siRNA knock-down of mutant torsinA restores processing through secretory pathway in DYT1 dystonia cells

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Most cases of the dominantly inherited movement disorder, early onset torsion dystonia (DYT1) are caused by a mutant form of torsinA lacking a glutamic acid residue in the C-terminal region (torsinAΔE). TorsinA is an AAA+ protein located predominantly in the lumen of the endoplasmic reticulum (ER) and nuclear envelope apparently involved in membrane structure/movement and processing of proteins through the secretory pathway. A reporter protein Gaussia luciferase (Gluc) shows a reduced rate of secretion in primary fibroblasts from DYT1 patients expressing endogenous levels of torsinA and torsinAΔE when compared with control fibroblasts expressing only torsinA. In this study, small interfering RNA (siRNA) oligonucleotides were identified, which downregulate the levels of torsinA or torsinAΔE mRNA and protein by over 65% following transfection. Transfection of siRNA for torsinA message in control fibroblasts expressing Gluc reduced levels of luciferase secretion compared with the same cells non-transfected or transfected with a non-specific siRNA. Transfection of siRNA selectively inhibiting torsinAΔE message in DYT fibroblasts increased luciferase secretion when compared with cells non-transfected or transfected with a non-specific siRNA. Further, transduction of DYT1 cells with a lentivirus vector expressing torsinA, but not torsinB, also increased secretion. These studies are consistent with a role for torsinA as an ER chaperone affecting processing of proteins through the secretory pathway and indicate that torsinAΔE acts to inhibit this torsinA activity. The ability of allele-specific siRNA for torsinAΔE to normalize secretory function in DYT1 patient cells supports its potential role as a therapeutic agent in early onset torsion dystonia.

INTRODUCTION

Early onset torsion dystonia (DYT1) is a dominantly inherited movement disorder characterized by sustained, involuntary muscle contractions and abnormal posturing (1). Most cases are caused by a specific deletional mutation (ΔGAG) in the DYT1 (TOR1A) gene encoding torsinA, which results in loss of a glutamic acid residue in the C-terminal region (2). TorsinA is a member of the superfamily of ATPases associated with a variety of activities (AAA+), a group of chaperone proteins involved in processing, degradation, movement and dynamic associations of other proteins (3,4). AAA+ proteins typically form homeric six member ring structures which bind substrate in the ATP bound state. The normal function of torsinA has not yet been delineated and different studies implicate various potential functions in mammalian cells, including the architecture of the nuclear envelope (NE) (5,6), neurite extension (7,8), cell adhesion (7), processing of proteins through the secretory pathway (9–11) and protection of cells from toxic insults and abnormal proteins (12–17). Many of these functions may involve protein interactions in the endoplasmic reticulum (ER). Further, a number of

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studies indicate that torsinΔE may act by inhibiting the normal function of torsinA (reviewed in 18). The presence of a specific mutation, a GAG deletion in the coding region of the DYT1 gene encoding torsinA (2), provides the opportunity for development of rational therapy for this disease using allele-specific silencing of the torsinΔE mRNA.

In the present study, torsinA function was monitored by assessing the status of the secretory pathway. Several studies support a function for torsinA in processing of proteins through the secretory pathway. Overexpression of torsinA in cultured cells inhibited processing of polytopic plasma membrane proteins, such as the dopamine transporter (DAT) (9), and overexpression of a torsinA homolog in nematodes decreased expression of a DAT–GFP fusion construct (17). Overexpression of torsinΔE also caused entrapment of the vesicular monoamine transporter in membranous inclusions (10) formed by overexpression of torsinΔE (19,20). In recent studies, we have shown that fibroblasts from DYT1 patients infected with a lentivirus vector encoding the secretory reporter protein, Gluc, had a reduced rate of secretion of luciferase into the medium when compared with control human fibroblasts (11).

RNA interference (RNAi) has proven to be an effective means to silence gene expression by catalyzing the degradation of mRNAs (21–23). RNAi can be mediated by chemically modified small interfering RNA (siRNA) oligonucleotides (typically about 22 bp) or by short hairpin (sh) RNAs delivered via viral vectors. Gonzalez-Alegre et al. (24) initially demonstrated allele-specific silencing of the torsinΔE mutant (mt) and torsinA wild-type (wt) mRNAs in cultured cells by co-transfection of specific siRNAs using expression cassettes for torsinA-GFP and torsinΔE-GFP fusion proteins. In subsequent studies, this group demonstrated allele-specific silencing using a lentivirus vector expressing shRNAs, including suppression of endogenous torsinA in mammalian neurons (25). Kock et al. (26) demonstrated that selective shRNA silencing of torsinA expression using a lentivirus vector could block the formation of inclusion bodies generated by overexpression of this mt protein in primary neurons.

In the present study, we evaluated the ability of siRNA to selectively downregulate endogenous levels of torsinA and torsinΔE in cultured fibroblasts from DYT1 patients and controls and the consequent effects on secretion of Gluc from cells. Inhibition of torsinA expression in control cells led to a decrease in Gluc secretion, whereas inhibition of torsinΔE or overexpression of torsinA normalized secretion in patient cells.

RESULTS

Measuring levels of torsinA and torsinΔE mRNAs

In order to quantitate the levels of torsinA and torsinΔE messages independently, we took advantage of the predicted difference in melting temperature conferred by the GAG deletion using the method of rapid cycle real-time PCR (27). Initially, PCR amplification was carried out across a 320 bp region using cDNA clones for human torsinA and torsinΔE so as to span the ΔGAG site (Fig. 1A). The PCR products were then hybridized to adjacent probes, an ‘anchor’ probe, WT Anc (21 bp) labeled at the 5’ end with LCRRed 640 and modified at the 3’ end by phosphorylation to block extension. The other ‘mutation detection’ probe, WT Sen (22 bp), was labeled at the 3’ end with fluorescein, and fluorescence emission conferred by energy transfer between adjacent probes was monitored over a melting curve. An increase in fluorescence conferred by melting of the probes occurred at 64.8°C for the wt cDNA sequence and at 53.7°C for the mt cDNA sequence (Fig. 1B and C, respectively). When RNA was extracted from control fibroblasts, RT–PCR of this fragment yielded a single peak with the appropriate melting temperature, whereas RNA from DYT1 fibroblasts gave two melting peaks corresponding to the torsinA and torsinΔE messages (Fig. 1D). In fibroblasts from two DYT1 affected individuals, the levels of mt and wt mRNA were roughly equivalent (data not shown). Western blot analysis of fibroblasts also yielded equivalent amounts of immunoreactive torsinA protein relative to α-tubulin in control and DYT1 lines (11).

SiRNAs targeting torsinΔE silence mt torsinA mRNA selectively and reduce protein levels

In order to inhibit the expression of torsinA and torsinΔE selectively, a series of eight siRNA oligonucleotides were tested for knock-down of wt and mt torsinA mRNAs. siRNAs (100 nM) were transfected into control and DYT1 fibroblasts and the levels of torsinA and GAPDH mRNAs were quantitated 48 h later by rapid cycle real-time PCR using a melting curve to distinguish wt and mt mRNAs. Two siRNAs, 1939 and 1952, were able to selectively decrease the levels of mt torsinA message by 80 and 50%, respectively, while having essentially no effect on levels of the wt mRNA in the same cells (Fig. 2A). Similarly, both siRNAs decreased the levels of total torsinA protein by 75 and 65%, respectively (Fig. 2B), consistent with the selective reduction of mt torsinA mRNA with the remaining protein comprising wt torsinA. Two other siRNAs, 1958 and 1963, were found to decrease the levels of torsinA message in control cells by ~80% with no change in levels of the non-targeted GAPDH mRNA (Fig. 3A). Seventy-two hours after transfection with these siRNAs, levels of immunoreactive torsinA protein in cells were evaluated by western blot analysis (Fig. 3B). Quantitative densitometry of bands showed a 70–80% decrease in levels of immunoreactive torsinA normalized to levels of α-tubulin, when compared with non-transfected cells. These same siRNAs (1958 and 1963) were also tested in DYT1 fibroblasts and found to decrease the levels of both mt and wt torsinA RNA (Fig. 4A) with levels of total torsinA protein decreased accordingly (Fig. 4B).

Gluc secretion is controlled by levels of wt and mt torsinA

In order to further evaluate the roles of torsinA and torsinΔE in regulating processing of Gluc through the secretory pathway, levels of Gluc in the medium were measured with and without transfection of Gluc-lentivirus-infected control and DYT1 fibroblasts with siRNAs selectively targeting torsinΔE or siRNAs targeting both wt torsinA and torsinΔE messages (Fig. 5A). A Cy3-labeled inactive siRNA was used as a negative control and to estimate transfection efficiency in both control and DYT1 fibroblasts. The transfection efficiency was high in both cell types (~90%) and the
control Cy3-labeled inactive siRNA did not alter Gluc secretion compared to untreated cells. When torsinA expression was decreased in control fibroblasts by siRNAs 1958 and 1963, levels of Gluc secretion were decreased 51% ($P < 0.01$ compared with non-transfected cells with two-tailed Student’s t-test) and 69% ($P < 0.01$), respectively. As predicted, siRNAs 1939 and 1952 which are selective for the mt message did not reduce Gluc secretion in control cells. In contrast, when mt torsinA levels were decreased selectively in DYT1 patient cells with siRNAs 1939 and 1952, levels of Gluc secretion were increased by 42% ($P < 0.02$) and 61% ($P < 0.003$), respectively, consistent with a role for wt torsinA in secretion. Statistical analysis for all different groups was also compared using ANOVA which gave a $P < 0.0001$.

Based on the siRNA results, it appears that the ratio of torsinA:torsinA$^{DE}$ in cells can regulate the secretion of Gluc. To further test this hypothesis, we infected DYT1 cells first with a lentivirus vector encoding Gluc and then 48 h later with a lentivirus vector expressing torsinA or torsinB which results in 3–4-fold increase in torsinA or torsinB above endogenous levels in fibroblasts (Fig. 5B). The Gluc secretion was assessed over a 24 h period (Fig. 5C). As predicted, overexpression of torsinA enhanced the secretion of Gluc by ~50%. Surprisingly, since torsinB is highly homologous to torsinA (2) and has a similar cellular localization (28), overexpression of torsinB did not increase the Gluc secretion in DYT1 cells. Evaluation of the effect of overexpression of torsinA$^{DE}$ was not performed as this results in ER-derived membrane inclusions (14,29) which are predicted to non-specifically interfere with processing through the secretory pathway.

**DISCUSSION**

Here we describe the studies that support an active role for torsinA in facilitating processing of proteins through the secretory pathway and indicate that torsinA$^{DE}$ interferes with this function. A novel luciferase reporter was used to monitor processing of proteins through the secretory pathway (30), which has previously revealed a reduced rate of secretion in fibroblasts from DYT1 patients compared with controls (11). When siRNA targeting the torsinA message was used to decrease the levels of wt torsinA in primary fibroblasts from control subjects, secretion of the reporter, Gluc, was reduced to levels seen in DYT1 fibroblasts. Previous studies have also shown that mouse embryonic fibroblasts from torsinA knock-out mice (−/−) that lack torsinA have a decreased rate of Gluc secretion when compared with those from wt littermates (+/+) (11). These results suggest that in DYT1 cells reduced activity of wt torsinA caused by mt torsinA decreases the processing of at least some proteins through the secretory pathway. Moreover, transfection of siRNAs that selectively downregulate torsinA$^{DE}$ mRNA, but not wt torsinA mRNA in DYT1 cells, can enhance the secretion of Gluc, supporting a dominant-negative action of mt torsinA on wt torsinA. This increase in secretion in DYT1 cells could also be effected by ectopic expression of torsinA via lentivirus vector transduction. These results highlight the potential of selective inhibition of the mt torsinA message as a therapeutic strategy to normalize DYT1 cell function, and application of siRNA therapeutics selectively targeting torsinA$^{DE}$ for the treatment of torsion dystonia.

The two siRNAs that selectively downregulate torsinA$^{DE}$ differed in their overall activity, with siRNA 1939 suppressing
torsinAΔE mRNA to a greater extent than siRNA 1952. siRNA 1939 has no chemical modifications for stabilization, whereas siRNA 1952 does, with 2'-O-methyl on selected pyrimidines and a phosphorothioate at each 3' end. Different patterns and types of chemical modifications (31) may be explored to increase the activity of siRNA 1952 while maintaining sufficient stability for *in vivo* administration, although chemical modifications may be added to siRNA 1939 to provide increased stability for *in vivo* administration while maintaining activity.

**Opposing roles of mt and wt torsinA in the secretory pathway**

Our findings are compatible with several other studies that have implicated torsinA in processing and movement of proteins through the secretory pathway. Torres *et al.* (9), showed in a cultured human cell line that overexpression of torsinA, but not of torsinAΔE, suppressed the processing of membrane proteins, such as the DAT and other polytopic membrane proteins to the cell surface. The possible role of
torsinA as a chaperone protein was supported by the finding that a mutation in the Walker A ATP-binding site blocked this inhibition, since AAA+ proteins typically bind their substrates in the ATP bound state. Involvement of torsinA in processing active DAT was also supported by studies in nematodes in which co-expression of the nematode homolog of torsinA, TOR-2, with a GFP–DAT fusion protein decreased processing of this protein (17). In contrast, studies by Misbahuddin et al. (10) showed that another membrane protein, vesicular monoamine transporter 2, is entrapped in membranous inclusions generated by overexpression of mt torsinA in human neuroblastoma cells, thereby restricting its ability to proceed through the secretory pathway to synaptic vesicles. In all three of these studies, the torsins were overexpressed and processing through the secretory pathway was evaluated visually in cell lines. In the present study processing was evaluated in primary human fibroblasts expressing endogenous levels of torsinA alone (control) or both torsinA and torsinAΔE (DYT1). In addition, by taking advantage of a sensitive and stable, naturally secreted enzymatic reporter we were able to quantify the extent of processing of Gluc to the active form with release into the extracellular space. Furthermore, by using siRNAs we were able to selectively and rapidly decrease levels of torsinA or torsinAΔE thereby modulating the secretory pathway while minimizing the possible development of compensatory mechanisms. Thus, the present study supports a role for endogenous torsinA in mediating processing of proteins through the secretory pathway in the ER and a role for mt torsinA in inhibiting this function.

**Implications for pathophysiology and treatment of dystonia**

One of the enigmas of torsion dystonia is the apparently healthy physical and mental condition of patients with the exception of a loss of motor control mediated, at least in part, through circuits involving the basal ganglia and globus pallidus (32). This enigma is compounded by the remarkable therapeutic effect of deep brain stimulation (DBS) in the globus pallidus of these patients, suggesting that the deficit can be overcome through electrical

**Figure 3.** siRNA transfected into control fibroblasts decreases levels of wt torsinA message and protein. (A) RT–PCR was used to quantitate levels of torsinA and GAPDH messages in control fibroblasts (HF18) 24 h following transfection of siRNA 1958 and siRNA 1963. (B) Levels of torsinA and α-tubulin in cell lysates were evaluated 72 h after transfection with these siRNAs when compared with non-transfected cells by gel electrophoresis, western blotting and densitometry analysis.
modulation of specific pathways in the brain (33–35). Particular neurons in the brain which are especially dependent on torsinA may be compromised in neurotransmission in DYT1 patients. For example, dopaminergic neurons in the human substantia nigra have high levels of torsinA message (36) and may require torsinA for normal cellular function, including processing of proteins through the secretory pathway and synaptic activity (37). Motor task and imaging studies suggest some alterations in motor learning paradigms, regional brain activity and microstructural features characteristic of both unaffected and affected DYT1 carriers, when compared with controls (38–40). The ability of siRNA selectively targeting mt torsinA to restore a more normal secretory phenotype to DYT1 cells supports the possibility of therapeutic intervention in torsion dystonia patients by delivery of siRNA targeting torsinAΔE to appropriate regions of the brain. The DBS paradigm offers a platform to evaluate this by catheter infusion of siRNAs into DYT1 patient brains through the same apparatus. Periodically, the DBS could be turned off to evaluate recovery of normal movement control mediated by siRNAs and then turned back on if no sustained therapeutic effect is seen.

**Use of RNAi strategies for therapeutic allele-specific inhibition**

A major advantage of an RNAi therapeutic is that selective inhibition of mutant torsinA can readily be achieved in contrast to small molecule drugs or biologics where very small differences between the molecular target of interest and closely related targets are usually not easily distinguished.
Both siRNAs and virally delivered shRNAs can mediate RNAi in normal rodents as well as in animal models of disease. Critical considerations for clinical translation are consistency and predictability of drug levels and ability to temporally regulate drug levels, which represent significant challenges for a gene therapeutic shRNA approach (41), in contrast to an siRNA approach. Multiple examples of successful in vivo delivery of synthetic siRNAs directly to the nervous system have been reported with naked (42–45) and cholesterol-conjugated (46) siRNAs formulated simply in phosphate-buffered saline, as well as with siRNAs formulated with lipids or polymers (47–52), resulting in substantial and rapid target suppression with reduction of pathological profiles in neurons in animal models of neurological diseases. Long-term infusion of siRNAs into the human brain may be feasible, as chronic direct CNS administration of candidate drugs and proteins has been used in clinical trials for Parkinson’s disease (53,54) and brain tumors (55). The proof-of-concept demonstrations of in vivo delivery of siRNAs to neurons in pre-clinical studies, the clinical precedent for chronic direct administration of small molecules to the brain, and the siRNAs described here that selectively and potently inhibit mt torsinA and normalize cellular function in DYT1 patient cells, support the exciting therapeutic potential of siRNAs targeting mutant torsinA for the treatment of torsion dystonia.

MATERIALS AND METHODS

Cell culture

The following fibroblast lines were generated from skin biopsies: human controls (HF18, HF24) and DYT1 ΔGAG affected carriers (HF41, HF48) generated in our laboratory (56). All cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Rockville, MD, USA) supplemented with: 4.5 g/l glucose, 2 mM glutamine, 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco). Cultures were maintained at 37°C in 95% air/5% CO₂.

Figure 5. Effects of siRNA and torsinA and B overexpression on secretion of Gluc from control and DYT1 fibroblasts. (A) Control fibroblasts (HF18) and DYT1 fibroblasts (HF41), which had been infected with a Gluc-lentivirus vectors 24 h previously, were either not transfected (untreated) or transfected with 25 nm siRNAs including: a control Cy3-tagged siRNA, siRNA 1939 or 1952 (targeting torsinADE selectively) or siRNA 1958 or siRNA 1963 (targeting both wt and mt torsinA). Seventy-two hours after transfection Gluc activity in the medium was measured over a 24 h period. (B and C) DYT1 fibroblasts (HF48) were first infected with a lentivirus vector expressing Gluc, then 24 h later with vectors expressing torsinA or torsinB. (B) Levels of endogenous and overexpressed torsinA and torsinB were evaluated 48 h later by western blotting and densitometry analysis. (C) Forty-eight hours after the second infection, luciferase activity in the medium was also measured. NS, non-significant; */P < 0.02, **P < 0.01 (with two-tailed Student’s t-test) compared with untreated cell and P < 0.0001 using ANOVA.
Vectors

Lentivirus vectors were derived from self-inactivating lentivirus (CS-CGW) (57). A cDNA encoding humanized Gluc (Prolume/Nanolight, Pinetop, AZ, USA) was inserted downstream of the CMV promoter followed by an internal ribosome entry site and cDNA for the optimized blue fluorescent protein, cerulean [(58); from Dr David Piston, Vanderbilt University, Med. Ctr., TN, USA; (11)]. DNAs for human torsinA and torsinB were also inserted into this vector construct under the CMV promoter (7). Vectors were produced by co-transfection of 293T cells with the lentivirus packaging plasmid (pCMVR8.91), envelope coding plasmid (pVSVG) and vector construct yielding typical titers of 108 transducing units (tu)/ml (57).

Gluc activity

To monitor Gluc secretion, cells were plated in 150 cm dish (1.5 million cells/dish) and infected with lentivirus vector encoding Gluc-CFP at a multiplicity of infection (MOI) = 50 to achieve infection of >90% of cells. The next day, infected cells were plated in a 12-well plate (25 000 cells/well) and transfected with 25 nm siRNA directed against wt and/or mt torsinA messages with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). To monitor secretion, luciferase activity was monitored in conditioned, cell-free medium at 72 h post-transfection. Activity was measured with a luminometer (Dynex Technologies, Chantilly, VA, USA) after adding 20 µM coelenterazine (Prolume/Nanolight) to the medium, with photon signals integrated over 10 s (59). Results are expressed ± SD (N) and significance was calculated using two-tailed Student’s t-test (Excel), as well as by ANOVA.

RNA

RNA was collected from cells using the RNAeasy Plus kit (Qiagen, Valencia, CA, USA). RT–PCR reactions were set up using the OligoScript RT kit (Qiagen) with 1 µg RNA, oligo dT and random hexamers. For QT-PCR, primers and probes were designed and supplied by TtB Molbiol (LLC), run on the Lightcycler (Roche Applied Sciences, Indianapolis, IN, USA) and subjected to a melting curve to distinguish the mt and wt torsinA sequences. Primers used for PCR of torsinA sequences were (5’–3’) torsin F1-gatcagagtgtgctgtgtgat and torsin R1-aacaccgttttgcagcctt. Fluorescent hybridization probes were WT Sen-cagagtgtgctgtgagatgca-FL labeled with fluorescein and WT Anc-ttcctcaagagagagagagaggtttc-FC labeled with LC red 640. Primers and probes used for PCR and hybridization of GAPDH were as per manufacturer’s instructions (TTB).

RNAi

For designing siRNA sequences that target torsinAΔE selectively, potential target sites not present in torsinA and encompassing the torsinAΔE mutation were used, whereas for designing siRNA sequences that target both torsinA and torsinAΔE, potential target sites common to torsinA and torsinAΔE were subjected to a homology search, in order to select siRNA sequences that avoid potential human off target transcripts. siRNA were synthesized on an ABI3900 DNA synthesizer according to standard procedures and purified by AEX HPLC. For fluorescently labeled siRNA directed against firefly luciferase, the Cy3 tag was attached to the 5’ end of the sense strand. Although the anti-sense strand of this fluorescently labeled siRNA is complementary to a region in luciferase mRNA, this siRNA is inactive and does not affect luciferase mRNA levels. siRNAs were generated by mixing an equimolar solution of complementary RNA strands in annealing buffer (20 mM sodium phosphate, pH 6.8; 100 mM sodium chloride), heating at 90–95°C for 3 min and cooling to room temperature over a period of 3–4 h. Unmodified nucleotides are indicated by capital letters, and thymidines are indicated by T. Chemical modifications comprised 2’-O-methyl on selected pyrimidines (indicated by lower case letters in sequences below), and a phosphorothioate (indicated by an s) between nucleotides 20 and 21 (counting from the 5’ end). siRNA antisense and sense strand sequences targeting torsinA and torsinAΔE selectively (1939 and 1952), or targeting both torsinA and torsinAΔE (1958 and 1963):

- 1939 antisense strand: 5’-UCUUCUCAGCCACUCUGCUCCUTTT-3’
- sense strand: 5’-AGCAGAGUUGGCUAGAUGATT-3’
- 1952 antisense strand: 5’-GUcAUcUcAGCcACUCUGCTTsT-3’
- sense strand: 5’-GcAGAGuGUcAGuGAcTsTsT-3’
- 1958 antisense strand: 5’-CUCCUcAGCcACUCUGCu UUTsTsT-3’
- sense strand: 5’-AAGCcAGAGuGGuGAGGAGGATsTsTsT-3’
- 1963 antisense strand: 5’-UCUCCUCAGCCACUCUGCUCCUTTT-3’
- sense strand: 5’-AGCAGAGUUGGCUAGGAGATsTsTsT-3’

Cy3-siRNA antisense and sense strand sequences:

- antisense strand: 5’-Cy3-UCGAAGuACUcAGCGuAAGTsTsTsT-3’
- sense strand: 5’-cuuAeGcuGAGuAcueGATsTsTsT-3’

Fibroblasts were transfected with siRNAs using Lipofectamine 2000 (Invitrogen). siRNAs were used at 100 nm/72 h for western blot analyses and 25 nm/72 h for the secretion assay.

Western blot analysis

Total cell lysates were prepared by washing the cells twice with PBS, and resuspending the cell pellet in lysis buffer containing 150 mM NaCl, 50 mM Tris, pH 8.0, 1% NP-40, 0.5% deoxycholate, 0.1% SDS and protease inhibitors (PI Complete; Boehringer Mannheim, Indianapolis, IN, USA). Protein concentrations were determined using the Coomassie plus protein assay (Pierce, Rockford, IL, USA) and a bovine serum albumin standard (Bio-Rad, Hercules, CA, USA). Cell
lysates were resolved by electrophoresis in 12.5% polyacryl-
amide gels according to the method of Laemmli (60). Proteins were transferred electrophoretically to nitrocellulose (Bio-Rad) and stained for protein with 0–2% Ponceau S (Sigma, St Louis, MO, USA). Then membranes were blocked overnight in 10% non-fat milk powder in TBST (150 mM NaCl, 50 mM TRIS, pH 7.9, 0.5% Tween). Blots were probed with antibodies to torsinA or torsinB [TB2; 1:500 (28)] [D-M2A8; 1:200 (14)] and α-tubulin (Sigma; 1:10 000); diluted in 2% milk in TBST and visualized with horseradish peroxidase (HRP) conjugated to secondary anti-
bodies and SuperSignal West Pico Chemiluminescent Sub-
strate™ (Pierce). Secondary antibodies for western blots were: sheep anti-mouse IgG-HRP (1:10 000) or donkey anti-
rabbit IgG-HRP (1:10 000) (Amersham Pharmacia Biotech, Piscataway, NJ, USA). For semi-quantitative analysis of protein levels, densitometry was performed on digital images obtained from western blots using Quantity one 4.6 (Bio-Rad).

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