A Unique Redox-sensing Sensor II Motif in TorsinA Plays a Critical Role in Nucleotide and Partner Binding*§

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Early onset dystonia is commonly associated with the deletion of one of a pair of glutamate residues (ΔE302/303) near the C terminus of torsinA, a member of the AAA+ protein family (ATPases associated with a variety of cellular activities) located in the endoplasmic reticulum lumen. The functional consequences of the disease-causing mutation, ΔE, are not currently understood. By contrast to other AAA+ proteins, torsin proteins contain two conserved cysteine residues in the C-terminal domain, one of which is located in the nucleotide sensor II motif. Depending on redox status, an ATP hydrolysis mutant of torsinA interacts with lamina-associated polypeptide 1 (LAP1) and luminal domain like LAP1 (LULL1). Substitution of the cysteine in sensor II diminishes the redox-regulated interaction of torsinA with these substrates. Significantly, the dystonia-causing mutation, ΔE, alters the ability of torsinA to mediate the redox-regulated interactions with LAP1 and LULL1. Limited proteolysis experiments reveal redox- and mutation-dependent changes in the local conformation of torsinA as a function of nucleotide binding. These results indicate that the cysteine-containing sensor II plays a critical role in redox sensing and the nucleotide and partner binding functions of torsinA and suggest that loss of this function of torsinA contributes to the development of DYT1 dystonia.

Torsion dystonia is an autosomal dominant movement disorder that causes the muscles to contract and spasm involuntarily. These muscle contractions force the body into repetitive movements and often twisted postures. The most common and severe early onset form of dystonia has been linked to the mutation in the human DYT1 (TOR1A) gene encoding the protein torsinA (1, 2). Although the function of torsinA and the mechanism underlying dystonia disease remain obscure, the sequence of torsinA is homologous to a large and diverse group of ATP-dependent oligomeric proteins named the AAA+ protein family. The AAA+ proteins typically form oligomeric rings that catalyze a variety of cellular processes, including protein translocation, membrane trafficking, and conformational remodeling of regulatory factors (3–5). TorsinA is the founding member of a subfamily of AAA+ proteins that includes torsinB (TOR1B) and two other related gene products (TOR2A and TOR3A) in mammals and OOC-5 in Caenorhabditis elegans (1, 6, 7).

The signal sequence and the following hydrophobic region at the N terminus (Fig. 1A) target torsinA to the lumen of the endoplasmic reticulum (ER). In the ER, torsinA perceives the redox status of the intraluminal environment (6, 8–10). Unlike ClpA and ClpB, torsinA lacks a substrate-binding domain or other functional region outside its ATPase domain (11). Experimental evidence suggests that torsinA mediates varied cellular activities, including protection from toxic cellular stress (12–15), trafficking of membrane-associated proteins (16), and synaptic vesicle recycling (17). In this regard, torsinA regulates trafficking of a G-protein-coupled receptor, dopamine transporters, and ion channels (16). The dominant-negative DYT1 mutation in transgenic mouse models causes impaired dopamine release (18) and a significant increase in dopamine turnover (19), likely contributing to the dopaminergic dysfunction and the neuropathology of this movement disorder (20). Recent evidence suggests that torsinA may act on substrate(s) both in the nuclear envelope (NE) and in the ER (21, 22). Two putative substrates have been identified: lamina-associated polypeptide 1 (LAP1) in the NE and the luminal domain like LAP1 (LULL1) in the ER (23–25). Consistent with the NE localization, torsinA has been reported to associate with outer nuclear membrane proteins, including SUNs (24) and nesprins (25) and some cytoskeletal components such as vimentin and actin (26), implicating its roles in NE structure regulation, cell adhesion, and neurite extension. Although the interaction of torsinA with these binding partners was reported to be impaired by the DYT1 mutation (24–26), the structural effect of the ΔE deletion has not been determined, nor have its effects on the nucleotide and redox sensing functions of torsinA been elucidated.

Low resolution structural models of human torsinA, based on an alignment with the closest homologs of known structure, ClpA and ClpB from Escherichia coli, revealed that torsinA...
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A position of conserved cysteines (red), residues required for nucleotide binding and hydrolysis (green and magenta), and mutated in dystonia (blue and cyan). B, close-up of the ATPase active site of the second AAA+ domain (D2) of E. coli ClpA structure (Protein Data Bank 1ksf). The conserved ATP-binding and hydrolytic residues are shown in green and magenta, respectively, as shown in A. Two conserved cysteines that form a disulfide bond in torsinA are located on the model using a multispecies alignment (29) and are highlighted by red spheres. The conserved Arg702 in the sensor II motif of ClpA is shown as a red stick. The positions of dystonia-associated mutations of torsinA, deletion of Glu302/303, and deletion of amino acids 323–328, are shown in different shades of blue. The ADP observed in the ClpA active site is shown in blue. The residues in parentheses are those in human torsinA.

Experimental Procedures

Reagents—The restriction enzymes and endoglycosidases were purchased from New England Biolabs (Beverly, MA). Tris, isopropyl β-D-thiogalactopyranoside, and DTT were from RPI Corp. (Mount Prospect, IL). GSH, N-ethylmaleimide (NEM), H2O2, Igepal CA-630, sodium deoxycholate, tris(2-carboxyethyl)phosphate, Triton X-100, and other reagents were obtained from Sigma-Aldrich. His-bind resin was from Novagen (Darmstadt, Germany). Prepacked columns were from GE Healthcare. 4-Acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid was from Molecular Probes (Invitrogen). Cell culture media and reagents were from Invitrogen. Twice-deionized water was used throughout.

Plasmids—Wild type torsinA was generated by amplifying the Clontech Human Universal Library with primers containing EcoRI and Xho restriction sites and ligating them into EcoRI/Xho linearized pcDNA3.1+ mammalian expression vector (Invitrogen). The ΔE302/303-torsinA and other single mutations as well as any combination of these mutations were prepared by site-directed mutagenesis using the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s protocol. An HA immunotag (YPYDVPDYA) was introduced at the C terminus of each construct for detection in cellular experiments. The 3× FLAG tag (MDYK-DHDGDYKHDIDYKDDDDY) was cloned into the Sall site introduced between positions 21 and 22 of torsinA, right after the N-terminal signal sequence cleavage site (22). Human LAP1 and LULL1 cDNA sequences were cloned into pCMV-Myc mammalian expression vector (Clontech), which contains the N-terminal c-Myc epitope tag for detections. The human LAP1 cDNA clone was obtained from the American Type Culture Collection mammalian genome collection with GenBank™ accession number BC023247. The human LULL1 cDNA sequence was amplified from HEK293 cell cDNAs, which were reversely transcribed from total RNAs purified by the NucleoSpin RNA II extraction kit (Clontech). Trimmed luminal domains of LAP1 (237LAP1) and LULL1 (236LULL1) were amplified from full-length cDNA and subcloned into the pSmt3 expression vector (34), a generous gift from C. Lima (Cornell University, Ithaca, NY), which contains a His/Smt3 tag at the N terminus.
terminus. All of the constructs were confirmed by DNA sequencing analysis.

Antibodies—Mouse monoclonal anti-torsinA (DM2A8) and rabbit polyclonal anti-torsinA (TAB1) were generous gifts from X. Breakefield (Harvard University, Boston, MA). Other primary antibodies used were mouse anti-HA (Covance, Berkeley, CA), mouse anti-Myc (Santa Cruz Biotechnology, CA), mouse anti-FLAG (Stratagene, La Jolla, CA), mouse anti-actin (Chemicon, Temecula, CA), rabbit anti-Grp78 (Grp78 also known as Bip; Stressgen, Victoria, Canada), and goat antilaminB1 (Santa Cruz Biotechnology). Secondary horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit, FITC-conjugated donkey anti-mouse, Cy5-conjugated donkey anti-rabbit, and Rhodamine Red-X-conjugated donkey anti-goat IgG were all purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell Culture and Transient Transfection—HeLa, COS-7, and HEK293 cells were all maintained in DMEM supplemented with antibiotics and serum at 37 °C with a 5% CO2 atmosphere in a humidified incubator. Transient transfections were performed using FuGENE 6 transfection reagent (Roche Applied Science) or Polyethylenimine, Linear (Polysciences) according to the manufacturer’s instructions. The cells were analyzed 40–48 h after transfection. All of the transfections were repeated at least three times with equivalent results.

Western Blotting and Glycosidase Treatment—Transfected HeLa and COS-7 cells were lysed with radioimmunoprecipitation buffer (50 mM Tris-HCl, 150 mM NaCl, 1% (v/v) Igepal CA-630, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) SDS, pH 8.0) containing Complete protease inhibitor tablets (Roche Applied Science) and then cleared by centrifugation. The solubilized cell extracts were boiled in 1× SDS sample buffer and treated with either endoglycosidase H or peptide:N-glycosidase F following the manufacturer’s directions. The samples were fractionated by SDS-PAGE and then transferred onto nitrocellulose membranes. Western blotting was performed using anti-HA or anti-torsinA, and the corresponding secondary antibody was conjugated with horseradish peroxidase and developed by chemiluminescence (ECL Plus; GE Healthcare).

Pulse-Chase Labeling—HeLa cells transiently transfected with torsinA constructs were starved in Met/Cys/Glu-free and serum-free minimal media for 30 min and then labeled with 200 μCi/well of [35S]Met/Cys (ICN Biochemicals) for 15 or 45 min. The cells were then chased with complete DMEM for different time periods. The supernatant fractions from cell lysates prepared in radioimmunoprecipitation buffer were immunoprecipitated using anti-HA antibody and protein G-agarose. Immunoprecipitated proteins were resolved by SDS-PAGE and visualized by phosphorimaging.

Protein Expression and Purification—The luminal domains of LAP1 and LULL1 were expressed as His/Smt3 fusion proteins in Rosetta-gami B (DE3) pLacI cells (Novagen), which facilitate expression of genes that encode rare E. coli codons and cytoplasmic disulfide formation. The cultures were grown at 37 °C to an A600 of 0.8−1.0, cooled to 15 °C, induced by the addition of 0.2 mM isopropyl β-D-thiogalactopyranoside, and allowed to express for 20 h. The cells were harvested and lysed by sonication in a buffer of 20 mM Tris, 200 mM NaCl, 10% glycerol, pH 7.8. The purification of the fusion proteins was followed by standard nickel affinity, a HiTrap Q ion exchange, and a HiPrep Sephacryl S-200 gel filtration. The final protein sample was concentrated and stored in the same buffer at −80 °C.

His Tag Pulldown Assay—Transiently transfected HEK293 cells with torsinA-HA constructs were lysed in the lysis buffer (50 mM Tris, 150 mM NaCl, pH 7.5, 1% Triton X-100) supplemented with Complete protease inhibitor tablets. The lysates were collected by centrifugation, and the supernatants were pulled down with purified His/Smt3 or His/Smt3 fusion proteins (either His/Smt3−237LAP1 or His/Smt3−236LULL1). They were then incubated with nickel-Sepharose 6 Fast Flow beads (GE Healthcare) pre-equilibrated in binding and washing buffer (40 mM imidazole, 50 mM Tris, 500 mM NaCl, pH 7.8). After 1 h of incubation at 4 °C, the beads were pelleted at 10,000 × g for 30 s and washed extensively with washing buffer. The bound proteins were eluted by incubating the beads in elution buffer (400 mM imidazole, 20 mM Tris, 200 mM NaCl, pH 7.8) for 5 min at room temperature. After a quick spin, the supernatant was boiled in 1× SDS loading buffer, subjected to SDS-PAGE, and immunoblotted with anti-HA antibody.

For assays with ATPγS, AMPNNP, or ADP, cell lysate was treated with apyrase (12 milliunits/μl) for 30 min on ice before each nucleotide was added to a final concentration of 2.5 mM and incubated for 15 min on ice. The treated samples were pulled down by adding His/Smt3−237LAP1 protein. For assays with DTT, GSH, or NEM, E171Q-torsinA transfected cell lysates were treated with a final concentration of 1 mM DTT, 5 mM DTT, or 5 mM GSH for 1 h on ice. NEM was added to 1 mM DTT-treated sample to a final concentration of 5 mM for 30 min before being pulled down by His/Smt3−237LAP1. For oxidative stress assay with H2O2, torsinA transfected cells were treated with a final concentration of 0.2 or 1 mM H2O2 in DMEM without serum for 30 min at 37 °C with a 5% CO2 atmosphere in a humidified incubator before being lysed in the lysis buffer and pulled down by His/Smt3−237LAP1.

Trypsin Digestion Assay—Transiently transfected HEK293 cells with torsinA constructs were lysed in the same lysis buffer as for pulldown assay. The lysates were collected by centrifugation, and the aliquots of supernatants were digested by trypsin at room temperature for 15 min. The digestion was stopped by boiling in 1× SDS loading buffer, subjected to SDS-PAGE, and immunoblotted with anti-HA antibody.

For assay with ATPγS, the treatment was the same as the assay with nucleotide for pulldown. The treated samples were digested with trypsin at room temperature for 15 min. For assays with DTT or NEM, the cell lysates were treated with a final concentration of 1 mM DTT for 1 h on ice. NEM was added to a DTT-treated sample to a final concentration of 5 mM for 30 min on ice before being digested by trypsin. Both assays have appropriate controls, such as apyrase treatment only, DTT, or NEM treatment only.

Immunofluorescence—COS-7 and HeLa cells grown on glass coverslips were transiently transfected with torsinA constructs. After fixation in 3.7% formaldehyde and permeabilization in 0.2% Triton X-100 and then blocking with 1% BSA and 1.35% fish skin gelatin, the cells were incubated for 1 h at room tem-
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RESULTS

Lack of Effect of Disease-causing Mutation and the RRS Mutation on the Stability of Human TorsinA—The cysteines at positions 280 and 319 in the C terminus of torsinA are predicted to form a regulatory disulfide bond (29). If the disulfide is strictly structural, it would be predicted to stabilize torsinA. When the two cysteines were changed to serines (CS mutant; Fig. 2A), the level of the CS mutant protein was only modestly reduced in HeLa (Fig. 2B, left panel) and COS-7 cells (Fig. 2C, left panel). Pulse-chase experiments also indicate that the disulfide does not significantly contribute to the stability of torsinA in HeLa cells. After 15-min (data not shown) and 45-min pulses (Fig. 2D), the turnover of the CS mutant was similar to that of WT torsinA.

One explanation suggested for the spheroid inclusion phenotype of the ∆E mutant is that the disease-causing mutation alters the stability of torsinA in the ER. However, levels of the ∆E mutant were the same as WT torsinA in both HeLa (Fig. 2B, middle panel) and COS-7 cells (Fig. 2C, middle panel). When the CS mutation was introduced with the ∆E-torsinA (Fig. 2A), expression was modestly decreased (Fig. 2B and C, middle panels). The WT, CS, ∆E, and the ∆E/CS double mutant (Fig. 2A) were all sensitive to digestion with both endoglycosidase H and peptide-N-glycosidase F (Fig. 2B and C), suggesting that the expressed torsinA proteins are all reaching the ER lumen, consistent with previous reports (35–38). Finally, the ∆E mutant turned over at a rate similar to WT after 45-min pulses (Fig. 2D).

The ∆E mutation has been reported to reduce torsinA protein stability in SH-SY5Y cells at a longer chase time, between 12 and 24 h (39), but this was not observed during the first 8 h (Fig. 2D). These results indicate that deletion of glutamate 302/303 in torsinA does not have profound effects on the stability of the protein inside cells. The similar metabolic turnover rates for the WT, the CS, and the ∆E torsinA suggest that neither the dystonia-causing mutation nor the site-directed CS mutation in RRS is a folding mutant trapped in the ER and targeted for degradation.

TorsinA Interacts with the Lumenal Domains of LAP1 and LULL1 in an ATP-dependent Manner—TorsinA has been reported to interact with proteins LULL1 and LAP1, which contain a single transmembrane span and cytoplasmic/nucleoplasmic and lumenal domains. Using immunoprecipitation strategy, full-length LULL1 (supplemental Fig. S1A) and LAP1 (supplemental Fig. S1B) were both co-immunoprecipitated with FLAG-tagged torsinA probed by anti-FLAG antibody. In the reverse experiment, torsinA was co-immunoprecipitated with Myc-tagged LULL1 by anti-Myc antibody (supplemental Fig. S1C). Noticeably, the association of WT torsinA with LULL1 (supplemental Fig. S1A) or LAP1 (supplemental Fig. S1B) was significantly weaker than EQ-torsinA, consistent with previous reports (23, 25).

Human LAP1 and LULL1 share 60% identity in their lumenal domains that interact with torsinA (23). To investigate the role of the RRS in the interaction of torsinA with the partners, the lumenal domains of LAP1 and LULL1 were expressed in E. coli and purified as His/Smt3 fusion proteins (supplemental Fig. S2, A and B). Far-UV CD spectra of purified His/Smt3–237LAP1 (supplemental Fig. S2C) indicate that it forms an α/β structure, with 36% α-helix and 16% β-structure estimated by on-line software, K2d (prediction of percentages of protein secondary structure from CD spectra). Each of the lumenal domains has conserved cysteine residues that are predicted to form a disulfide bond in the oxidizing ER lumen. Like torsinA, disulfide formation is not required for maintaining the secondary structure of the lumenal domain of LAP1 or LULL1 because the

FIGURE 2. The effects of the dystonia-causing mutation and the RRS mutation on the stability of torsinA. A, diagram of different versions of torsinA for stability studies, including WT, the C280S/C319S mutant (CS), the ∆E mutant (∆E), and the ∆E with the CS mutant (∆E/CS) of torsinA. B and C, steady state level of wild type torsinA and mutants in HeLa (B) and COS-7 (C) cells. Supernatant fractionation of cells expressing the WT, the CS mutant, the ∆E mutant, and the ∆E/CS mutant of torsinA digested with peptide-N-glycosidase F (PNGase F) or endoglycosidase H (Endo H) and immunoblotted with anti-HA antibody is shown. Molecular weight markers are shown on the left of images. Immunoblot of endogenous actin is the loading control. D, pulse-chase analysis of WT torsinA, the CS mutant, and the ∆E mutant in HeLa cells. Supernatant fractionation of cells expressing the WT, the CS mutant, the E mutant (Fig. 2A), expression was modestly decreased (Fig. 2B and C, middle panels). The WT, CS, ∆E, and the ∆E/CS double mutant (Fig. 2A) were all sensitive to digestion with both endoglycosidase H and peptide-N-glycosidase F (Fig. 2B and C), suggesting that the expressed torsinA proteins are all reaching the ER lumen, consistent with previous reports (35–38). Finally, the ∆E mutant turned over at a rate similar to WT after 45-min pulses (Fig. 2D).

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presence of reductant did not change the CD spectrum (supplemental Fig. S2C).

Purified His/Smt3–237LAP1 and His/Smt3–236LULL1 were used to pull down torsinA from HEK293 extracts containing the WT or various mutants of torsinA (Fig. 3A). The “substrate trap” mutant, EQ-torsinA, which binds but is incompetent for ATP hydrolysis (21–23, 25), was pulled down with LAP1 or LULL1 (supplemental Fig. S2D, lanes 5 and 8). In contrast, neither WT nor the ATP-binding mutant (KA) of torsinA was pulled down by LAP1 or LULL1 (supplemental Fig. S2D, lanes 4, 6, 7, and 9). Thus, torsinA, when bound to ATP, forms a physical complex with the luminal domain of LAP1 and LULL1. These results confirmed the above co-immunoprecipitation (supplemental Fig. S1) and previously reported interaction of the LAP1 luminal domain with the EQ-torsinA observed by immunofluorescent labeling and co-immunoprecipitation (21, 23).

Because AAA+ proteins typically bind substrates when bound to ATP (40), binding partners of torsinA would be expected to bind more tightly to the EQ mutant. To more directly examine whether binding of torsinA to its partners is ATP-dependent, the ability of nucleotide analogs to mediate the interaction between torsinA and LAP1 was tested. ATP and ADP in the cell extracts were depleted by preincubation with apyrase (41). Incubation of His/Smt3–237LAP1 with ATP and ADP in the cell extracts were depleted by preincubation (21, 23).

The Redox-sensitive Nucleotide Sensor II regulates the interaction between torsinA and Its Binding Partners—Previous studies of the torsinA homolog OOC-5 indicated that the torsin family proteins function as integrators of cellular nucleotide and redox status to regulate the specific action on unknown substrates. The redox-sensing cysteine is located in the nucleotide sensor II motif, which has been termed the redox-regulated sensor (29). To assess the sensitivity of the interaction between torsinA and LAP1, cell lysates expressing the EQ-torsinA was first treated with DTT, GSH, or buffer and then incubated with NEM for 1 min DTT-treated sample prior to the pulldown assays. B, the interaction between torsinA and LAP1 is decreased by oxidative stress. Cells expressing the EQ-torsinA were first treated with 0.2 or 1 mM H2O2 and then lysed for the pulldown assays. C, mutation of the sensor II motif in torsinA interferes with the interaction of torsinA with LAP1. Cell lysates expressing EQ, EQ/C319S, EQ/C319A, and EQ/K320R of torsinA were subjected to the pulldown assays. D, the two cysteine residues in the C-terminal domain of torsinA, but not the N-terminal cysteines, are critical for the interaction between torsinA and LAP1. Cell lysates expressing EQ, EQ/C945S/C950S, EQ/C445/C162S, EQ/CS, and EQ/4CS of torsinA were subjected to the pull-down assays. EQ+NEM represents the cell lysate expressing EQ-torsinA treated by NEM only prior to the pulldown assay. IB, immunoblot.

The Redox-sensitive Nucleotide Sensor II regulates the action between torsinA and LAP1. A, blockage of redox sensing decreases the interaction between torsinA and LAP1. Cell lysates expressing the EQ-torsinA was first treated with DTT, GSH, or buffer and then incubated with NEM for 1 min DTT-treated sample prior to the pulldown assays. B, the interaction between torsinA and LAP1 is decreased by oxidative stress. Cells expressing the EQ-torsinA were first treated with 0.2 or 1 mM H2O2 and then lysed for the pulldown assays. C, mutation of the sensor II motif in torsinA interferes with the interaction of torsinA with LAP1. Cell lysates expressing EQ, EQ/C319S, EQ/C319A, and EQ/K320R of torsinA were subjected to the pulldown assays. D, the two cysteine residues in the C-terminal domain of torsinA, but not the N-terminal cysteines, are critical for the interaction between torsinA and LAP1. Cell lysates expressing EQ, EQ/C945S/C950S, EQ/C445/C162S, EQ/CS, and EQ/4CS of torsinA were subjected to the pull-down assays. EQ+NEM represents the cell lysate expressing EQ-torsinA treated by NEM only prior to the pulldown assay. IB, immunoblot.

The Redox-sensitive nucleotide sensor II motif, which has been termed the redox-regulated sensor (29). To assess the sensitivity of the interaction between torsinA and Its partners to changes in redox potential, pulldown assays were performed using extracts from the cells expressing EQ-torsinA. In the presence of reductant, the EQ-torsinA was pulled down by LAP1 (Fig. 4A, lanes 3–5, and supplemental Fig. S3A), similar to the control (Fig. 4A, lane 2). When the cell extract was first reduced and then blocked by NEM, the pulldown of torsinA was decreased (Fig. 4A, lane 6), whereas NEM treatment only did not alter the binding of torsinA to LAP1 (Fig. 4D, lane 6). In contrast, upon oxidative stress, such as H2O2 treatment on intact cells for 30 min, the
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ability of the EQ-torsinA to interact with LAP1 or LULL1 was significantly decreased (Fig. 4B, lanes 2 and 3, and supplemental Fig. S3A). These results indicate that nonmodified sulfhydryls are critical for torsinA to interact with LAP1 or LULL1.

To further test the requirement for free sulfhydryls in the RRS motif of torsinA for the interaction with its partners, CysS119 was mutated to serine or alanine in the EQ-torsinA background (Fig. 3A). Interaction between torsinA and LAP1 was significantly diminished in both the C319S and the C319A mutants (Fig. 4C, lanes 2 and 3). The role of other conserved residues was also assessed because the amino acid sequence GCK in sensor II is highly conserved in the torsin protein family (29).

Surprisingly, when Lys320 was substituted by arginine (Fig. 3A), as found in ClpA, the interaction between torsinA and LAP1 was impaired (Fig. 4C, lane 4). Therefore, the RRS motif of torsinA subfamily is essential for partner binding, whose sequence is distinct from that of the ClpA/Hsp100 family of AAA+ protein.

Each of the four cysteines on the N-terminal domain of torsinA was mutated to serine, and double serine mutations were made to mutate predicted disulfides in the EQ-torsinA background (Fig. 3A), including C44S/C162S and C49S/C50S, for pulldown assays (Fig. 4D and supplemental Fig. S3B). Mutants C49S (supplemental Fig. S3B), C49S/C50S (Fig. 4D, lane 2), and C44S/C162S (Fig. 4D, lane 3) as well as all four cysteines in the N-terminal domain (Fig. 4D, lane 5) did not alter the interaction of torsinA with its binding partners. By contrast, the disulfide in the C-terminal domain formed between Cys280 and Cys319 is redox-sensitive (Fig. 3A), because the interaction between torsinA and LULL1 or LAP1 was significantly diminished when they were mutated to serines (Fig. 4D, lane 4). Thus, the loss of the ability of torsinA to interact with LAP1 and LULL1 by alterations of the cysteines in the C-terminal domain parallels the observed loss of the function of OOC-5 during embryo development in C. elegans when the C-terminal cysteines were mutated (29).

The Dystonia-causing Mutation Impairs the Interaction between TorsinA and Its Binding Partners—The effects of the ΔE mutation on the ability of torsinA to bind LAP1 or LULL1 were investigated using the pulldown assay. When Glu292/303, which is between Cys280 and Cys319, is redox-sensitive (Fig. 3A), because the interaction between torsinA and LULL1 or LAP1 was significantly diminished when they were mutated to serines (Fig. 4D, lane 4). Thus, the loss of the ability of torsinA to interact with LAP1 and LULL1 by alterations of the cysteines in the C-terminal domain parallels the observed loss of the function of OOC-5 during embryo development in C. elegans when the C-terminal cysteines were mutated (29).

The Dystonia-causing Mutation Increases the Protease Susceptibility of TorsinA, although It Causes Spheroid Inclusion Phenotype—Immunofluorescence of HeLa and COS-7 cells showed large spheroid inclusions formed by both ΔE-torsinA and EQ/ΔE-torsinA (Fig. 5, A and B), which recapitulates previous reports (25, 28). Similar to WT torsinA, the CS-torsinA was located predominantly in the ER (Fig. 5, A and B). Moreover, the CS mutation could rescue the spheroid inclusion phenotype caused by the ΔE mutation (Fig. 6, A and B), suggesting that the C-terminal region of torsinA is essential for partner binding.

The conformational consequences of nucleotide binding and redox sensing of ΔE-torsinA were determined by susceptibility of a sentinel tryptic site between the N- and C-terminal

![FIGURE 5. The dystonia-causing mutation impairs the interaction between torsinA and the luminal domains of LAP1 and LULL1. A, diagram of different mutations of torsinA for the interaction studies, including wild type (WT), the ΔE mutant (ΔE), and a series of mutations on the ΔE or the EQ background. B, the interaction between torsinA and LAP1 is dramatically reduced by introducing the ΔE mutation into the EQ-torsinA. Cell lysates expressing WT, EQ, ΔE, ΔE/EQ, ΔE/CS, and ΔE/KA of torsinA were subjected to pulldown assays by His/Smt3–237LAP1. C, the interaction between torsinA and LULL1 as well as LULL1 is also reduced by another natural mutation on DYT1 gene, ΔL323–328, but is unaffected by the missense mutation at Glu292 or Glu303. Cell lysates expressing EQ, ΔE, EQ/EQ, EQ/E302A, EQ/E303A, EQ/T324V, and EQ/ΔL323–328 of torsinA were subjected to the pulldown assays by His/Smt3–237LAP1 and His/Smt3–236LULL1. IB, immunoblot.](https://example.com/figure5.png)
domains of torsinA, similar to OOC-5 (29). In the absence of any supplemental reductant or nucleotide, trypsin accessibilities of the ΔE-torsinA and the EQ/ΔE-torsinA (Fig. 6, C, lanes 6 and 16, and D) were remarkably increased compared with those of the WT torsinA and the EQ-torsinA (Fig. 6, C, lanes 5 and 15, and D), respectively. By contrast, neither E302A nor E303A (Fig. 6, C, lanes 17 and 18, and D) altered the trypsin cleavage of the EQ-torsinA, consistent with the lack of an alteration of the interaction of torsinA with LAP by these missense mutations (Fig. 5C). Significantly, susceptibilities for the CS (Fig. 6, C, lane 7, and D), EQ/CS (Fig. 6, C, lane 20, and D), and EQ/C319A (Fig. 6, C, lane 19, and D) mutants were increased in comparison with that of the WT torsinA and the EQ-torsinA, respectively. Double mutation of ΔE and CS caused the increased protease susceptibility with significant difference to the WT torsinA, although no additive effect was observed (Fig. 6, C, lane 8, and D). Therefore, the dystonia-causing mutation (ΔE) alters the conformation of torsinA, as reflected by increasing the protease susceptibility with significant difference from the WT torsinA, although no additive effect was observed (Fig. 6, C, lane 8, and D). Therefore, the dystonia-causing mutation (ΔE) alters the conformation of torsinA, as reflected by increasing the protease susceptibility, although it forms spheroid inclusion around ER and NE. The CS mutation not only alters the conformation but also rescues the spheroid inclusion phenotype of the ΔE mutation, suggesting that the CS mutation has a significant impact on the local conformation of both WT torsinA and ΔE-torsinA.

The Dystonia-causing Mutation Interferes with Nucleotide Binding and Redox Sensing Activities of TorsinA—To more directly examine whether binding of nucleotide impacts the conformation/protease cleavage of torsinA, ATP and ADP in the cell extracts were first depleted by preincubation with apyrase (41). Supplemented ATPγS significantly stabilized WT torsinA (Fig. 7A, left top, lanes 3 and 4) and EQ-torsinA (Fig. 7, A, right top, lanes 7 and 8, and B; ATPγS stabilization is statistically significant at p < 0.01) simply by the ligand-
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binding induced stabilization, whereas it did not alter the trypsin digestion of ΔE-torsinA (Fig. 7, A, left center, and B), EQ/ΔE-torsinA (Fig. 7, A, right center, and B), CS-torsinA (Fig. 7, A, left lower, and B), and EQ/CS-torsinA (Fig. 7, A, right lower, and B). Thus, both the ΔE and CS mutations impair the conformational change that reflects the nucleotide binding activity of torsinA.

In addition, both ΔE and CS mutations decreased the redox sensing ability because reduction and subsequent alkylation increased the stability of WT torsinA (Fig. 7C, left top, lane S) and EQ-torsinA (Fig. 7, C, right top, lane 10, and D); DTT and NEM treatment is statistically significant different from DTT treatment only at p < 0.05), whereas it did not alter the trypsin digestion of ΔE-torsinA (Fig. 7C, left center), EQ/ΔE-torsinA (Fig. 7C, right center), CS-torsinA (Fig. 7C, left lower), and EQ/CS-torsinA (Fig. 7C, right lower). This stabilization is most simply explained by a local conformational change around the trypsin cleavage site for wild type and the E171Q mutant, by contrast to the ΔE and CS mutants which lose the ability to change their conformations induced by reduction and alkylation. Therefore, the dystonia-causing mutation, ΔE, may interfere with both redox sensing and nucleotide binding activities by altering the significant conformational change otherwise correlated with the interaction of torsinA with its binding partners, LAP1 and LULL1.

DISCUSSION

The torsinA-interacting proteins, LAP1 and LULL1, contain a transmembrane span, and the N terminus of LULL1 is predicted to localize in the cytoplasm, whereas the N terminus of LAP1 is in the nucleoplasm (23). Our preliminary results indicate that the N-terminal parts of LAP1 and LULL1 are highly disordered, whereas the C-terminal luminal domains assume α/β structures (supplemental Fig. S2C). In the lumen of the ER and the NE, torsinA, associated with the ER/NE membrane by its N-terminal hydrophobic region (42), interacts with the luminal domains of LAP1 or LULL1 when the binding conformation is induced by ATP binding and appropriate redox state. Thus, the substrate trap mutant, E179Q-torsinA, whose substitution in Walker B motif inhibits ATP hydrolysis, binds LAP1/LULL1 very tightly. If the ATP-binding site is disrupted by the mutation K108A, the interaction of torsinA with LAP1/LULL1 is weakened. Mutating the cysteine at position 319 or both Cys280 and Cys319 to serine also diminishes the interaction, demonstrating that the unique cysteine in nucleotide sensor II plays a central role in the interaction between torsinA and its binding partners. These results are consistent with the hypothesis proposed based on results with OOC-5 from C. elegans, that the torsin family proteins integrate the nucleotide binding and redox sensing functions through its RRS (29).

The dystonia-causing mutation (ΔE) impairs the interaction between torsinA and LAP1/LULL1 without dramatically affecting the stability of full-length torsinA (Fig. 2) and the oligomerization of the isolated C-terminal domain (supplemental Fig. S4B). In contrast, CD studies of the isolated C-terminal domain of torsinA revealed a redox-dependent conformational change that was impacted by the ΔE mutation (supplemental Fig. S4C). These data indicate that the dystonia-causing mutation alters the local conformation, which is critical for both nucleotide binding and redox sensing and which in turn mediates the interaction between torsinA and its partners. Taken together, these results demonstrate that the C-terminal domain of torsinA plays a critical role in regulating the interaction with its binding partners and that the unique sensor II in the C-terminal domain mediates redox regulation of the N-terminal AAA+ functions of torsinA.

4 L. Zhu, J. O. Wrabl, and P. J. Thomas, unpublished data.
LAP1 proteins are associated with both type A and B lamins, as well as mitotic chromosomes (43). Although studied extensively, the function of LAP1 remains unclear. A recent report utilizing torsinA mutant mice showed that LAP1 and torsinA act in the same biological pathway required for normal nuclear membrane morphology in both neuronal and non-neuronal cells (44). The C-terminal luminal domain of LAP1B is highly conserved, but its function is unknown; the N-terminal nucleoplasmic domain and the transmembrane span of LAP1B are necessary and sufficient for the nuclear localization of the molecule (45). If the N-terminal nucleoplasmic domain is responsible for the interaction of LAP1s with lamins and chromosomes, the C-terminal luminal domain is likely to play a regulatory role for the maintenance of the nuclear architecture and organization, which is likely to be modified by the AAA+ activity of torsinA. Therefore, the impaired interaction between the ΔE-torsinA and the luminal domain of LAP1 (Fig. 5), probably as well as other NE proteins such as SUNs and nesprins (24, 26), would explain the dystonia-associated disruption of the neuronal nuclear envelope (44, 46) arising from the inability of LAP1 to maintain nuclear structure during neuronal maturation. LULL1 shares remarkable similarities with LAP1, suggesting that the N-terminal cytoplasmic domain of LULL1 may interact with some unknown molecules in the cytoplasm, i.e. cytoskeleton components (27), whereas the C-terminal luminal domain may regulate this interaction through the modification by the AAA+ activity of torsinA. The diversified structures and functions between the N-terminal regions of LAP1 and LULL1 need further investigation, which may provide new clues regarding the function of torsinA in neurons.

Wild type torsinA (data not shown) and the EQ mutant (supplemental Fig. S4E, left panel) have two labile disulfide bonds that can be gradually reduced and modified by 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid after DTT titration on the intact HEK293 cells (47), whereas the EQ/CS have only one (supplemental Fig. S4E, right panel). Thus, consistent with the disulfide mapping results for the homologous nematode protein OOC-5 (29), human torsinA can exist in an oxidized state with two pairs of disulfide inside the cells, and two cysteines in the C-terminal domain have been shown to form one of these disulfide bonds. These results are also consistent with the predominant location of torsinA within the oxidizing, continuous lumen of the ER and NE (48, 49) and the hypothesis that torsin family proteins are regulated by the redox state of the ER or involved in the redox regulation of the other proteins in the ER and NE (29).

Pulldown experiments suggest that torsinA interacts with LAP1 or LULL1 in its reduced form because the binding of torsinA to LAP1 or LULL1 was significantly decreased after oxidative stress (Fig. 4B), whereas little alteration was observed with DTT treatment (Fig. 4A and supplemental Fig. S3A). This seems to be improbable because torsinA and LAP1/LULL are both localized to the oxidizing environment of the ER and NE. However, it is consistent with nucleotide-induced conformational change of torsinA homolog, OOC-5, under reducing conditions revealed by the protease accessibility (29). Both of these experiments suggest that the nucleotide-binding state of torsinA is coupled to its redox status by its RSS. It is interesting to consider the possibility that the function of torsinA is regulated by redox and nucleotide status in the context of its proposed role as an ER chaperone (9, 16) and putative involvement in the ER stress response (10). In addition, torsinA and LAP1 have six and three conserved cysteines, respectively; thus the formation of a disulfide-linked species of torsinA-LAP1/LULL1 could happen transiently and may be mediated by reduced glutathione inside cells under appropriate redox conditions. Future work will identify which cysteines on LAP1 or LULL1 are responsible for the interaction with torsinA.

Other protein activities regulated by redox and nucleotide status include ATP-sensitive potassium channels (50, 51) and vascular H+-ATPases (52) as well as Bacillus subtilis αα′ transcription factor (53). TorsinA is extensively expressed in certain neurons in the brain, including dopaminergic innervating and cholinergic interneurons within the basal ganglia, as well as neurons in the cerebral cortex, hippocampus, thalamus, and cerebellum (2). Similar to muscle where redox-regulated ATP-sensitive potassium channels are expressed, these tissues are highly metabolically active and consume much more O2 than other organs. Because metabolic and redox signals are closely related and are recognized to play a critical role in stress responses, it is reasonable to hypothesize that redox regulation of the AAA+ activity of torsinA may be central under some stress conditions during the early stages of brain development. Imaging studies report subtle alterations in microstructure and metabolism in certain regions of the brain in DYT1 patients (54, 55). Therefore, alteration of nucleotide binding and redox regulation of the AAA+ function of torsinA caused by the DYT1 mutation could contribute to abnormal neuronal structure and function underlying the dystonic symptoms.

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