteins have shown that both forms of torsinA are glycosylated, can be proteolyzed and solubilized in the presence detergents, and exhibit similar migration patterns on sucrose gradients (*15*). Collectively, these preliminary findings suggested that both WT- and ΔE-torsinA proteins are membrane-associated, lumenally oriented glycoproteins of similar oligomeric states. To further investigate the biochemical properties of torsinA and its ΔE mutant form, an expression strategy was required that would afford large amounts of purified recombinant proteins. Here we describe a baculovirus expression system for the production and purification of milligram quantities of WT- and ΔE-torsinA and report an initial comparative characterization of these purified proteins.

EXPERIMENTAL SECTION

Generation of TorsinA Expression Constructs. Constructs coding for the various torsinA fusion proteins were generated by either direct subcloning (as for Δ20aa-torsinA by using a natural PstI site located 60 bp from the start codon) or by PCR from the previously cloned torsinA cDNAs (*15*). The following primers were used for Δ40aa-torsinA: sense primer: 5′-GCCTCGAGCGTCTCTACTGCCTCTTCGCC-3′; antisense primer: 5′-GCGAATTCTCAATCATCGTAG-TAATAATC-3′. Δ20aa-torsin constructs containing an N-terminal export sequence (MAIMAPRTLVLLLSGALALTQ-TWAGSHSRG) followed by an internal His_6-tag were generated by PCR (sense primer: 5′-GCCCGCGGACAC-CACCACCACCACCACGTGGAGCCCATCAGCCTGG-3′; antisense primer: 5′-GCGTCGACTCAATCATCGTAG-TAATAATC-3′) and subcloning into the 477 vector [provided by Dr. Luc Teyton (*18*, *19*)]. For insect cell expression, these Δ20aa-torsin constructs were subcloned into the multicloning site of transfer vector pvL1393 (Pharmingen) for baculovirus expression in Sf9 cells. All constructs were confirmed by sequencing. Subcellular localization of torsinA proteins was determined by immunofluorescence analysis of COS7 cells transiently transfected with torsinA constructs subcloned into pcDNA3, performed as described previously (*15*).

Production of Recombinant Baculovirus for Torsin Protein Expression. Recombinant transfer vectors containing the Δ20aa-WT- and ΔE-torsinA inserts in pVL1393 were cotransfected with linearized bacmid DNA (Pharmingen) on a 10 cm^2 layer of monolayer 40% confluent *Spodoptera frugiperda* (Sf9) cells with 1 mL of serum-free insect media (Gibco SF900 II SFM), 30 μL of CELLfectin (Gibco), and 2 μg of transfer vectors. These mixtures were incubated with rocking at room temperature for 4 h, and then serum-free media (4 mL) was added and transfections were incubated for 5 days at 27 °C in a humid chamber. Supernatants were harvested and 100 μL was used to infect a fresh batch of cells. Infection was observed in 3 days, and expression of protein was checked by Western analysis of the lysed cell pellet with anti-torsinA antibodies (*15*). Recombinant baculovirus was purified from single plaques according to manufacturer's directions, and clones were checked by Western blotting. The virus was then amplified once 25-fold, and twice 100-fold. Viral titer was checked by plaque assay and stocks were kept at approximately 1×10^8 plaque-forming units (pfu)/mL.

Optimization of the Expression of WT- and ΔE-TorsinA Proteins in Baculovirus-Infected Sf9 Cells. Cells were infected in suspension culture (Gibco SF900 II SFM with 2 mM L-glutamine and 100 U penicillin/100 μg of streptomycin/0.25 μg of fungizone) at a multiplicity of infection (moi) of 2 and a cell density of 2×10^6 cells/mL. Cells were harvested at 36, 48, 54, and 60 h post-infection in 25 mL aliquots from a 100-mL culture. Cell pellets were isolated by centrifugation (1000*g*, 5 min), and resuspended in 5 mL of 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 cocktail (Roche). Cells were lysed by sonication (30 s) and the cytosolic and membrane fractions were separated by centrifugation (100000*g*, 1 h). Proteins from membrane fractions were solubilized in 5 mL of buffer A + 1% Triton X-100 by rotation at 4 °C for 1 h. Aliquots (20 μL) of each cellular fraction were run on SDS−PAGE (reducing conditions) and analyzed by Western blotting with anti-torsin antibodies. From these studies, it was determined that 54 h represented an optimum infection period for production of torsin proteins.

Purification of WT- and ΔE-TorsinA Proteins from Sf9 Cells. Larger scale preparations of infected cells (500 mL) were pelleted at 1000*g* for 20 min. The cell pellet was washed with 10 mL of phosphate buffered saline (PBS) and pelleted again at 1000*g* for 10 min. The cells were lysed in 30 mL of buffer B (0.1 M Tris-HCl, pH 7.4, 400 mM sucrose) containing a protease inhibitor cocktail (Roche) by dounce homogenization (25×) and probe sonication (1 min) on ice. The resulting cell lysate was subjected to sequential centrifugation: 3000*g* spin (15 min) to pellet nuclei and heavy membranes, from which the supernatant was further subjected to a 100000*g* spin (1 h) to isolate torsin-enriched microsomes. TorsinA proteins were solubilized from cell microsomes by douncing (25×) in 30 mL of buffer B + 2% LDAO (Fluka), probe sonication (1 min) on ice, and rotation at 4 °C for 1 h. Following a 100000*g* spin (1 h), solubilized torsinA protein was treated with 10 mM imidazole and the solution was applied to a 1 mL bed volume of Talon cobalt affinity resin (Clontech). The mixture was incubated at 4 °C with rotation for 1 h, after which the beads were collected in a fritted column, washed with 50 mL of buffer C (50 mM Tris pH 7.4, 150 mM NaCl with detergent, 0.1% LDAO), and eluted in 5 mL of buffer C + 500 mM imidazole. The eluted fractions were concentrated by centrifugal filtration (Amicon Ultra, Millipore) and torsinA protein further purified by gel filtration (Superose 6, AKTA purifier, Amersham Pharmacia Biotech). The protein sample (0.4 mL, 2.5 mg of protein/mL) was loaded onto the gel filtration column

[1] Abbreviations: AAA: ATPases associated with a variety of cellular activities; ATP: adenosine triphosphate; ER: endoplasmic reticulum; GdmCl: guanidine hydrochloride (guanidinium chloride); LDAO: lauryldimethylamine *N*-oxide (*N*,*N*-dimethyldodecylamine *N*-oxide).

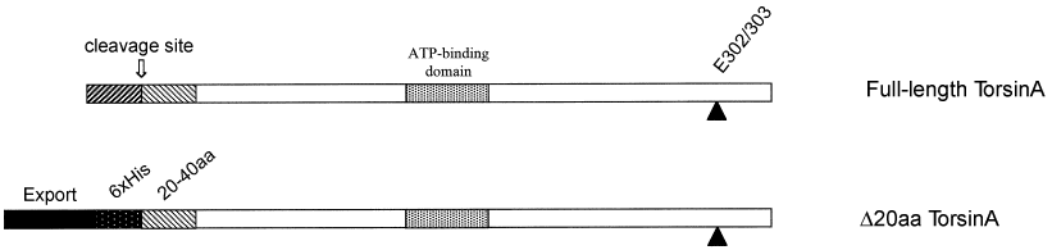

FIGURE 1: A comparison of full-length and Δ20aa-torsinA expression constructs. The predicted Mg-dependent ATP binding domain and the position of the deleted glutamate ($\Delta E_{302/303}$) are indicated.

equilibrated with buffer C at a flow rate of 0.4 min/mL, and the eluted protein was monitored by UV absorbance at 280 nm. Fractions confirmed to contain torsinA protein by staining (Coomassie blue) and Western blotting were pooled together and concentrated by centrifugal filtration. The concentrations of purified torsinA proteins were measured with the D_c Protein Assay kit (Bio-Rad) using a serial dilution of bovine serum albumin as a standard. PNGase F studies were performed according to manufacturer's specifications (New England Biolabs).

UV-Circular Dichroism Spectroscopy of TorsinA Proteins. Protein samples were prepared for circular dichroism (CD) experiments by exchange into buffer D (50 mM Tris pH 7.4, 150 mM NaCl with detergent, 0.01% LDAO) by repeated (3×) concentration and dilution using centrifugal filtration (Amicon Ultra, Millipore). The protein was diluted to 0.1 mg/mL and CD measurements were recorded at 25 °C in a 0.1-cm path length Suprasil quartz cell (Hellma) on an Aviv model 202SF stopped-flow CD spectrometer.

Thermal and Guanidinium Chloride (GdmCl) Denaturation Studies of TorsinA Proteins. Thermal denaturation of torsinA proteins (0.1 mg/mL) was carried out in buffer D and evaluated by monitoring reductions in the far-UV CD signal at 220 nm. The CD signal was recorded from 2 to 98 °C in 2 °C steps, equilibrating proteins at each temperature for 1 min. Denaturation of torsinA proteins as a function of GdmCl concentration was measured in buffer D at pH 7.4. Each protein (0.04 mg/mL) was incubated for 10 min at 25 °C with freshly prepared solutions of GdmCl at concentrations ranging from 0 to 7 M. The tryptophan fluorescence spectrum of each torsinA protein was recorded over the range of 310−410 nm with an ATF 105 Aviv spectrofluorometer with excitation at 295 nm using a 1 × 0.6 cm cuvette. Native torsinA proteins (both WT- and ΔE-) exhibited a fluorescence maximum between 335 and 340 nm, whereas unfolded proteins showed a maximum emission between 359 and 365 nm. The 359/339 nm emission intensity ratio was used to follow the denaturation of each torsinA protein as a function of GdmCl concentration.

Sucrose Gradient Analysis of TorsinA Proteins. Purified WT- and ΔE-torsinA proteins (50 μg in 100 μL) were preincubated at 30 °C for 10 min with ATP (5 mM), ADP (5 mM), or buffer alone in buffer E (50 mM Tris pH 7.4, 150 mM NaCl, 0.01% LDAO, 20 mM $MgCl_2$). These solutions were then applied to 3−12% linear sucrose gradients (12 mL) poured in buffer E containing ATP (5 mM), ADP (5 mM), or buffer alone, respectively. Sucrose gradients were centrifuged at 39 000 rpm at 4 °C for 17 h using an SW 40 Ti rotor (Beckman). Fractions were removed from the top of the gradient in 1 mL aliquots. A sample (25 μL) of each aliquot was analyzed by SDS−PAGE (reducing conditions) and Western blotting with anti-torsin antibodies.

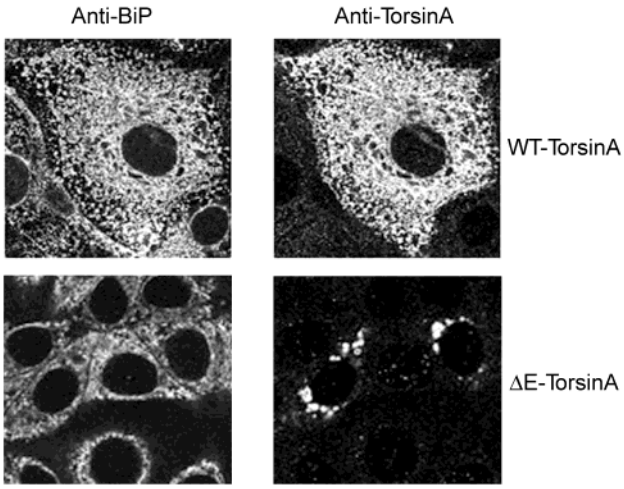

FIGURE 2: Immunofluorescence microscopy of Δ20aa-WT- and ΔE-torsinA expressed in COS7 cells by transient transfection. Left panel, localization of the resident endoplasmic reticulum (ER) protein BiP (anti-BiP antibodies, StressGen); right panel, localization of torsinA proteins [anti-torsin antibodies (*15*)]. As was observed for full-length WT- and ΔE-torsinA (*6*, *15*), Δ20aa-WT- and ΔE-torsinA localized to the ER and to large spheroid intracellular structures exclusive of BiP immunoreactivity, respectively.

ATPase Assays of TorsinA Proteins. Purified WT- and ΔE-torsinA proteins (5 μM in 18 μL) were preincubated at 30 °C in buffer F (50 mM Tris pH 7.4, 150 mM NaCl, 0.01% LDAO, 2 mM $MgCl_2$) for 5 min. Triplicate reactions were initiated with 2 μL of 0.1−10 mM ATP (Fisher) with an added constant amount (3.3 μM) of [α^{32}P]ATP (30 Ci/mmol, Amersham Life Science) in a final volume of 20 μL. At the time points, 1, 5, 10, 30, and 60 min, samples (1 μL) from the incubation mixtures were applied directly onto polyethyleneimine (PEI)-cellulose thin-layer chromatography sheets (Fisher). Substrate and products of the reaction were separated by development of the sheets in 1 M formic acid (Fisher) and 0.5 M LiCl (Aldrich) as previously described (*20*, *21*). The ATP and ADP spots were identified by chromatography with unlabeled standards and formation of [α^{32}P]-ADP was quantified using a phosphorimager (Cyclone Storage Phosphor system; Packard Biosciences). TorsinA proteins exhibited saturation kinetics and K_m and v_{max} were calculated by a nonlinear regression routine (GraphPad Prism version 3.0c for Macintosh, GraphPad Software, San Diego, www.graphpad.com). Background ATP hydrolysis was measured with reactions that contained denatured torsinA proteins, and these values were found to be less than 1% of torsinA-catalyzed rates. Baculovirus containing empty vector was used to mock infect an equal number of cells (2×10^6

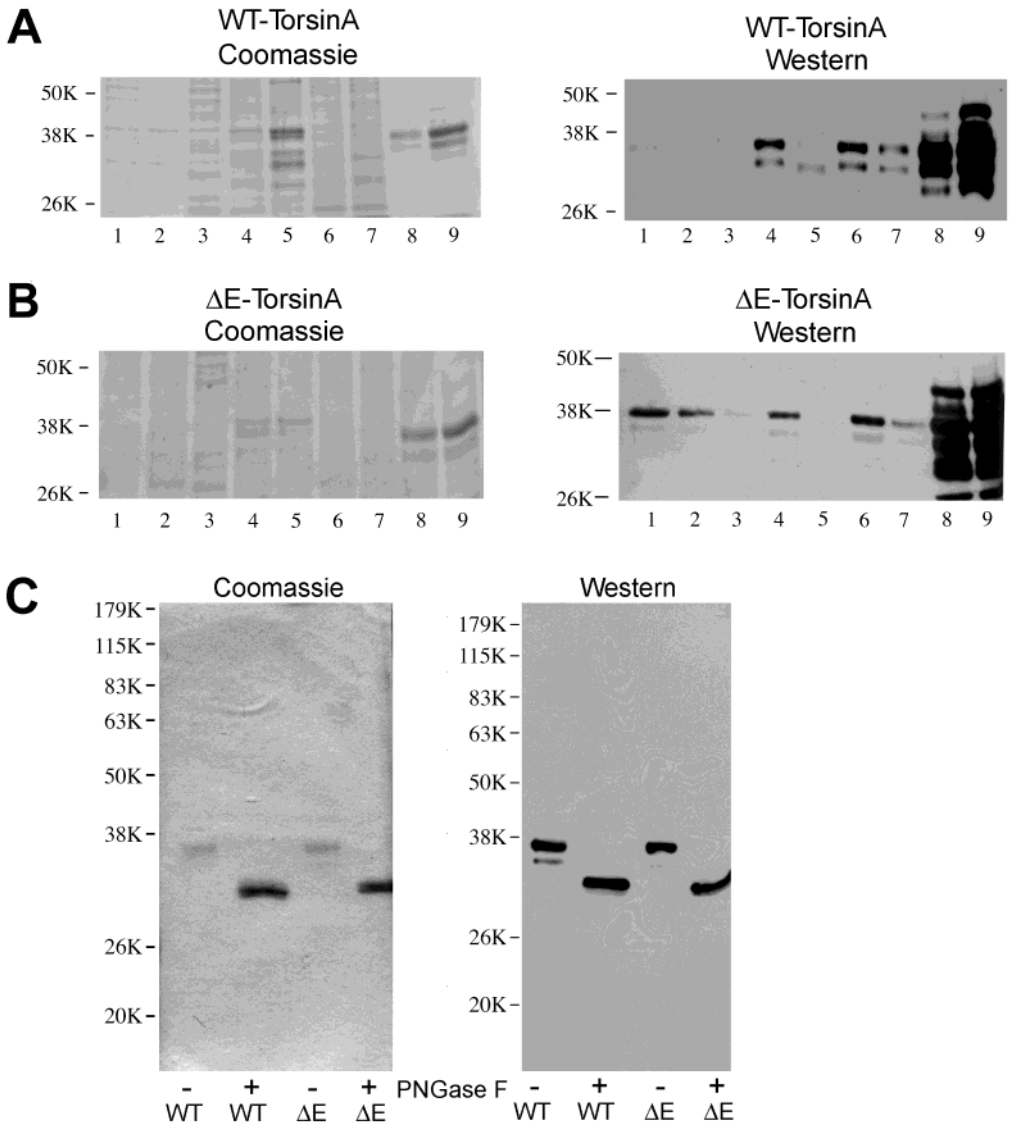

FIGURE 3: Expression and purification of recombinant torsinA. (A) and (B), Lane numbers: (1) whole cell homogenate; (2) pellet from 3000*g* spin; (3) soluble protein fraction; (4) microsome enriched fraction; (5) pellet from 100000*g* spin following solubilization in buffer B with LDAO (2%); (6) supernatant from 100000*g* spin (pre-Talon); (7) flow-through after Talon binding; (8) elution from Talon binding; (9) elution from gel filtration (for representative elution profiles from gel filtration, see Figure 6A). Lanes were loaded with equal concentrations of total protein (15 μg/lane) and analyzed by staining (Coomassie blue) and Western blotting with anti-torsin antibodies. (C) Analysis of purified recombinant torsinA proteins. Equal amounts (2 μg) of purified WT- and ΔE-torsinA were incubated with or without PNGase F, separated by SDS−PAGE, and analyzed by staining (Coomassie blue) and Western blotting with anti-torsinA antibodies. Prior to treatment with PNGaseF, each torsinA protein migrated as a diffuse series of bands ranging from 32 to 40 kDa [also see lanes 8 and 9 of (A) and (B)]; following treatment with PNGase F, these bands converged to single strongly stained protein bands corresponding to each torsinA variant.

cells/mL) as previously infected with torsin-containing virus at an equal titer according to the protocol described earlier. The cells from this mock infection were processed through the same purification procedure as cells infected by torsinA-containing virus. The resulting "mock-purified" sample possessed very low protein concentration and therefore was tested for ATPase activity (5 mM ATP) at equal volumes to the volume of purified torsinA protein utilized in the ATPase assays. Mock-purified samples exhibited less than 10% of the ATPase activity of purified torsinA samples.

RESULTS

Recombinant Expression of TorsinA Proteins in Sf9 cells by Baculovirus Infection. Initial attempts to express torsinA proteins in *Escherichia coli* were unsuccessful, producing only insoluble protein products that could not be solubilized with detergents (data not shown). Therefore, several candidate constructs for eukaryotic expression of torsinA proteins were generated and initially tested by transient transfection in COS7 cells. A C-terminal myc-epitope construct failed to produce detectable levels of torsinA proteins, while an N-terminal FLAG-epitope construct generated torsinA proteins that displayed abnormal subcellular distributions (data not shown). In contrast, a construct in which the first 20 amino acids of torsinA (Δ20aa) were replaced with a cleavable export sequence (*18*, *19*) followed by a His_6-tag (Figure 1) produced high levels of WT- and ΔE-torsinA fusion proteins that exhibited similar subcellular localizations to their full-length counterparts (Figure 2). Similar constructs in which the first 40 amino acids of torsinA proteins (Δ40aa)

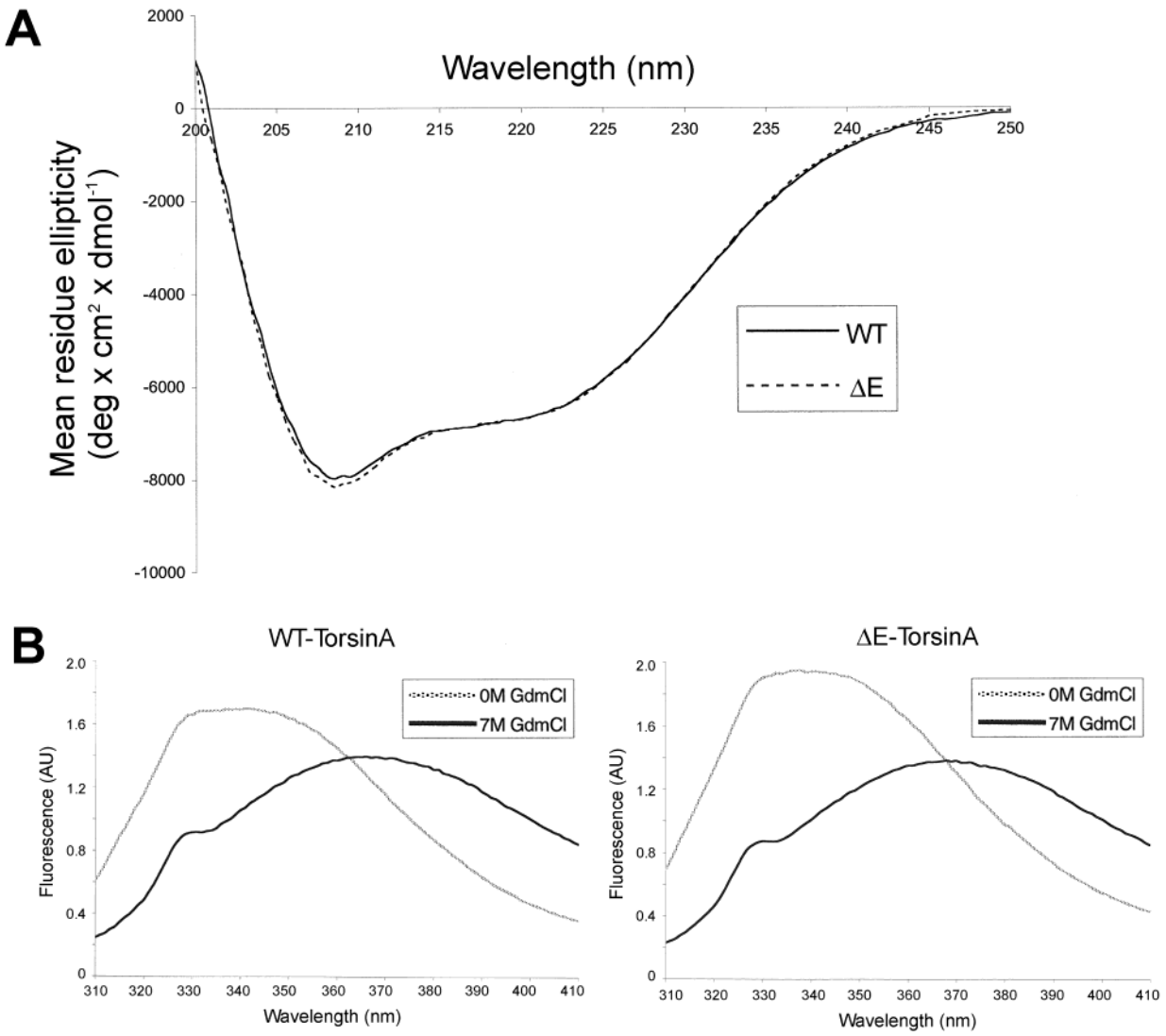

FIGURE 4: Evidence for secondary and tertiary folding of purified torsinA proteins. (A) The far-UV CD spectra of WT-torsinA (solid line) and ΔE-torsinA (dashed line). CD spectra were taken in buffer D with 0.01% LDAO at 0.1 mg of protein/mL at 25 °C. (B) Equilibrium fluorescence data of WT-torsinA (left panel) and ΔE-torsinA (right panel) obtained under native [0 M guanidinium chloride (GdmCl), dotted lines] and denaturing conditions (7 M GdmCl, solid lines) at 25 °C. In the presence of 7 M GdmCl, the tryptophan emission maxima of torsinA proteins shifted from 339 to 368 nm and showed a decrease in fluorescence intensity.

were replaced with the export sequence-His_6 tag yielded low levels of protein with abnormal subcellular distributions (data not shown).

The Δ20aa-WT- and ΔE-torsinA constructs were inserted into the baculovirus transfer vector pVL1393 and these vectors cotransfected in the presence of linearized baculovirus DNA into Sf9 cells to produce recombinant baculovirus constructs, which were purified by plaque assay and amplified to high titer. Western blotting with anti-torsinA antibodies (*15*) detected both WT- and ΔE-torsinA variants in whole cell homogenates of baculovirus-infected cells, with the highest concentration of protein being found in the microsomal fraction (Figure 3A). No torsinA protein was detected in the conditioned media of Sf9 cultures. TorsinA proteins were solubilized by treatment of microsomal fractions with detergent (2% LDAO) and purified sequentially using Talon Metal Affinity (Clonetech) and gel filtration (Superose 6, Amersham Pharmacia Biotech) chromatography (Figure 3A). Attempts to remove the LDAO during the purification process resulted in precipitation of torsinA proteins (data not shown), and therefore at least 0.01% LDAO was maintained in buffers throughout the purification procedure. Although purified WT- and ΔE-torsinA proteins migrated as predominantly single 38-kDa bands by SDS−PAGE, longer exposure times of Western blots uncovered several additional molecular species ranging from 35 to 40 kDa (Figure 3A,B, lanes 8 and 9). Treatment of purified torsinA proteins with PNGase F reduced these multiple species to a single 33-kDa band, indicating that torsinA heterogeneity was due to glycosylation [e.g., Figure 3C, where PNGaseF treatment of WT and ΔE-torsinA converted each of these proteins from a faint, diffuse 37-kDa band (with other glycosylated forms below the detection of staining) to single strong 33-kDa band]. The identities of the deglycosylated WT- and ΔE-torsinA proteins were confirmed by tryptic digestion and mass spectrometry analysis, followed by database searches with the resulting tryptic peptide maps (data not shown). Typical yields of purified WT- and ΔE-torsinA ranged from 0.8 to 1 mg per liter of culture volume.

Relative Structural Stabilities of Recombinant WT- and ΔE-Torsins. Purified WT- and ΔE-torsinA exhibited similar far-UV CD spectra indicative of folded proteins with

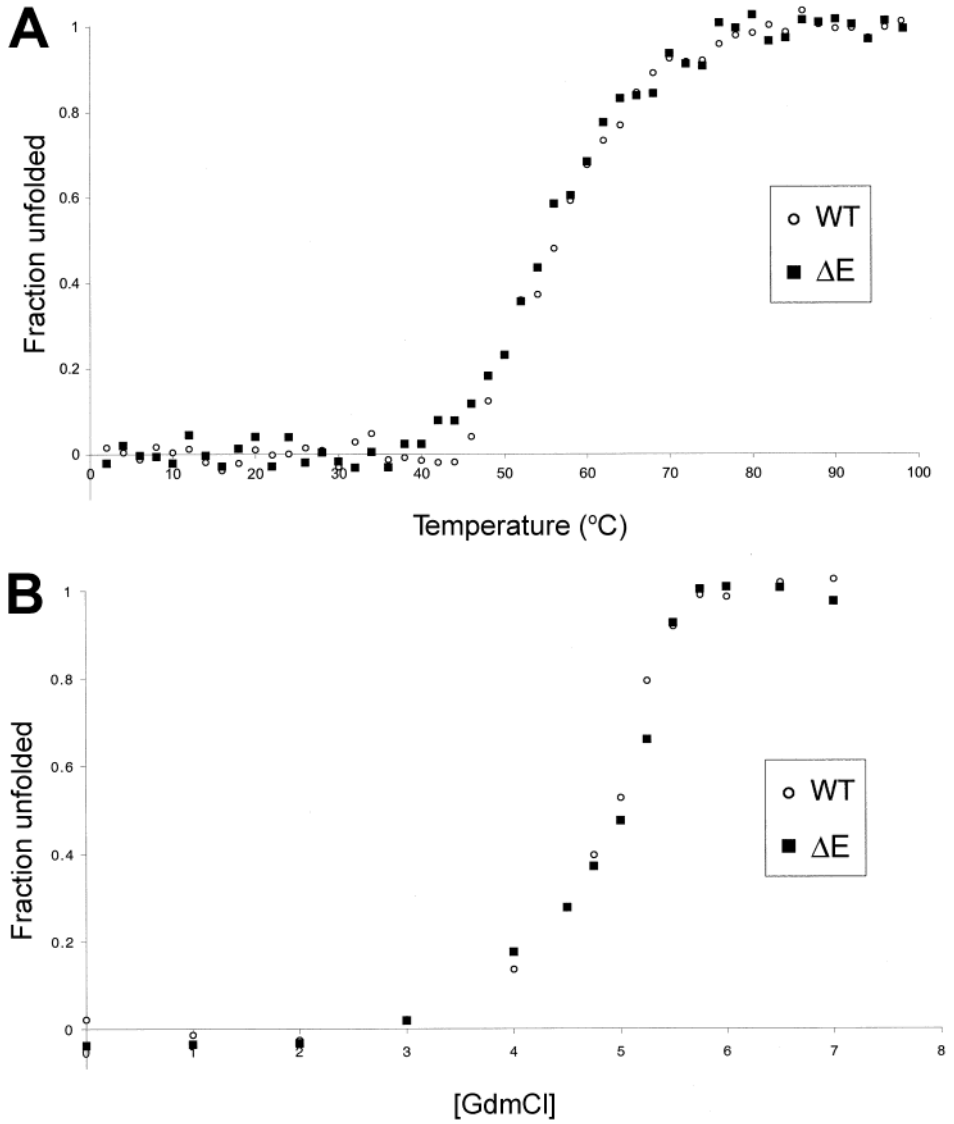

FIGURE 5: (A) Thermal denaturation curves for WT and ΔE-torsinA. Denaturation of torsinA proteins was monitored by CD spectroscopy as a decrease in UV absorbance at 220 nm. (B) GdmCl denaturation curves for WT and ΔE-torsinA. Denaturation of torsinA proteins was measured by tryptophan fluorescence (359 nm/339 nm emission intensity ratio). Data represent average values where $n = 3$/group.

moderate helical content (Figure 4A). Following heating (98 °C, 5 min), both torsinA proteins showed decreases in intensity of the negative CD bands consistent with a loss of secondary structure (data not shown). Similarly, exposure of WT- and ΔE-torsinA proteins to denaturing concentrations of guanidinium chloride (GdmCl; 7.0 M) resulted in a decrease in fluorescence emission intensity and a red shift in emission maximum characteristic of loss of tertiary structure (i.e., exposure of buried tryptophan residues to the polar environment of solvent) (Figure 4B).

The relative structural stabilities of WT and ΔE-torsinA were examined by two methods, thermal and GdmCl denaturation. For thermal denaturation curves, increases in the far-UV CD absorbance of torsin proteins at 220 nm were monitored by CD spectroscopy following exposure to temperatures ranging from 2 to 98 °C (Figure 5A). GdmCl denaturation curves for torsin proteins were determined by monitoring changes in tryptophan fluorescence intensity ratio (359 nm/339 nm) over a concentration range of GdmCl (0−7 M) (Figure 5B). The thermal and GdmCl denaturation curves for WT- and ΔE-torsinA proteins were fit using a linear extrapolation model with a two-state assumption (*22*) by taking into account sloping of the folded and unfolded baselines using fitting software (Kaleidagraph, Synergy Software). Both WT- and ΔE-torsinA displayed similar T_m and ΔG_U values for unfolding (estimated from thermal and GdmCl denaturation curves, respectively) (Table 1). The GdmCl denaturation curves of WT- and ΔE-torsinA also showed equivalent midpoints of unfolding ($c_{1/2}$). Collectively, these studies indicate that recombinant WT- and ΔE-torsinA are folded proteins of comparable global structural stability.

Table 1: Comparison of the Thermal and GdmCl Denaturation Curves of Recombinant WT- and ΔE-TorsinA[a]

	T_{melt} (°C)	ΔG_U (kcal/mol)	$c_{1/2}$ (M)
WT	57.3 ± 0.3	8.90 ± 0.78	5.0 ± 0.1
ΔE	55.1 ± 0.3	8.33 ± 1.08	5.2 ± 0.1

[a] Means ± SD; $N = 3$.

Comparative Characterization of the Oligomerization States of WT- and ΔE-TorsinA Proteins. The oligomeric states of WT- and ΔE-torsinA were studied by gel filtration chromatography and sucrose gradient analysis and data obtained with each method indicated that the torsinA variants

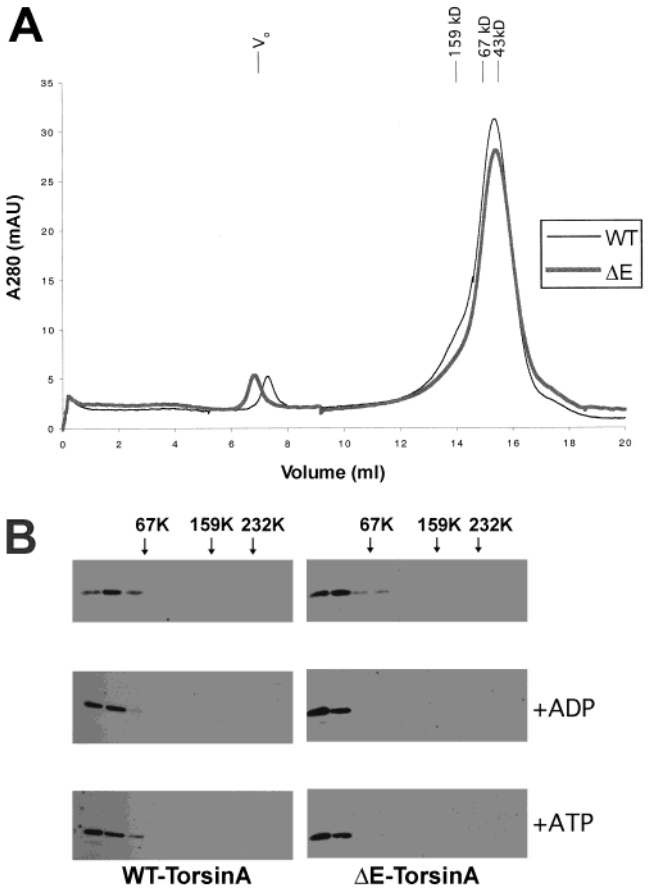

FIGURE 6: Evidence that WT- and ΔE-torsinA are monomeric proteins. (A) Size exclusion chromatography of WT- (thin line) and ΔE-torsinA (thick line). Peak at 7 mL represents the void volume (V_o). Protein standards: aldolase, 159 kDa; albumin, 67 kDa; ovalbumin, 43 kDa. (B) Sucrose gradient analysis of WT- (left column) and ΔE-torsinA (right column), in the absence (top row) and presence of 5 mM ADP (middle row) or 5 mM ATP (bottom row). Protein samples were run on 3−12% linear sucrose gradients in 50 mM Tris pH 7.4, 150 mM NaCl, 0.01% LDAO, 20 mM $MgCl_2$ (± 5 mM adenosine nucleotide) and detected by Western blotting using anti-torsinA antibodies. Protein standards: albumin, 67 kDa; aldolase, 159 kDa; catalase, 232 kDa.

existed primarily as single, monomeric species in solution (Figure 6A and B, respectively). Other members of the AAA family of ATPases have been shown to assemble into oligomeric ring structures in the presence of ATP or nonhydrolyzable ATP analogues (*23*−*25*). Therefore, the effect of adenosine nucleotides on torsinA oligomerization was examined by sucrose gradient analysis. Neither WT- nor ΔE-torsinA exhibited a detectable change in migration on a 3−12% sucrose gradient in the presence of ATP or ADP (Figure 6B), indicating that these nucleotides did not cause a stable shift in the oligomerization of these proteins. Collectively, these data indicate that both WT- and ΔE-torsinA exist primarily as monomers in solution; however, these results do not exclude the possibility that torsinA proteins homooligomerize either transiently or stably under other conditions (e.g., on membranes).

Examination of the ATPase Activity of WT- and ΔE-TorsinA Proteins. We next examined whether the torsinA proteins displayed ATPase activity by following the conversion of [α-^{32}P]-ATP to [α-^{32}P]-ADP using thin-layer chromatography (Figure 7A). Both WT and ΔE-torsinA proteins hydrolyzed ATP, and these activities were abrogated by heat-denaturation of the protein samples. Additionally, protein preparations from mock-purified Sf9 cells, in which equivalent quantities of mock-infected cells (infected with baculovirus derived from empty vector) were carried through the purification process, showed less than 10% of the hydrolysis rates of WT- and ΔE-torsinA proteins, indicating that these activities were due to torsinA proteins rather than potentially contaminating ATPases. Both WT- and ΔE-torsinA displayed saturation kinetics (Figure 7B) and showed similar K_m and V_{max} values for ATP hydrolysis. The V_{max} values for torsinA proteins were slightly lower (2.5−10-fold) than those displayed by other members of the HSP100/Clp ATPase family in the absence of their cognate protein substrates (Table 2) (*26*−*30*).

DISCUSSION

Torsin proteins possess several unusual characteristics that make these proteins a challenge for biochemical analysis. First, in contrast to other AAA family members, which are mostly soluble, cytosolic proteins, torsins are lumenally oriented, membrane-associated glycoproteins with six conserved cysteine residues, indicating that these proteins are subjected to extensive posttranslational modifications and may possess up to three disulfide bonds. These special biochemical features might be anticipated to hinder the development of prokaryotic expression systems for torsin proteins. Consistent with this notion, several attempts to produce recombinant torsinA proteins in *E. coli* failed to yield appreciable quantities of folded protein. In contrast, significant quantities of recombinant WT- and ΔE-torsinA were produced in Sf9 cells using a baculovirus expression system. The first 20 amino acids of each of these proteins were replaced with an N-terminal, cleavable export sequence followed by a His_6-tag to facilitate expression and purification. Like their full-length counterparts, these Δ20aa, tagged-forms of WT and ΔE-torsinA were membrane-associated glycoproteins that exhibited distinct subcellular distributions (Figure 2).

Both WT- and ΔE-torsinA proteins were expressed to high levels in Sf9 cells. Optimal protein expression was dependent on the time of infection, and, after 54 h, cellular levels of torsinA proteins dropped precipitously. TorsinA proteins were enriched in microsomes of Sf9 cells and could be solubilized from this cellular fraction with the detergent LDAO and purified by sequential metal affinity and gel filtration chromatography. Notably, attempts to remove LDAO from buffers during the purification process resulted in the loss of soluble torsinA proteins. This finding, in combination with the complete association of Δ20-torsinA proteins with the particulate fraction of the cell, suggests that these truncated torsinA variants still possess regions that interact with membranes/detergents. It is possible that amino acids 20−40 of torsinA may serve as a membrane-binding domain. Consistent with this notion, a recent study has shown that the first 20 amino acids of the N-termini of WT- and ΔE-torsinA are processed by *Drosophila* S2 cells, resulting in truncated proteins that remain associated with membranes (*31*). Overall, using this Sf9-baculovirus expression system, we consistently obtained ~1 mg of purified torsinA protein per liter of cell culture volume.

Purified, recombinant WT- and ΔE-torsinA proteins behaved as folded, monomeric species in solution as judged

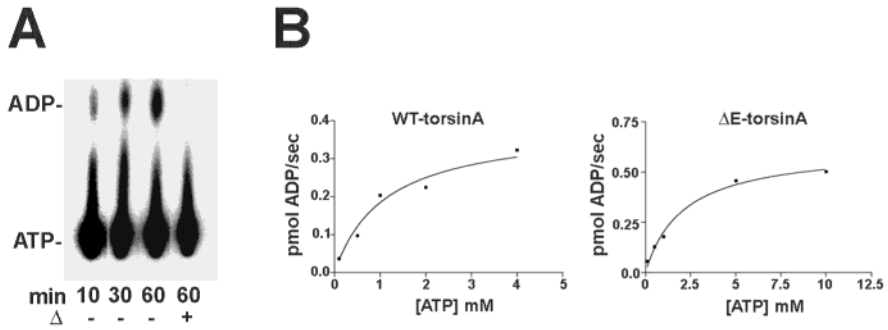

FIGURE 7: Recombinant WT and ΔE-torsinA are active ATPases. (A) Radiolabeled ATPase assay, where conversion of [α^{32}P]-ATP to [α^{32}P]-ADP is followed by thin-layer chromatography and quantified by phosphorimaging. Representative assay shown for ΔE-torsinA. Time is shown in minutes; Δ, heat-denatured control. (B) WT- and ΔE-torsinA show saturation kinetics for conversion of ATP to ADP. Each point represents the average of three independent trials.

Table 2: K_m and V_{max} Values for WT- and ΔE-TorsinA, Assayed at 30°C[a]

	K_m (mM)	V_{max} (nmol min^1 μg^{-1})
WT	1.1 ± 0.2	0.11 ± 0.01
ΔE	2.1 ± 0.4	0.22 ± 0.01
HSP104[b]	5	1
ClpA[c]	0.2	0.7−0.9[f]
ClpB[d]	1.1	0.8[f]
ClpX[e]	0.5	0.5[f]

[a] Data were obtained by nonlinear regression to the one-site binding model using Prism (Graphpad). Reference values for HSP100/Clp were obtained from the cited literature. [b] Schirmer et al., 1998 (*28*). [c] Hwang et al., 1988 (*27*) and Maurizi et al. 1994 (*26*). [d] Woo et al., 1992 (*30*). [e] Wawrzynow et al., 1995 (*29*). [f] Assayed at 37 °C.

by a combination of CD and fluorescence spectroscopy (Figure 4), thermal and chemical denaturation studies (Figure 5), and analyses of estimated solution molecular weight (size exclusion chromatography, sucrose gradients; Figure 6). The $E_{302/303}$ deletion did not appear to markedly affect the global structural stability of torsinA, as WT- and ΔE-torsinA exhibited similar heat and GdmCl denaturation curves (Figures 5 and Table 1). Finally, both WT- and ΔE-torsinA proteins were active as ATPases (Table 2), consistent with their classification by sequence analysis as members of the Clp family of AAA proteins. WT-torsinA exhibited a slightly lower V_{max} and K_m values for ATP compared to ΔE-torsinA; however, the significance of these modest differences in catalytic properties remains unknown.

Many HSP00/Clp family members engage in homo- and/or heterotypic interactions in the presence of ATP or ATP analogues (*8*, *9*, *23*). Oligomerization of HSP100/Clp family members appears to be mediated by the C-terminal regions of these proteins (*32*, *33*), which for torsinA would encompass the $E_{302/303}$ deletion. Thus, while this mutation did not appear to negatively impact the structural stability of the torsinA monomer, it could affect the formation of higher oligomeric states that are important for cellular function. However, neither WT- nor ΔE-torsinA showed evidence of oligomerization in the presence of ATP or ADP, as judged by sucrose gradient analysis (Figure 6B). It is possible that other protein partners are required to support the formation of higher-order complexes of torsinA. Alternatively, the presence of the detergent LDAO may have disrupted protein contacts required for nucleotide-induced oligomerization. Collectively, these findings highlight the need for further studies aimed at elucidating the potential protein−protein and/or protein−lipid interactions of WT- and ΔE-torsinA. Indeed, it remains possible that the $\Delta E_{302/303}$ mutation could promote or inhibit specific homo- or heterotypic protein−protein interactions that went undetected in these studies.

The overall similarity in the biophysical and catalytic properties of WT and ΔE-torsinA, in combination with the dominant expression of the $\Delta E_{302/303}$-induced torsion dystonia phenotype (*5*), are consistent with a hypothesis in which the $\Delta E_{302/303}$ mutation in torsinA promotes torsion dystonia through a gain-of-function mechanism (*6*, *15*, *34*). However, a loss-of-function mechanism cannot be excluded, especially considering recent cell biological studies indicating a selective ability of WT- but not ΔE-torsinA to reduce α-synuclein aggregates in neuroglioma cells (*14*). Regardless, the recombinant expression system described herein for the production of large quantities of WT- and ΔE-torsinA protein should facilitate further investigations of these hypotheses, which may in turn provide a molecular and cellular framework for understanding the basis of early onset torsion dystonia.

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REFERENCES

1. Eidelberg, D., Moeller, J. R., Antonini, A., Kazumata, K., Nakamura, T., Dhawan, V., Spetsieris, P., deLeon, D., Bressman, S. B., and Fahn, S. (1998) *Ann. Neurol. 44*, 303−12.
2. Risch, N. J., Bressman, S. B., deLeon, D., Brin, M. F., Burke, R. E., Greene, P. E., Shale, H., Claus, E. B., Cupples, L. A., and Fahn, S. (1990) *Am. J. Hum. Genet. 46*, 533−8.
3. Fahn, S. (1988) *Adv. Neurol. 50*, 1−8.
4. Hedreen, J. C., Zweig, R. M., DeLong, M. R., Whitehouse, P. J., and Price, D. L. (1988) *Adv. Neurol. 50*, 123−132.
5. Ozelius, L. J., Hewett, J. W., Page, C. E., Bressman, S. B., Kramer, P. L., Shalish, C., de Leon, D., Brin, M. F., Raymond, D., Corey, D. P., Fahn, S., Risch, N. J., Buckler, A. J., Gusella, J. F., and Breakefield, X. O. (1997) *Nat. Genet. 17*, 40−8.
6. Hewett, J., Gonzalez-Agosti, C., Slater, D., Ziefer, P., Li, S., Bergeron, D., Jacoby, D. J., Ozelius, L. J., Ramesh, V., and Breakefield, X. O. (2000) *Hum. Mol. Genet. 9*, 1403−13.
7. Schirmer, E. C., Glover, J. R., Singer, M. A., and Lindquist, S. (1996) *Trends Biochem. Sci. 21*, 289−96.
8. Parsell, D. A., Kowal, A. S., and Lindquist, S. (1994) *J. Biol. Chem. 269*, 4480−7.
9. Maurizi, M. R., Singh, S. K., Thompson, M. W., Kessel, M., and Ginsburg, A. (1998) *Biochemistry 37*, 7778−86.

10. Parsell, D. A., Kowal, A. S., Singer, M. A., and Lindquist, S. (1994) *Nature 372*, 475−8.
11. Sanchez, Y., and Lindquist, S. L. (1990) *Science 248*, 1112−5.
12. Hoskins, J. R., Pak, M., Maurizi, M. R., and Wickner, S. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 12135−40.
13. Caldwell, G. A., Cao, S., Sexton, E. G., Gelwix, C. C., Bevel, J. P., and Caldwell, K. A. (2003) *Hum. Mol. Gen. 12*, 307−19.
14. McLean, P. J., Kawamata, H., Shariff, S., Hewett, J., Sharma, N., Ueda, K., Breakefield, X. O., and Hyman, B. T. (2002) *J. Neurochem. 83*, 846−54.
15. Kustedjo, K., Bracey, M. H., and Cravatt, B. F. (2000) *J. Biol. Chem. 275*, 27933−9.
16. O'Farrell, C., Hernandez, D. G., Evey, C., Singleton, A. B., and Cookson, M. R. (2002) *Neurosci. Lett*. 327, 75−8.
17. Leung, J. C., et al. (2001) *Neurogenetics 3*, 133−43.
18. Matsumura, M., Saito, Y., Jackson, M. R., Song, E. S., and Peterson, P. A. (1992) *J. Biol. Chem. 267*, 23589−95.
19. Jackson, M. R., Song, E. S., Yang, Y., and Peterson, P. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 12117−21.
20. McClellan, A. J., Endres, J. B., Vogel, J. P., Palazzi, D., Rose, M. D., and Brodsky, J. L. (1998) *Mol. Biol. Cell 9*, 3533−45.
21. Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C., and Zylicz, M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2874−8.
22. Santoro, M. M., and Bolen, D. W. (1988) *Biochemistry 27*, 8063−8.
23. Vale, R. D. (2000) *J. Cell Biol. 150*, F13−9.
24. Hoskins, J. R., Sharma, S., Sathyanarayana, B. K., and Wickner, S. (2001) *Adv. Protein. Chem. 59*, 413−29.
25. Krzewska, J., Konopa, G., and Liberek, K. (2001) *J. Mol. Biol. 314*, 901−910.
26. Maurizi, M. R., Thompson, M. W., Singh, S. K., and Kim, S. H. (1994) *Methods Enzymol. 244*, 314−31.
27. Hwang, B. J., Woo, K. M., Goldberg, A. L., and Chung, C. H. (1988) *J. Biol. Chem. 263*, 8727−34.
28. Schirmer, E. C., Queitsch, C., Kowal, A. S., Parsell, D. A., and Lindquist, S. (1998) *J. Biol. Chem. 273*, 15546−52.
29. Wawrzynow, A., Wojtkowiak, D., Marszalek, J., Banecki, B., Jonsen, M., Graves, B., Georgopoulos, C., and Zylicz, M. (1995) *EMBO J. 14*, 1867−77.
30. Woo, K. M., Kim, K. I., Goldberg, A. L., Ha, D. B., and Chung, C. H. (1992) *J. Biol. Chem. 267*, 20429−34.
31. Liu, Z., Zolkiewska, A., Zolkiewski, M. (2003) *Biochem. J. 374*, 117−122.
32. Mogk, A., Schlieker, C., Strub, C., Rist, W., Weibezahn, J., and Bukau, B. (2003) *J. Biol. Chem. 278*, 17615−24.
33. Bochtler, M., Hartmann, C., Song, H. K., Bourenkov, G. P., Bartunik, H. D., and Huber, R. (2000) *Nature 403*, 800−5.
34. Breakefield, X. O., Kamm, C., and Hanson, P. I. (2001) *Neuron 31*, 9−12.

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