Relative tissue expression of homologous torsinB correlates with the neuronal specific importance of DYT1 dystonia-associated torsinA

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Received September 22, 2009; Revised and Accepted December 14, 2009

A three base-pair deletion in the widely expressed TOR1A gene causes the childhood onset, neurological disease of DYT1 dystonia. Mouse Tor1a gene knockout also specifically affects the developing nervous system. However, in both cases, the basis of neuronal tissue specificity is unknown. TorsinA is one of four predicted mammalian torsin ATPases associated with assorted cellular activities (AAA+) proteins, raising the possibility that expression of a functionally homologous torsin compensates for torsinA loss in non-neuronal tissues. We find that all four mammalian torsins are endoplasmic reticulum resident glycoproteins. TorsinA, torsinB and torsin2 are all present in large M complexes, which suggests that each assembles into an oligomeric AAA+ enzyme. Introducing a mutation (WBEQ) that typically stabilizes AAA+ proteins in a substrate-bound state causes torsinA and torsinB to associate with a shared nuclear envelope (NE) binding partner and this NE localization requires the torsinA interacting protein, lamina associated polypeptide 1. Although torsin proteins are widely expressed in the adult mouse, we identified that embryonic neuronal tissues contain relatively low torsinB levels. Therefore, our results reveal that torsinB expression inversely correlates with the cell and developmental requirement for torsinA. In conclusion, multiple cell types appear to utilize torsin AAA+ proteins and differential expression of torsinB may contribute to both the neuronal specific importance of torsinA and the symptom specificity of DYT1 dystonia.

INTRODUCTION

DYT1 dystonia is an autosomal dominant disease caused by heterozygosity for an in-frame, three base-pair deletion in the TOR1A gene that encodes torsinA (1). This specifically neurological disease is characterized by childhood or adolescent onset of abnormal, involuntary twisting movements that develop in the absence of other symptoms or gross abnormalities in brain structure (2,3). The deletion that causes DYT1 dystonia is a loss-of-function mutation in mice (4) and therefore a partial loss of torsinA activity is expected in the disease condition. Furthermore, the involvement of torsinA loss in disease pathogenesis is also suggested by the specifically neuronal abnormalities caused by mouse Tor1a gene deletion (4).

TorsinA is an endoplasmic reticulum (ER) targeted member of the ATPases associated with assorted cellular activities (AAA+) superfamily. As their name suggests, AAA+ proteins operate on a wide variety of substrates and AAA+ activity is required for cellular functions as diverse as DNA replication, cytoskeletal remodeling, vesicular trafficking and protein degradation. AAA+ proteins typically couple the energy of ATP hydrolysis to conformational change in binding partners. This activity requires that AAA+ proteins assemble into ring structures that contain six or more ATP hydrolyzing modules and, in turn, these bind and generate structural rearrangements in specific partners upon ATP hydrolysis (5,6).

The binding partners modified by AAA+ ATPase activity are regarded as the enzyme substrates and their cellular roles usually explain the biological importance of a AAA+ protein. Currently, neither the substrate nor cellular roles of torsinA are well understood. Several lines of evidence indicate that torsinA acts upon a protein, or protein complex, specifically localized in the nuclear envelope (NE) subdomain of the main ER system. This subcellular site of activity was first identified when an ATP hydrolysis deficient ‘substrate trap’, torsinA isoform specifically concentrated in the NE...
(7–9), and was further supported by finding an NE localized torsinA interacting protein, lamina associated polypeptide 1 (LAP1; TOR1AIP1) (8,10). It has also been shown that torsinA loss causes nuclear membrane abnormalities to develop in neuronal cells (4), again localizing torsinA activity to the NE lumen. The NE is continuous with the main ER compartment and luminal proteins are believed to freely exchange between the two. However, the NE also possesses a specialized proteome (11) that includes membrane proteins associated with transcriptional control, providing resistance to mechanical stress and connecting the nuclear interior with cytoskeletal networks.

Why Torlα gene deletion specifically affects the nuclear membrane of neurons is unclear. TorsinA is widely expressed (4), LAP1 is present in most cell types (4) and torsinA associates with the NE in most cell types (7,9,12). Although it is possible that torsinA has a unique neuronal function, this is doubtful given that over-expression of a ‘dominant negative’ torsinA causes nuclear membrane abnormalities to develop in a non-neuronal cell line (9). One possible explanation for these apparently contradictory results is that non-neuronal cells have a unique ability to compensate for torsinA loss, but that this mechanism is surmounted by a dominant negative torsinA isoform. Since the human genome contains four homologous torsin genes, TOR1A (torsinA), TOR1B (torsinB), TOR2A (torsin2) and TOR3A (torsin3), we hypothesize that non-neuronal cell types express a torsin protein that compensates in the absence of torsinA.

To date, the expression and subcellular localization of torsinB, torsin2 and torsin3 proteins remain poorly characterized and, with the exception of torsinB (13,14), it is not clear that all are either expressed or reside within the ER system. Furthermore, although the glycosylation state of torsinA indicates that it is an ER resident protein (13,15–17), even this well-characterized torsin has been variously described in post-ER secretory pathway compartments (18) and possessing cytosolic binding partners (19–24). Thus, further examination of the organelar location of torsinA protein is needed in order to clarify the importance of some previous observations. In addition, it is also unknown whether each torsin is an active, and/or independently operating, AAA+ enzyme. Recently, torsinA was shown to assemble into the classic hexameric structure of an active AAA+ protein (12). However, it is unclear whether other torsin proteins assemble into distinct AAA+ oligomers or co-oligomerize and potentially act as torsinA ‘co-factors’. Therefore, although mammalian genomes possess multiple torsin genes, it is far from clear that torsinB, torsin2 or torsin3 proteins are functionally homologous to torsinA.

RESULTS

Signal sequence bearing AAA+ proteins are encoded by all animal genomes and we subsequently refer to such proteins as torsins. The human genome contains four torsin genes that vary in the length and nature of their amino terminal domains. TOR1A/torsinA and TOR1B/torsinB have similar predicted relative molecular mass (Mr) and both possess short hydrophobic sections, TOR3A/torsin3 is larger and contains a unique extended amino terminus, and the shorter TOR2A/torsin2 lacks a distinguishing amino terminal domain between the signal sequence and AAA module (Fig. 1A and B).

We extended this comparison of torsin proteins to include the predicted torsins of mouse (Mus musculus), chicken (Gallus gallus), zebrafish (Danio rerio), Caenorhabditis elegans and Drosophila melanogaster (Fig. 1A). Many of these torsin proteins contain amino terminal or internal hydrophobic domains, in addition to ER targeting sequences, suggesting that membrane association is a semi-conserved feature of torsin family proteins. ClustalW2 analysis grouped the majority of vertebrate torsins into torsinA, torsinB, torsin2- or torsin3-type classes. This categorization into torsin subgroups is also supported by both the exon–intron organization and chromosomal location of each vertebrate torsin gene. TorsinA- and torsinB-type genes are immediately adjacent, and oppositely oriented; a torsin2-type gene is located in a nearby chromosomal region, and all three possess a five-exon organization (Fig. 1A). In contrast, the longer torsin3-type torsins are encoded by an alternative six-exon/five-intron structure and are located on a distinct chromosome to the cluster of five-exon torsin genes.

All four human torsin genes are present in mouse and chicken, although only torsin2 and torsin3 are also present in zebrafish, which instead contains two zebrafish-specific torsin gene clusters on chromosomes 2 and 6. In addition to several ‘pseudo’ torsin genes that lack predicted signal sequences, each zebrafish torsin gene cluster also possesses hydrophobic domain containing torsins that could be functionally analogous to the torsinA- and torsinB-type torsins (Fig. 1A). This analysis suggests that torsin2 and torsin3 are ancient vertebrate torsin genes that pre-date separation of fish from mammalian/avian lineages, whereas more recent gene duplication events at the original torsin2 locus generated a cluster of five-exon torsins. Although the torsins of C. elegans and D. melanogaster do not fall into the vertebrate torsin sub-categories, they share several torsin specific sequence elements that suggest a single ancestral AAA gene gave rise to the metazoan torsin family.

The mammalian ER contains four torsin AAA+ proteins

TorsinA and torsinB both contain hydrophobic amino terminal domains and the human proteins are 84% similar overall. Although torsin2 and torsin3 are less similar to torsinA (62 and 50%, respectively), all four predicted proteins are homologous in the core AAA domain and the functionally important region affected by the DYT1 dystonia mutation (Fig. 1B). In order to determine whether torsin protein expression is overlapping, and consistent with redundant function in mammalian cells, we first tested the antigen specificity of a panel of rabbit polyclonal anti-torsin antibodies. We found that each detects a protein with the predicted Mr, for the respective torsin antigen, although anti-torsin3 only detects such a protein in human cell lysates. In each case, transfecting cell lines with siRNA that targets individual torsins specifically affects the immunoreactivity (IR) of the appropriate anti-torsin antibody; thus, verifying that each antibody specifically detects an endogenously expressed torsin protein (Fig. 2A and B). We proceeded to...
examine the glycosylation state of each torsin protein in order to monitor ER targeting and movement through the secretory pathway. Endoglycosidase H (EndoH) cleaves the N-linked oligosaccharides that are added during translocation into the ER lumen and therefore monitors the efficiency of this process. In addition, since oligosaccharides become resistant to EndoH once mannose residues are removed by cis-Golgi localized enzymes, assessment of EndoH sensitivity also provides a read-out of protein trafficking through the secretory pathway (25). We find that EndoH digestion reduces the Mr of all torsinA, torsinB, torsin2 and torsin3 protein present in whole cell preparations (Fig. 2C). Since any non-translocated, cytosolic torsin protein is expected to exist as a smaller, EndoH insensitive species, this suggests that all four torsins...
are efficiently targeted into the ER lumen in vivo. Furthermore, the absence of EndoH insensitive torsin proteins also strongly suggests that each torsin is retained within the ER system, consistent with the possibility of functional redundancy within this protein family.

We also found that the torsins present in liver microsomes, or in digitonin permeabilized HEK293T cells, are entirely protected from trypsin protease digestion, therefore suggesting that all are located within the ER lumen (Fig. 2D). This biochemical assessment also shows that multiple torsins are co-expressed in mouse liver and we subsequently expanded this analysis to other adult mouse tissues. In general, torsin protein levels vary with those of the ER chaperone calreticulin (calret), suggesting that cell-type differences in ER volume account for some of the variability in torsin expression level. There are also some tissue types where certain torsin concentrations appear particularly high or low; one notable example is the high torsinB expression in striated muscle (Fig. 2E).

Since anti-torsin3 fails to detect mouse protein, we examined torsinA, torsinB and torsin3 levels in commonly used human cell lines (Fig. 2F) and found that both epithelial and fibroblast-type cells also express multiple torsin proteins. Taken together, this analysis of torsin protein levels indicates that the majority of cell types express disease-associated torsinA alongside other torsin proteins.

Multiple torsin proteins independently interact with LAP1

Impairment of torsinA specifically affects nuclear membrane structure (4,9), strongly suggesting that torsinA AAA+ activity is directed toward a NE lumen-localized substrate. In addition, mutating the Walker B box (WB; E to Q mutation) of AAA+ proteins typically stabilizes many AAA+ enzymes in an ATP bound, substrate-trapped state (26–32). As previously described, introducing the WBEQ mutation into torsinA causes redistribution to the NE (Fig. 3A) (7,9,10), as expected if a torsinA substrate is located in this ER subdomain. In order to examine whether other torsins also operate in this ER region, we mutated the WB box of torsinB (E178Q), torsin2 (E162Q) and torsin3 (E236Q).

Figure 2. The mammalian torsins are widely expressed ER resident proteins. (A) Specificity of rabbit anti-mouse torsin antibodies assessed by western blotting of 50 µg of 1% SDS extracted NIH-3T3 cell lysates. Anti-torsinA, anti-torsinB and anti-torsin2 antibodies each recognize a protein with the predicted Mr for the specific torsin antigen. In each case, this immunoreactivity (IR) is specifically reduced by transfection with siRNA that targets the appropriate torsin antigen. (B) Western blotting with rabbit anti-human torsin3 antibody detects a 45 kDa protein in 20 µg of 1% SDS extracted HeLa cell lysates that is absent from TOR3A siRNA transfected cells (upper panel), whereas β-actin levels in 5 µg protein remain unchanged (lower panel). (C) Glycosylation state of torsins assessed by SDS–PAGE and anti-torsin western blotting of 50 µg of 1% SDS extracted mouse liver protein lysate (torsinA, torsinB and torsin2), or HeLa cell lysate (torsin3), that was mock treated (left lanes) or digested with EndoH (right lanes). (D) Protease protection of torsins present in mouse liver microsomes (torsinA, torsinB, torsin2) or digitonin permeabilized HEK293 cells (torsin3). Preparations were incubated with trypsin in the presence or absence of 1% Triton X-100 detergent and then SDS–PAGE and anti-torsin western blotting assessed protein digestion (50 µg protein in each condition). (E and F) Torsin and calret protein levels in 50 µg of 1% SDS extracted adult mouse tissue and human cell lysates.
WB_{EQ}-torsinB and WB_{EQ}-torsin2 mutants both concentrate in the NE of BHK21 cells compared with wild-type (WT) proteins (Fig. 3B and C, compare left and right panels). In contrast, WT and WB_{EQ}-torsin3 proteins were similarly distributed and were predominantly located in the main ER compartment, although each displayed some NE enrichment compared with the ER localized, protein disulphide isomerase (PDI; Fig. 3D).

Although this finding suggests that torsinA, torsinB and torsin2 all operate on NE localized binding partners, it is possible that the distinct torsin monomers assemble into mixed heteromeric AAA⁺ enzymes, and thereby operate in concert. TorsinA migrates as a /C2437 kDa M_r protein in SDS–PAGE, although mild non-ionic detergents extract a /C24240 kDa torsinA species when assessed by Blue Native PAGE (BN-PAGE) (12). This is consistent with torsinA monomers existing within a typical hexameric AAA⁺ enzyme. We confirmed that BN-PAGE separates monomeric and oligomeric torsinA species by detecting torsinA monomers within a /C24240 kDa complex when BN-PAGE separated proteins are subjected to a second dimension of SDS–PAGE (Fig. 4A).

Since we previously established that both U2OS cells and mouse liver contain multiple torsin proteins, we proceeded to examine and compare the oligomeric states of torsinA, torsinB, torsin2 and torsin3. We find that /C2437 kDa torsinB also migrates within a /C24240 kDa complex when BN-PAGE separated proteins are subjected to a second dimension of SDS–PAGE (Fig. 4A). Since we previously established that both U2OS cells and mouse liver contain multiple torsin proteins, we proceeded to examine and compare the oligomeric states of torsinA, torsinB, torsin2 and torsin3. We find that /C2437 kDa torsinB also migrates within a /C24240 kDa complex when BN-PAGE separated proteins are subjected to a second dimension of SDS–PAGE (Fig. 4A). Since we previously established that both U2OS cells and mouse liver contain multiple torsin proteins, we proceeded to examine and compare the oligomeric states of torsinA, torsinB, torsin2 and torsin3. We find that /C2437 kDa torsinB also migrates within a /C24240 kDa complex when BN-PAGE separated proteins are subjected to a second dimension of SDS–PAGE (Fig. 4A).

Together, these findings suggest that the majority of torsinA and torsinB subunits oligomerize into distinct complexes, although does not exclude the possibility of some heterooligomerization. We also examined whether torsinB concentrates in the NE in the absence of torsinA and found similar NE localization of WB_{EQ}-torsinB in Tor1a⁻/⁻ and Tor1a⁺/⁺ mouse embryonic fibroblast cells (Fig. 4F).

We proceeded to test whether torsinA and torsinB interact with a common NE binding partner. We initially approached this question by developing an in-cell competition assay that examined whether different WB_{EQ} torsins compete for NE association. We first confirmed competition between myc-tagged and GFP-tagged WB_{EQ}-torsinA and, as expected, found loss of NE localized GFP signal in cells co-expressing myc-WB_{EQ}-torsinA [Fig. 5A, cell highlighted by white arrow; Fig. 5B, *** indicates significantly (P<0.001) fewer cells with NE concentrated GFP-WB_{EQ}-torsinA compared with DsRed control]. We subsequently extended this assay to assess competition between different WB_{EQ} torsins. This found that WB_{EQ}-torsinB also significantly inhibits the NE association of GFP-WB_{EQ}-torsinA.

**Figure 3.** NE accumulation is a conserved characteristic of torsin proteins. (A–D) Subcellular localization of transfected GFP tagged WT and WB_{EQ} mutant torsins (top row; green) compared with the ER protein, PDI (middle row; red) in BHK21 cells. Merged images (bottom row) show co-localization of torsin and PDI signal in the main ER (yellow), and torsin protein concentration in the NE (green perinuclear signal). Torsin proteins are detected using rabbit anti-GFP or rabbit anti-torsin2, and the ER is visualized using mouse anti-PDI.
compared with the DsRed control (Fig. 5A and B, \(P < 0.001\)). In contrast, WB EQ-torsin2 and WB EQ-torsin3 do not affect the degree to which GFP-WB EQ-torsinA associates with the NE, although conversely, it appeared that the presence of GFP-WB EQ-torsinA prevented the NE association of WB EQ-torsin2. This result suggests that torsinA and torsinB associate with a common NE binding partner, although torsin2 either interacts more weakly with this partner or at a distinct site within the NE lumen.

TorsinA interacts with the inner nuclear membrane protein LAP1 (8,10), but it is unknown whether this association solely underlies the NE accumulation of WB EQ-torsins. In addition, it remains unclear whether LAP1 is a torsinA substrate or a cofactor that modulates torsinA activity. It is previously established that LAP1 overexpression alone is sufficient to increase the levels of NE localized WT torsinA (8,10) and we now tested whether LAP1 is required for the NE association of WB EQ-torsinA. Transfection of NIH-3T3 cells with LAP1 siRNA caused a greater than 80% reduction in LAP1 protein levels (Fig. 5C). As expected, the predominantly ER localization of WT-torsinA was unchanged by LAP1 depletion (Fig. 5D, top row). However, the normal NE accumulation of WB EQ-torsinA was significantly reduced in LAP1 depleted cells (Fig. 5D, bottom row; Fig. 5F, ** indicates \(P < 0.01\)), revealing that this torsinA interacting protein is required for the NE localization of WB EQ-torsinA, consistent with previous studies (8,10). We subsequently extended this analysis to examine whether the NE association of other torsin proteins also requires LAP1. We found that significantly fewer cells displayed NE concentrated WB EQ-torsinB when LAP1 is removed (Fig. 5E; Fig. 5F, * indicates \(P < 0.05\)). In contrast, the subcellular localization of WB EQ-torsin2 and WB EQ-torsin3 was unaffected by LAP1 depletion, again suggesting that these torsins interact with distinct binding partners. The similar sensitivity of WB EQ-torsinA and WB EQ-torsinB suggests that they both interact with LAP1 and this is again consistent with functional redundancy between these two mammalian torsin proteins.

**TorsinB expression inversely correlates with the neuronal specific torsinA requirement**

The DYT1 dystonia mutation generates a hypoactive torsinA isoform and torsinA activity is at least partially lost in DYT1 dystonia (4). However, given that torsinA is widely expressed in both mouse and human tissues (1,4) and appears to interact with NE substrates in many cell types (7,9), it is unclear why DYT1 dystonia symptoms are specifically neurological or why Tor1a gene deletion in mice specifically affects nuclear membranes of neuronal cells. We examined torsinA glycosylation state in neuronal and non-neuronal tissues to assess the possibility that neuronally...
expressed torsinA also performs roles outside the ER system. However, this found that torsinA present in adult mouse cortex and cerebellum has the same sensitivity to EndoH treatment as non-neuronal tissues (Fig. 6A), revealing that, independent of cell type, all torsinA enters and resides in the ER system.
Our data have shown that torsinA and torsinB share considerable sequence homology, appear to assemble into complexes with typical AAA+ enzyme structure, and the ATP bound form of both proteins appear to interact with LAP1 in the NE. We now proceeded to examine whether expression of homologous torsins can explain the neuronal specific importance of torsinA activity. This required that we assess torsin protein expression in late embryonic stage mice where it is previously established that brain and spinal cord neurons require torsinA activity, while liver, kidney, skeletal muscle, cardiac muscle and spleen are unaffected by torsinA loss (4; and unpublished data). We first determined whether Tor1a gene deletion affects either torsinB or torsin2 expression. Although this analysis found unaltered torsinB and torsin2 protein levels (Fig. 6B, compare ++/+ versus −/−), and revealed that torsinA and torsin2 are similarly expressed by neural and non-neural tissue-types, it also appeared that the central nervous system (CNS) contained considerably lower amounts of torsinB than liver or muscle (TorsinB panel of Fig. 6B).

We subsequently extended this analysis and examined torsinA and torsinB levels in additional embryonic tissues. This found uniformly low levels of torsinB across the embryonic CNS when compared with several different non-neuronal tissues (Fig. 6C, middle row). In contrast, torsinA levels are relatively equal between neuronal, epithelial and mesenchymal tissue types (Fig. 6C, upper row), although some enrichment is seen in neural tissues. In order to confirm the single embryo analysis of torsinA and torsinB levels, we also quantified CNS, muscle and kidney IR relative to that of embryonic liver, in samples prepared from several late-embryonic stage animals. This verified that relative torsinB IR is significantly lower in the CNS compared with muscle (P<0.01) or kidney (P<0.05).

![Figure 6. TorsinB expression inversely correlates with the neuronal-specific requirement for torsinA activity.](https://academic.oup.com/hmg/article-abstract/19/5/888/584464/1)
DISCUSSION

A vast number of cellular processes utilize members of the AAA+ protein family. These enzymes exist in all kingdoms of life and several eukaryotic organelles also contain specialized AAA+ family members. However, despite the generally ubiquitous presence of AAA+ proteins, the animal specific torsins are the only members with signal sequences that direct the AAA module into the secretory pathway. Our examination of torsin protein glycosylation state now reveals that in vivo translated torsin gene products both enter and are retained within the ER system. Therefore, it is likely that torsin binding partners and torsin AAA+ activity are restricted to within this organelle. In addition, since we also find broad torsin gene expression, we conclude that the ER of most mammalian cell types contains four distinct torsin proteins.

Torsin proteins were previously placed in the AAA+ superfamily on the basis of sequence analysis. Our finding that multiple torsins are present in complexes with appropriate sizes for classic hexameric AAA+ enzymes strongly supports this categorization. In addition, the torsins share particular homology with Clp/HSP100 AAA+ proteins (1,33,34) that are stabilized in a hexameric, substrate-associated state by WBEG mutations (26,35). Therefore, finding that WBEG mutations cause torsinA, torsinB and torsin2 to associate with NE binding partners also argues that these are functional AAA+ enzymes. Although WT- and WBEG-torsinA are similarly distributed in the mammalian ER system, and we failed to detect a higher-order torsin3-containing complex, we cannot exclude the possibility that torsin3 is also a functional AAA+ enzyme. TORS3 is the only interferon-regulated torsin gene (36) and torsin3 possesses a unique N-terminal extension. These features suggest that torsin3 is distinct from the NE associated torsins that are encoded by a cluster of five-exon torsin genes. The invariably presence of torsin3-type genes in vertebrates also suggests that torsin3 performs an important biological function. Therefore, rather than signifying that torsin3 is not a true AAA+ enzyme, the mixed ER/NE localization of WBEG-torsin3 could reflect binding to substrate protein(s) located in both the main-ER and NE.

The mature forms of mouse torsinA and torsinB are 82% similar, and human are 84% similar, after signal sequence cleavage. TorsinA and torsinB both possess a hydrophobic amino terminus, enter and reside within the ER lumen, associate with LAP1 in an ATP stabilized state and are present within a ~240 kDa complex. When considered together, these similarities suggest that torsinA and torsinB both possess a NE localized AAA+ activity. Consequently, the variations we find in torsin protein expression may suggest that embryonic neurons mainly depend upon torsinA activity, although both torsinA and torsinB are available in other cell types. This hypothesis provides a plausible explanation for why Tor1a gene deletion specifically affects neuronal cells, even though a great deal of experimental evidence points to a general cellular need for NE localized torsin AAA+ activity (4,7,9).

Since torsinA activity is also reduced in DYTI dystonia, and this disease is characterized by childhood-onset of specifically neurological symptoms, variations in torsinA and torsinB expression during human development could also underlie the symptom specificity of DYTI dystonia. Although torsinB expression has previously been examined during mouse and human brain development (37,38), this is the first study to assess expression between neuronal and non-neuronal tissues and it remains to be determined whether human neurons also possess relatively low torsinB levels. In addition, although the TOR1A mutation is causative for DYTI dystonia, only ~30% of DYTI1 mutation carriers develop disease (1). It is unknown whether the basis for this resistance to TOR1A mutation occurs at the molecular, cellular or neurobiological systems level. However, the multiple similarities between torsinA and torsinB raise the possibility that variable expression of either protein affects disease penetrance.

It was previously unclear whether mammalian torsins operate as individual AAA+ enzymes, or co-assemble into mixed oligomers and work in concert. Our finding that torsin2 containing complexes are smaller than those containing torsinA and torsinB, reveals that torsin2 exists as a distinct, possibly homomeric, AAA+ enzyme. This is in agreement with a previous study where only torsinB and torsin3 co-immunoprecipitate with WBEG-torsinA (8). This work also suggests that torsinA, torsinB and torsin3 are co-assembled, which contrasts with our failure to co-immunoprecipitate torsinA and torsinB, or detect torsin3-containing complexes. One possible explanation for this discrepancy is that the WBEG mutation stabilizes mixed torsin oligomers that rarely form between physiological concentrations of WT proteins. This conclusion is also supported by a previous work showing that physiologically expressed torsinA and torsinB fail to co-immunoprecipitate, despite interaction between the two when expression is increased (13). However, our data does not exclude the possibility of co-oligomerization and there is evidence that this may depend on cell-type and expression level (13). Nevertheless, independent of the degree to which torsinA and torsinB co-oligomerize, torsinA is not required for WBEG-torsinB binding to the shared NE partner. Although binding does not necessarily reflect function, this interaction suggests that torsinA and torsinB may independently operate in the NE and is consistent with the hypothesis that torsinB expression underlies non-neuronal tissue resistance to torsinA loss.

We demonstrate here that several mammalian torsins appear to operate on a NE localized substrate or substrates. In addition, a C. elegans torsin, OOC-5, also localizes to the NE upon loss of an ER localized interacting protein (39) and ooc-5 deficient animals fail to perform a developmentally essential nuclear rotation (40). Therefore, the ability to generate conformational change in NE localized substrate(s) may ultimately be found to underlie the evolution of the animal specific torsin proteins. The identity of ooc-5 NE substrate(s) remains unknown and therefore the cellular role of torsins also remains to be determined. LAP1 is a torsinA interacting protein that interacts more strongly with WBEG-torsinA than WT torsinA (8,10). Considering this alongside our finding that LAP1 is required for the NE association of WBEG-torsinA and WBEG-torsinB, again suggests that LAP1 may be a NE localized torsin enzyme substrate. However, as a counter point, we also note that the LAP1 insensitive NE concentration of WBEG-torsin2 suggests other torsin interacting proteins may be present in this region. Although widely expressed, and one of the first inner nuclear membrane proteins to be
identified (10,41–43), the function of LAP1 remains unknown and torsins are the only identified luminal interacting proteins. Other NE proteins operate in transcriptional regulation, nuclear–cytoplasmic transport, nuclear–cytoskeletal coupling, lipid and membrane biogenesis, nuclear subdomain organization and maintain the structural integrity of the nucleus (44–47). Notably, some studies have suggested a relationship between torsin protein function and nuclear–cytoskeletal connections (12,40,48,49) and, consequently, it is attractive to hypothesize that animals require NE AAA+ activity in order to modify the developmentally important complexes that couple nucleus to cytoskeleton.

MATERIALS AND METHODS

Plasmid construction

PCR amplification used Pfx high fidelity PCR (Invitrogen) and site-directed PCR mutagenesis used Quikchange (Stratagene), both according to the manufacturer’s instructions. In all circumstances, the entire coding domain was sequenced to ensure fidelity of amplification. Cloning of human TOR1A/torsinA constructs and epitope tagging has been previously described (7). The human TOR1B/torsinB cDNA was amplified from HeLa cell cDNA using ATGTTGCAGGCTGGTG GGCT and TCAGTGAAATCCAGCCGC primers, followed by TOPO cloning into pCR II (Invitrogen) and subsequent subcloning into pcDNA (Invitrogen). Site-directed mutagenesis was used to insert a NheI restriction site between codons 25 and 26 and to mutate glutamic acid residue 178 in order to produce the E178Q Walker B substrate trap protein. The human TOR1B/torsinB and torsin2 were generated by inoculating animals with peptides (Tor1b CKTVQRSLDFH; Tor2a CKTVASRL TFFL) conjugated to Keyhole Limpet Hemocyanin (KLH; Pierce) in Freud’s Complete Adjuvant, followed by a series of booster inoculations with peptide-KLH conjugate and Freud’s Incomplete Adjuvant. TorsinB and torsin2 affinity columns were generated by covalently coupling peptides to Sulfolink Coupling resin (Pierce) and antibodies were affinity purified from serum as described above. Antibodies were also purchased from the following vendors: rabbit anti-human torsin3 (Aviva Systems Biology), mouse anti-PDI (Stressgen), rabbit anti-calret (Sigma), mouse anti-myc clone 9E10 (ATCC), chicken anti-GFP (Chemicon) and mouse anti-β actin (Sigma). All animals were used in accordance with the National Institutes of Health guidelines for the use of live animals and the animal protocol was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Cell culture and transfection

BHK21 fibroblasts, U2OS, HEK 293T/17, HeLa and NIH-3T3 cells were obtained from ATCC and were cultured using recommended conditions. The generation and culturing of a BHK21 cell line that stably expresses GFP-WBEQ-torsinA, and generation and culturing of Tor1a+/− and Tor1a−/− MEF cell lines, are previously described (4,7). Cells destined for immunofluorescent labeling were cultured on collagen (Sigma) coated coverglasses (Carolina Biological Supply). Plasmid transfections were performed using Lipofectamine and Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer’s instructions. Transfected cells were fixed at 24 h post-transfection by 20 min incubation with 4% formaldehyde, followed by washing with 1× PBS. Torsin siRNA molecules were transfected into NIH-3T3 cells using RNAiMAX (Invitrogen) according to the manufacturers’ instructions. Cells were collected at 48 h post-transfection, washed once with 1× PBS and prepared for anti-torsin western blotting as described below. The following duplexed oligonucleotides were used to reduce mouse torsin protein expression. TorsinA: CCG GAA CCA CAU AGA UUA U and UUA AUG UAU GAG GGU CCG G. TorsinB: CCG UGC CAC CAG AGU GUA U and AAU AAC UUC GGU GGC AAG G. Torsin2: GCC CUA CUC GGG UUG UGU A and UAC ACA ACC CAA GAA GGG C. Torsin3: GGU AGU CCA GGU UCU GGU and AAAG CCA ACU CAG GAC UAC C. Human torsin3 expression was reduced using: AAG UUG UUG GAG AUU CUG C and GCA GAA UCU CCA ACA ACU U. Combined siRNA and plasmid transfection of NIH-3T3 cells was performed using Lipofectamine.
2000 reagent (Invitrogen). These cells were either fixed 48 h post-transfection, or trypsinized, pelleted, washed with 1× PBS and lysed as described below. LAPI knockdown was performed using siRNA oligonucleotides CCA CUG CCG UUC AAG AAU U & AAU UCU UGA ACG GCA GUG G and control oligonucleotides CCA CCG UUG AAC AGC UAU U & AAU AGC UGU UCA ACG GUG G.

**Protein characterization**

Torsin protein levels and glycosylation states were assessed by extracting proteins from BHK21, NIH-3T3, HeLa cells and mouse tissues by homogenization in extraction buffer (1% SDS, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM PMSF, (Sigma), 1× Protease Inhibitor Cocktail (Sigma)). Proteins were further solubilized by 20 min incubation with gentle rocking, and then insoluble material removed by centrifugation for 10 min at 20 000g. Protein concentrations were determined using the BCA Protein Assay (Pierce). The glycosylation state of torsins present in 50 μg of 1% SDS extracted mouse liver or HeLa cell lysates were assessed using EndoH (New England Biolabs) according to the manufacturer's instructions, followed by SDS–PAGE and anti-torsin western blotting.

Microsomal preparations were generated by disrupting mouse liver in at least 10 volumes of 250 mM sucrose, 10 mM HEPES (pH 7.3), 10 mM KCl, 100 mM NaCl, 1.5 mM MgCl2, 5 mM EDTA, 1 mM PMSF, 1× Protease inhibitor cocktail, using six strokes of a glass-Teflon homogenizer. The homogenate was centrifuged twice at 10 000g for 5 min, twice at 10 000g for 10 min and then the light membrane fraction and cytosol separated by 90 min centrifugation at 100 000g. Protease protection was performed after resuspending the microsomal pellet at 1 μg/μl in microsome buffer lacking protease inhibitors. Suspended microsomes were then incubated for 30 min on ice with 0 or 50 ng trypsin/μg protein, in the presence or absence of 1% Triton X-100 detergent (Pierce). Protease digestion were halted by adding PMSF to a final 1 mM concentration, followed by an equal volume of 2× Laemmli sample buffer and heating to 95°C for 10 min. Protease protection of HEK293 cells permeabilized by 10 min incubation in microsome buffer including 40 μg/ml digitonin (Sigma) was similarly performed, except that digests were performed for 45 min in the presence of 100 ng trypsin/μg protein. SDS–PAGE and anti-torsin western blotting were then used to analyze protein degradation. Mice were used in accordance with the National Institutes of Health guidelines for the use of live animals and the animal protocol was approved by the University of Tennessee Institutional Animal Care and Use Committee.

Blue Native PAGE (BN-PAGE) was performed essentially as described in (50) and (12). Microsomal pellets were solubilized for 20 min in BN-PAGE buffer (50 mM imidazole (pH 7.0), 50 mM NaCl, 4 mM MgCl2, 2 mM ATP, 2 mM 6-aminocaproic acid (Fisher), 1 mM PMSF and 1× protease inhibitor cocktail including either 0.1% n-Dodecyl-β-D-maltoside (Invitrogen), 0.5 or 1% digitonin). U2OS cell proteins were also extracted and prepared for BN-PAGE by homogenization and gentle rocking in BN-PAGE buffer. Insoluble material was removed from microsome preparations by two, 30 min centrifugations at 100 000g and from U2OS cell lysates by two, 10 min centrifugations at 20 000g. Following BN-PAGE separation, entire lanes of electrophoresed samples were excised and prepared for SDS–PAGE as described in the instructions for NativePage Gel System (Invitrogen). Following SDS–PAGE, gels were processed for western blotting using standard conditions. TorsinA was immunoprecipitated from U2OS lysates prepared as described for those used in BN-PAGE. Lysates were then precleared by 30 min incubation with ProteinA-agarose (Pierce), followed by 1 h incubation with anti-torsinA, and complete immune complexes captured by 3 h incubation with additional ProteinA-agarose. Complexes were washed by four, 5 min incubations in fresh solubilization buffer and then immune complexes eluted by addition of 2× Laemmli sample buffer and 10 min incubation at 95°C. The levels of torsin proteins in U2OS cell lysates and immune complexes were determined by SDS–PAGE and anti-torsinA or anti-torsinB western blotting.

Western blotting of nitrocellulose membranes (Biorad) used standard blocking, primary antibody and washing steps. Primary antibodies were detected using peroxidase-conjugated Immunopure secondary antibodies (Pierce) or light chain specific anti-rabbit (Jackson Immunoresearch). Peroxidase activity was assessed using Supersignal West Dura reagent (Pierce) and visualized by exposure to Hyblot film (Denville Scientific). Immunoreactive band optical density measurements were acquired using Photoshop. A region of interest was first sized so that it encompassed the largest immunoreactive band area present on a film. The average pixel intensity within this fixed area was then determined for immunoreactive bands from each tissue type. These measurements were normalized to the pixel intensity of liver IR; therefore generating a ‘relative IR’ versus that of liver and decreasing inter-experiment variation due to film background or exposure length. A mean CNS IR value was generated for embryos where multiple CNS regions were assessed. Statistical analysis of data was performed using GraphPad Prism.

**Immunolabeling**

Cells cultured on cover-glasses were transferred to a ParaFilm support within a humidified chamber, incubated for 1 h at room temperature in normal donkey serum (NDS; Jackson Immunoresearch) block solution (1× PBS, 0.25% Triton X-100, 10% NDS). Cells were then incubated overnight at 4°C in NDS-blocking solution including primary antibodies. The following day, samples were washed six times with 1× PBS, incubated for 1 h with secondary antibodies diluted in NDS-blocking solution, washed and prepared for fluorescent microscopy by inverting coverslips into ‘Vectashield with DAPI’ (Vector Laboratories) on a microscope slide. Fluorescent secondary antibodies used were donkey anti-rabbit FITC or Rhodamine Red X, anti-chicken FITC, anti-mouse Rhodamine Red X (all from Jackson Immunoresearch). Labeled samples were examined using a Nikon Eclipse Ti and imaged with Nikon Digital Sight DS-QiMc and NIS-elements BR3.0. The percentage of cells containing NE concentrated GFP-WB5O-torsinA was assessed by blinded quantification of GFP subcellular localization in 50 cells that were also immunoreactive for myc, torsinB, torsin2 or

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torsi
orsin3 or were positive for red fluorescent DsRed signal. The effects of LAP1 siRNA knockdown on the NE accumulation of EQ-torsins was determined by blinded quantification of GFP localization in at least fifty cells. Statistical analysis of cell quantification data was performed using GraphPad Prism.

ACKNOWLEDGEMENTS

We thank Dauer Lab members, and Bill Dauer in particular, for the anti-torsinA antibody, multiple plasmids and years of torsin-protein related discussion. We also wish to acknowledge Xandra Breakefield’s kind gift of an anti-torsinB antibody used in preliminary experiments and technical support provided by Rex Rui and Kirsten Raby.

Conflict of Interest statement. None declared.

FUNDING

This work was partly funded by the Dystonia Medical Research Foundation and we would like to thank the foundation for their general and wide-ranging support.

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