TorsinA binds the KASH domain of nesprins and participates in linkage between nuclear envelope and cytoskeleton

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Summary
A specific mutation (ΔE) in torsinA underlies most cases of the dominantly inherited movement disorder, early-onset torsion dystonia (DYT1). TorsinA, a member of the AAA+ ATPase superfamily, is located within the lumen of the nuclear envelope (NE) and endoplasmic reticulum (ER). We investigated an association between torsinA and nesprin-3, which spans the outer nuclear membrane (ONM) of the NE and links it to vimentin via plectin in fibroblasts. Mouse nesprin-3α co-immunoprecipitated with torsinA and this involved the C-terminal region of torsinA and the KASH domain of nesprin-3α. This association with human nesprin-3 appeared to be stronger for torsinAΔE than for torsinA. TorsinA also associated with the KASH domains of nesprin-1 and -2 (SYNE1 and 2), which link to actin. In the absence of torsinA, in knockout mouse embryonic fibroblasts (MEFs), nesprin-3α was localized predominantly in the ER. Enrichment of yellow fluorescent protein (YFP)-nesprin-3 in the ER was also seen in the fibroblasts of DYT1 patients, with formation of YFP-positive globular structures enriched in torsinA, vimentin and actin. TorsinA-null MEFs had normal NE structure, but nuclear polarization and cell migration were delayed in a wound-healing assay, as compared with wild-type MEFs. These studies support a role for torsinA in dynamic interactions between the KASH domains of nesprins and their protein partners in the lumen of the NE, with torsinA influencing the localization of nesprins and associated cytoskeletal elements and affecting their role in nuclear and cell movement.

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Key words: Nesprin, Dystonia, Cell migration, Nuclear polarization, DYT1, Vimentin, Actin

Introduction
Early-onset generalized torsion dystonia (DYT1) is a severe, dominantly inherited movement disorder with onset in childhood and limited therapeutic options (Breakefield et al., 2008; Kamm et al., 2008). Most cases of DYT1 are caused by a specific mutation (AGAG) resulting in loss of a glutamic acid in the C-terminal region of torsinA (Ozelius et al., 1997). The mutant form of torsinA, torsinAΔE, appears to have a loss of function and to act in a dominant-negative manner to decrease function of wild-type torsinA (Goodchild et al., 2005; Dang et al., 2006; Pham et al., 2006; Hewett et al., 2008). Dystonic symptoms are believed to arise from abnormal functioning of neurons in the brain with no apparent neuronal loss (Rostasy et al., 2003; McNaught et al., 2003), and presumably interacts with transmembrane proteins concentrated in these locales. TorsinA is associated with lamin-associated polypeptide 1 (LAP1; TOR1AIP1) in the inner nuclear membrane (INM) of the NE and with LULL1 (TOR1AIP2) in the ER/NE (Goodchild and Dauer, 2005; Naismith et al., 2004), as well as with conventional kinesin light chain 1 (KLC1) (Kamm et al., 2004), vimentin and actin (Hewett et al., 2006) in the cytoplasm. The function of torsinA is unknown, but studies support a role in the structure of the NE (Goodchild et al., 2005; Naismith et al.,...
The present study was initiated based on our findings of coordinated movement of torsinA and vimentin during cellular recovery from microtubule dissociation (Hewett et al., 2006). Vimentin intermediate filaments (IFs) form a net around the NE and are involved in defining nuclear shape and mediating nuclear movement (Djabali, 1999). Cells lacking vimentin have abnormally shaped nuclei with invaginations (Sarria et al., 1994) and a reduced rate of migration as compared with control cells in wound assays (Eckes et al., 1998). Vimentin also participates in the distribution of NE membrane/LAP1-containing vesicles during mitosis (Maison et al., 1997), in the movement of the ER during cell migration (Eckes et al., 1998), and in early neurite outgrowth (Dubey et al., 2004). Loss of a nematode ortholog of torsinA, OOC-5, disrupts nuclear rotation during early embryogenesis (Basham and Rose, 2001).

The structure, shape and movement of the nucleus is maintained, at least in part, by interactions between proteins, such as the SUNs, which span the INM and interact with the IF lamins in the nucleus, and the nesprins (SYNEs), which span the outer nuclear membrane (ONM) and link to cytoskeletal elements, including IFs, microtubules and actin microfilaments, in the cytoplasm (Crisp et al., 2006; Starr and Fischer, 2005; Wilhelmsen et al., 2006). The SUN domain of INM proteins interacts with the KASH domain of nesprins in the luminal space. Nesprin-1 and -2 (SYNE1 and 2) interact with actin microfilaments, UNC-83 (in nematodes) interacts with microtubules, and mouse nesprin-3α (the homolog of human nesprin-3) interacts with the cytolinker/plakin, plectin (Wilhelmsen et al., 2005; Wilhelmsen et al., 2006), which in turn links to IFs (Sonnenberg and Liem, 2007). Like the other nesprins, nesprin-3 is a type II transmembrane protein and contains a C-terminal KASH domain that lies within the luminal space of the NE. Unlike nesprin-1 and -2, nesprin-3 lacks an actin-binding domain (ABD) and is therefore unable to associate with actin directly, but forms an interconnected mesh with actin microfilaments, microtubules and IFs through plakins (Wilhelmsen et al., 2006; Chang and Goldman, 2004; Herrmann et al., 2007). Recent commentaries on the links between the NE and cytoskeleton have suggested a possible role for torsinA in modulating the interaction between SUNs and nesprins within the NE lumen (Gerace, 2004; Worman and Gundersen, 2006).

In this study, we have investigated the association between torsinA in the NE and plectin/vimentin in the cytoplasm in primary cultures of torsinA-null and control mouse embryonic fibroblasts (MEFs), human skin fibroblasts from DYTI1 patients and controls, mouse pup fibroblasts (MFs) and human 293T cells, using recombinant proteins, immunoprecipitation, pull-downs, immunocytochemistry and confocal microscopy. We found that torsinA associates with the C-terminal KASH domains of nesprin-3α, nesprin-1 and nesprin-2. Under some conditions, the DYTI1 mutant form of torsinA, torsinAΔE, associated more strongly with human nesprin-3 than did torsinA. TorsinA-null MEFs showed reduced polarization of nuclei relative to the centrosome, and delayed migration in wound-healing assays. These studies support a role for torsinA in modulating dynamic interactions between the KASH domain of nesprins and their binding partners in the lumen of the NE, with torsinA loss causing an enrichment of nesprin-3 in the ER.

Results

Interaction between torsinA and mouse nesprin-3α, plectin and vimentin

Immunoprecipitations carried out in previous studies showed associations between torsinA, vimentin and actin (Hewett et al., 2006) and between nesprin-3α, plectin and vimentin (Wilhelmsen et al., 2005; Wilhelmsen et al., 2006). In order to evaluate whether endogenous torsinA participates in a composite complex that includes these proteins, we carried out immunoprecipitations of torsinA using lysates of wild-type MFs (Andrà et al., 2003). Immunoprecipitations were performed in RIPA buffer using antibodies to torsinA (D-M2A8 and D-MG10, 1:1) and to immunoglobulin G (anti-IgG) as a control. Proteins in lysates and precipitates were resolved by SDS-PAGE and blots probed with antibodies to plectin (300 kDa), nesprin-3α (120 kDa), vimentin (55 kDa), torsinA (37 kDa) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 36 kDa) (Fig. 1). Nesprin-3α, plectin and vimentin co-immunoprecipitated with torsinA, with very little or no co-immunoprecipitation of the cytoplasmic control protein GAPDH. This demonstrates an association between endogenous torsinA, nesprin-3α, plectin and vimentin in fibroblasts.

Association of torsinA and torsinAΔE with human nesprin-3

In order to evaluate the potential relevance of the torsinA-nesprin-3 association in DYTI1 dystonia, we evaluated the relative association of torsinA and torsinAΔE in this complex. Human nesprin-3 exhibits 94.5% homology with mouse nesprin-3α, and shares the plectin-binding domain (PBD) (supplementary material Fig. S1). As no anti-human-nesprin-3α antibody is available, we used a tagged version consisting of human nesprin-3 fused in-frame at the N-terminus to yellow fluorescent protein (YFP), termed YFP-nesprin-3α. When this fusion protein was expressed in control primary human fibroblasts via a lentivirus vector, most fluorescence was found around the nucleus, typical of the localization of nesprin-3α described in mouse cells (Wilhelmsen et al., 2005) (Fig. 2A,D). Immunocytochemical staining of control cells expressing YFP-nesprin-3α (ΔE) with human nesprin-3 expressed in 293T cells. These results suggested that the presence of torsinAΔE inhibited the interaction of YFP-nesprin-3α with other binding partners in the NE, leading to an aggregation of overexpressed YFP-nesprin-3α, cytoskeletal binding partners and torsinA-immunoreactive protein primarily in the ER.

To further evaluate the association of YFP-nesprin-3α and torsinA we carried out immunoprecipitation from 293T cells co-transfected with YFP-nesprin-3α and torsinA or torsinAΔE expression cassettes. Forty-eight hours post-transfection, cell lysates were immunoprecipitated with antibodies to green fluorescent protein (GFP) (which also recognize YFP) and IgG (as a control), followed by SDS-PAGE and immunoblotting with antibodies for torsinA and GAPDH. Because ATP is involved in the interaction of AAA+ proteins with their substrates, immunoprecipitations were carried out in the presence of 1 mM ATP, non-hydrolysable ATP, or EDTA (with no added ATP) to evaluate the role of ATP in torsinA-nesprin-3α interactions. Both torsinA and torsinAΔE were more efficiently immunoprecipitated with YFP-nesprin-3α in the presence of ATP or non-hydrolysable ATP than of EDTA (Fig. 3A). TorsinA and
torsinAΔE were not found to associate with IgG and showed no co-immunoprecipitation with GAPDH (Fig. 3A). In the presence of ATP, less torsinA (8±0.57%, n=3) was immunoprecipitated with YFP-nesprin-3 than torsinAΔE (25±0.88%, n=3, P<0.0001, two-way ANOVA). These findings suggest that the association of both torsinA and torsinAΔE with YFP-nesprin-3 is enhanced by ATP, and that there is a tighter association of torsinAΔE than of torsinA with YFP-nesprin-3.

Our laboratory has previously shown that overexpression of torsinB, which is highly homologous to torsinA (Ozelius et al., 1997), or of torsinAΔE, leads to an enrichment of immunoreactive protein in the ER and perinuclear region in large inclusion bodies (Hewett et al., 2004). We evaluated the association between endogenous torsinB and YFP-nesprin-3 in 293T cells (supplementary material Fig. S3). Antibodies to torsinB were able to immunoprecipitate YFP-nesprin-3, indicating that both torsinB and torsinA can associate with nesprin-3 and thus might carry out related functions in the NE.

To evaluate whether the C-terminal region of torsinA is involved in the association with nesprin-3, 293T cells were co-transfected with YFP-nesprin-3 and torsinA, torsinAΔE or torsinAΔ312-332 (Fig. 3B). Forty-eight hours post-transfection, cell lysates were immunoprecipitated with antibodies to GFP and IgG (as a control), followed by SDS-PAGE and immunoblotting with antibodies for torsinA and GAPDH. Again, torsinAΔE associated more tightly with YFP-nesprin-3 than did torsinA, whereas there was no association of YFP-nesprin-3 with torsinAΔ312-332, indicating that the C-terminal region of torsinA is necessary for this association. To certify that torsinA binds specifically to nesprin-3 and not to YFP, 293T cells were also co-transfected with YFP and torsinA or torsinAΔE (Fig. 3C). Forty-eight hours post-transfection, cell lysates were immunoprecipitated with antibodies to torsinA followed by SDS-PAGE and immunoblotting with antibodies for torsinA and GFP. TorsinA or torsinAΔE did not immunoprecipitate YFP alone. Our recent results, and those by Phyllis Hanson’s group (School of Medicine, Washington University in St Louis, MO; personal communication), suggest that torsinA has two independent binding domains: one in the N-terminal α-β sub-domain of the AAA+ domains that binds LULL1 and LAP1 (transmembrane proteins in the INM), and one in the C-terminal α-helical sub-domain that binds the KASH domain of nesprins. It remains to be tested whether torsinA can bind both types of partners simultaneously.

A difference in the binding of torsinA and torsinAΔE to nesprin-3α was also detected in pull-down experiments using recombinant maltose-binding protein (MBP) fusion proteins of torsinA. Both MBP-torsinA and MBP-torsinAΔE fusion proteins were able to pull down endogenous nesprin-3α in lysates from MFs in the presence of ATP.
with MBP-torsinAΔE consistently pulling down ~threefold more than torsinA (3.0±0.88, n=3, P<0.003, two-way ANOVA) (Fig. 4A).

Association of the KASH domain of nesprin-3 with torsinA

If torsinA associates with nesprin-3 it must do so through its KASH domain, which is the only portion of nesprin-3 located in the lumen of the NE, where torsinA resides. To evaluate whether torsinA binds directly to nesprin-3 KASH sequences, MBP-torsinA and MBP-torsinAΔE recombinant proteins were mixed 1:1 with GST or GST-KASH-3α and bound to glutathione-Sepharose TM4B beads in the presence of ATP. The mouse nesprin-3α KASH domain exhibits a high degree of homology with that of human nesprin-3 (88%) and the two proteins also share a PBD domain in the N-terminal region (supplementary material Fig. S1). Unbound material was removed by centrifugation and washing, and bound proteins resolved by SDS-PAGE and immunostained with antibodies to MBP and GST (Fig. 4B). Direct binding of wild-type and mutant torsinA fusion proteins to GST-KASH-3α was observed, but with no apparent binding differential between mutant and wild-type torsinA under these in vitro conditions.

Nesprin-3α has an alternatively spliced exon 1 variant, nesprin-3β, which retains the KASH domain that determines NE localization but is unable to associate with plectin (Wilhelmsen et al., 2005). Consistent with this, nesprin-3β also binds to torsinA (supplementary material Fig. S4).

To further evaluate the involvement of the KASH domain in the nesprin-3-torsinA complex, 293T fibroblasts were transfected with expression cassettes for GFP-nesprin-3α or GFP-nesprin-3αKASH (Wilhelmsen et al., 2005). SDS-PAGE and immunoblotting of lysates of transfected cells revealed comparable expression levels of both fusion proteins 48 hours post-transfection (data not shown). Immunoprecipitation of torsinA was carried out in cell lysates, and precipitates were resolved by SDS-PAGE and subject to western blotting using antibodies to plectin, GFP, vimentin, torsinA and GAPDH. GFP-nesprin-3α showed the previously observed association with torsinA, but GFP-nesprin-3αKASH did not (Fig. 5A). Similar amounts of torsinA were precipitated with torsinA antibodies from non-transfected cells
Expression cassettes for GFP-KASH fusion proteins derived from mouse nesprin-3 α were transfected into 293T fibroblasts and immunoprecipitated with torsinA antibodies to plectin, GFP, vimentin, torsinA and GAPDH. Similar amounts of torsinA were precipitated from non-transfected cells and cells transfected with GFP-nesprin-3αΔKASH constructs. By contrast, no detectable plectin was immunoprecipitated with torsinA from cells expressing GFP-nesprin-3αKASH, although some was immunoprecipitated from cells expressing GFP-nesprin-3α, nesprin-1 and nesprin-2. Immunoprecipitation with torsinA antibodies and immunoblotting for GFP revealed an association of torsinA with all three KASH domains (Fig. 5B).

Effect of torsinA status on the distribution of endogenous nesprin-3α

Since torsinA appears to be associated with nesprin-3α in the ONM, the absence of torsinA might affect the cellular distribution of nesprin-3α. In most torsinA+/− MEFs, nesprin-3α immunoreactivity was detected in the perinuclear region, as described for other cells (Wilhelmsen et al., 2005), as judged by co-immunocytochemistry for phosphodisulfide isomerase (PDI; red), a soluble chaperone in the lumen of the ER (Fig. 6Aa-c). By contrast, in most torsinA−− MEFs, a substantial proportion of the nesprin-3α immunoreactivity appeared to be in the ER, as confirmed by relative co-localization with PDI (Fig. 6Ad-f). The relative proportion of nesprin-3α in the perinuclear region versus the ER proper was quantitated by evaluating co-localization coefficients for ER staining of PDI and nesprin-3α using a confocal microscope (Fig. 6B-D). TorsinA+/− cells showed a predominantly perinuclear localization of nesprin-3α (co-localization coefficient with PDI of 14±5%, n=3), whereas in torsinA−− cells nesprin-3α had extensive overlap with the ER (co-localization coefficient of 87±12%, n=3; P<0.0001, two-way ANOVA), indicating that interaction with torsinA is a factor in localizing nesprin-3 in the NE.

Effect of PMA treatment on the cytoskeletal network and torsinA distribution

Treatment of human fibroblasts with phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC), causes a collapse of vimentin into the perinuclear region (Djabali, 1999). PKC is a serine-threonine kinase that phosphorylates vimentin at multiple sites, leading to partial filament disassembly (Eriksson et al., 2004). Plectin is also phosphorylated by PKC with a consequent weakening, but not elimination, of plectin-vimentin connections (Foisner et al., 1991). We confirmed that treatment of control primary human fibroblasts with PMA led to a concentration of vimentin immunoreactivity in the perinuclear region within 30 minutes (Fig. 7E,K), with no concomitant collapse of microfilaments or microtubules (supplementary material Fig. S5), and with rapid recovery upon removal of PMA and no loss of viability (data not shown). In addition to vimentin, torsinA and plectin also collapsed into the perinuclear region in PMA-treated fibroblasts (Fig. 7D,J). PMA-induced perinuclear vimentin collapse was also observed in torsinA+/− MEFs, but not in torsinA−− MEFs, consistent with a role for torsinA in maintaining nesprin-3α in the ONM and with nesprin-3α linking vimentin to the NE (data not shown).

Effects of torsinA status on nuclear structure, cell migration and nuclear polarization

Given the influence of vimentin on nuclear shape (Sarria et al., 1994) and the involvement of vimentin and plectin in cell migration (Eckes et al., 1998; Andrä et al., 1998), the question remained as to the possible functions of the complex formed between torsinA, nesprin-3α, plectin and vimentin in fibroblasts (see above). Ultrastructural evaluation of the apposition of the INM and ONM in the NE in torsinA−− MEFs did not reveal any apparent abnormalities (supplementary material Fig. S6). To evaluate whether this torsinA-nesprin association was involved in cell migration, we carried out a wound-healing assay in which swaths were made in confluent monolayers of MEFs and the rate of migration of the leading edge...
was evaluated using both a standard assay (Gomes and Gundersen, 2006) and a new method employing microfabricated coverslips. In the latter method, cells are plated on coverslips that are partially covered by a thin polydimethylsiloxane (PDMS) membrane coincident with micropatterned marks, and then covered with a thin layer of chrome (Fig. 8A) that does not affect cell adhesion or movement (Hunter et al., 1995). Cells were allowed to form a confluent monolayer on this surface and then a wound was initiated by removal of the PDMS membrane, made visible microscopically by the absence of chrome in the wound region. A significant delay in the migration of torsinA–/– cells was noted, with only 23±7% of the distance covered by torsinA+/+ cells at 4 hours post-wounding, 31±4% at 8 hours and 47±3% at 26 hours (n=7; P<0.0001 at all time points, two-way ANOVA) (Fig. 8B,C).

As an initial step in cell migration, we also evaluated polarization of the nucleus relative to the wound edge by immunocytochemistry for pericentrin, as to determine the position of the centrosome relative to the nucleus (DAPI staining), as described (Gomes et al., 2005). At 3 hours post-wounding, there was a significant difference between the extent of nuclear polarization, with 85±5% of torsinA+/+ cells and only 38±7% of torsinA–/– cells (n=3; P<0.001, two-way ANOVA) showing positioning of the nucleus behind the centrosome relative to the wound edge (Fig. 9). Together, these findings suggest that torsinA is not crucial to the structure of the NE per se in fibroblasts, but does affect the positioning of the nuclei within migrating fibroblasts and the rate of movement of these cells.

Discussion

This study demonstrates an association between torsinA in the lumen of the NE and the transmembrane protein nesprin-3 in the ONM of the NE, which in turn links to plectin (Wilhelmsen et al., 2005) and vimentin (Sonnenberg and Liem, 2007) in the cytoplasm. Using recombinant proteins, this association appears to involve a direct interaction between the KASH domain of nesprin-3 and the C-terminal region of torsinA. TorsinA also binds to the KASH domains of nesprin-1 and -2, which interact with cytoplasmic actin (Crisp et al., 2006; Starr and Fischer, 2005) (see Fig. 10A). TorsinA appears to play a role in the localization of nesprin-3 because in fibroblasts lacking torsinA, nesprin-3α is concentrated in the ER (Fig. 10B, diagram), whereas in wild-type cells it is localized predominantly in the NE region. Furthermore, the collapse of vimentin into the perinuclear region in response to PKC activation was abrogated in torsinA-null cells, consistent with vimentin being normally anchored to the NE by nesprin-3/plectin. The accumulation of nesprin-3α in the ER was also observed in a previous study upon overexpression of GFP-nesprin-3αΔPPPT (nesprin-3α deleted for the SUN-binding domain) (Ketema et al., 2007), consistent with the interaction of nesprin-3α with both torsinA and SUN proteins. The dystonia-associated inactive mutant form of torsinA, torsinAΔE, was found under some conditions to bind more tightly to nesprin-3 than did torsinA. Thus, when YFP-nesprin-3 was overexpressed in control fibroblasts, it showed a predominantly NE localization, whereas...
in fibroblasts from DYT1 subjects it resulted in YFP-nesprin-3 globular structures located predominantly in the ER compartment that were enriched in torsinA, vimentin and actin (Fig. 10C). This torsinA-nesprin-cytoskeletal association does not appear to be involved in maintaining the structure of the NE in fibroblasts per se, but torsinA-null fibroblasts showed a reduced ability to position nuclei behind the centrosome in relation to the leading edge of cell migration in a wound assay, and migration itself was delayed in torsinA-null as compared with wild-type fibroblasts.

Relationship between torsinA and the cytoskeleton
TorsinA has been hypothesized to be involved in modulating connections between transmembrane proteins in the INM, such as LAP1 and SUNs, and those in the ONM, such as nesprins, which in turn connect with cytoskeletal elements (Gerace, 2004; Worman and Gundersen, 2006). The present study supports a complex of torsinA-nesprin-3-pectin-vimentin in fibroblasts, with torsinA also associating with the KASH domains of nesprin-1 and -2 and hence presumably with complexes involving actin microfilaments. As nesprin-3 is primarily localized in the NE (Wilhelmsen et al., 2005), and torsinA is found in both the NE and ER, torsinA must have other partners in the ER. Since both SUNs (Starr and Fischer, 2005) and torsinA (this study) bind to the KASH domain of nesprins, it is hypothesized that, as an AAA+ protein, torsinA might confer a dynamic aspect to the interaction between nesprin and SUN proteins in the lumen of the NE.

Vimentin dynamics
Vimentin, a member of the large family of IF proteins, is especially abundant in fibroblasts and extends out from the nucleus to the plasma membrane in a dense filamentous network interlinked to actin filaments and microtubules through plakins (Wiche, 1998). Vimentin-knockout mice do not have an obvious phenotype (Colucci-Guyon et al., 1999), but their fibroblasts have impaired mechanical stability and contractility with poorly organized actin.
filaments and reduced directional migration (Eckes et al., 1998). A similar cellular phenotype was observed in plectin-knockout fibroblasts, again implicating an effect on actin dynamics (Andrä et al., 1998). Vimentin is also involved in initial neurite outgrowth from neurons (Dubey et al., 2004) and in retrograde signaling following nerve injury and regeneration (Perlson et al., 2005), with vimentin-null mice having abnormal brain morphology (Colucci-Guyon et al., 1999). Thus, changes in IF dynamics caused by loss of, or mutations in, torsinA could affect a wide range of neuronal properties, including process extension, response to injury and embryonic migration.

Involvement of torsinA in nuclear dynamics

A role for torsinA in nuclear dynamics was initially indicated by a nematode homolog, OOC-5, which has a perinuclear/ER localization and is involved in rotation of the nucleus during early embryogenesis (Basham and Rose, 2001). Other studies indicate that an E171Q mutation in the WalkerB ATP-binding domain of torsinA, which blocks ATPase activity, results in accumulation of torsinA near the NE, presumably owing to prolonged interaction with substrate(s) there and abnormalities in the structure of the NE (Naismith et al., 2004; Goodchild et al., 2005). High-level expression of torsinAΔE or torsinA produces whorled membrane inclusions that appear to ‘spin off’ the NE/ER (Bragg et al., 2004; Gonzalez-Alegre and Paulson, 2004; Grundmann et al., 2007). These observations are

Fig. 9. Nuclear polarization in migrating torsinA+/+ and torsinA−/− MEFs. To evaluate nuclear polarization, confluent monolayers of MEFs were serum-starved for 24 hours, then a wound was made through the monolayer and cells were stimulated with 2 μM lysophosphatidic acid (LPA; Sigma-Aldrich) (Gomes and Gundersen, 2006). Three hours later, the cells were fixed and immunostained for pericentrin to label the centrosome (green) and for β-tubulin to label microtubules (red), with DAPI staining (blue) for nuclei. The nucleus was scored as polarized (+) when the centrosome was localized between the nucleus and the leading wound edge and as non-polarized (−) in any other location. Representative images are shown for torsinA+/+ (left) and torsinA−/− (right) cells along the wound edge. One hundred cells of each type were evaluated for orientation of the centrosome between the nucleus and the leading edge in each of three experiments using two different MEF preparations of each type. TorsinA+/+ cells showed 85±5% nuclei in the polarized location, whereas torsinA−/− cells showed only 38±7% in the polarized position at this time point (values are mean percentage ± s.e.m., n=3 experiments, P<0.001). Magnification: 20×.

Fig. 10. Theoretical consequences of torsinA status for nesprin-SUN interactions. Nesprins span the outer nuclear membrane (ONM) and interact with cytoskeletal elements in the cytoplasm and with inner nuclear membrane (INM) proteins, such as SUNs, in the lumen of the nuclear envelope (NE). TorsinA is hypothesized to act as an AAA+ protein in the disassembly/reassembly of these nesprin-SUN interactions, for example, during movement of the nucleus in cell migration. (A) In wild-type cells, nesprins and associated cytoskeletal elements are localized to the ONM by interactions with INM proteins, such as SUNs, and this interaction (at least for nesprin-3) is modulated by torsinA (depicted as an oligomer in cross-section, two circles). (B) In torsinA-null cells, nesprin-3 and linked cytoskeletal elements are located predominantly in association with the endoplasmic reticulum (ER) (see Fig. 6), as the association of nesprins with INM proteins is predicted to be compromised. (C) Cells from DYT1 subjects express both torsinA and torsinAΔE, with the mutant (mt) torsinA hypothesized to form inactive oligomers with torsinA (depicted as X-shaped structures). When YFP-nesprin-3 is overexpressed in DYT1 cells, torsinA oligomers containing torsinAΔE, which binds more tightly than torsinA to nesprins, reduce the interaction of YFP-nesprin-3 with INM proteins, such that YFP-nesprin-3 accumulates in the ER, where, together with torsinA/torsinAΔE and associated cytoskeletal elements, it forms globular structures (see Fig. 2).
consistent with torsinA regulating the interaction between the luminal domains of transmembrane proteins that span the INM and ONM and are involved in NE structure.

The interaction of NE proteins with cytoskeletal elements contributes to intracellular movement of the nucleus during cell migration (Gomes et al., 2005; Wilhelmsen et al., 2006). For example, SUN1 (UNC84A) is involved in linkage between the centrosome and the NE (Malone et al., 2003) and other SUN proteins interact with nesprins, which link to actin microfilaments and IFs (Crisp et al., 2006; Ketema et al., 2007). Several components might underlie the reduced rate of cell migration in torsinAΔ cells and torsinA-null MEFs, including disorientation of vimentin or actin microfilaments and/or a reduced rate of nuclear polarization. Polarization of the nucleus during cell migration is thought to be dependent primarily on actin microfilaments (Gomes et al., 2005). Nuclear polarization in torsinAΔ cells might be compromised by localization of nesprin-3 in the ER, with associated plakin-mediated interconnections between IFs and actin microfilaments (Chang and Goldman, 2004). The potential mislocalization of nesprin-1 and -2 in cells lacking torsinA was not investigated in this study, so it is not known whether the decrease in cell migration observed in torsinAΔ MEFs involves a disturbance in nesprin-1 and -2 connections to actin microfilaments (Crisp et al., 2006).

Interestingly, BPAG1 (dystonin), which is responsible for dystonic movements in a mouse model termed dystonia musculorum deformans (di/di) is, like plectin, a member of the plakin family of proteins that link together elements of the cytoskeleton (Sonnenberg and Liem, 2007). Recent studies show that an isoform of BPAG1 is associated with the NE and affected neurons in BPAG1-deficient mice have neurofilament aggregates and improper placement of nuclei (Young et al., 2006). Thus, alterations in nuclear dynamics during neuronal migration and cell migration during development and thus reduced numbers of neurons in specific regions of the brain. This would predict a subtly altered neuronal circuitry in the brains of DYT1 subjects that contributes to an abnormal neurotransmission underlying the dystonic symptoms.

Materials and Methods

Cell culture

Cells were primary human skin fibroblasts as control (HF19 and HF27, Breakefield Laboratory) and from DYT1 subjects (FFF13111983, from M. Filocamo, L’Instituto Gianna Gaslini, Genoa, Italy; HF48 and HF49, Breakefield Laboratory) and human embryonic kidney fibroblasts line 293T (from D. Baltimore, MIT, Cambridge, MA). Primary MEFs were generated from individual E13-14 embryos, genotyped (Dang et al., 2005), treated with Plasmocin (InvivoGen) to avoid mycoplasma contamination and used within the first four passages. MEFs were generated from mouse pups (Andrà et al., 1998). Cells were maintained in culture as described (Hewett et al., 2007).

Vectors and constructs

Lentivirus vectors were derived from self-inactivating lentivirus (CS-CGW) (Sena-Esteves et al., 2004), in which the cDNA under control of the cytomegalovirus promoter (hCMV) immediately early promoter is followed by an internal ribosomal entry site (IRES) and a reporter cDNA (Hewett et al., 2006). Fusion constructs were used a modified version lacking the IRES.

cDNAs for human torsinA and torsinAΔ (Ozelius et al., 1997) were cloned downstream of the CMV promoter yielding CS-CGW-TorA and CS-CGW-TorAΔ, respectively (Hewett et al., 2006). A C-terminal-deleted version of torsinA containing amino acids 1-311 (torsinAΔ312-322) was amplified from the cDNA and inserted into the CFP-containing PCS-CGW backbone. To generate YFP-human-nesprin-3, human nesprin-3 cDNA was amplified from single-stranded DNA that had been reverse-transcribed from human fibroblast (HF19) RNA. PCR was performed with Phusion Hot Start (Finnzymes) using primers upstream of the start codon (5’-GCCCCTGTGAGGTCCACATAGCTGAG-3’) and downstream of the stop codon (5’-CTGATATGGATGCGTCTGCAGT-3’). Nested forward (5’-ATGATACATGACGCCCCAGGAGCAG-3’) and reverse (5’-TTAGGTGGTTGGCCGATGTTA-3’) primers were used to amplify just the CDFN. YFP sequences were PCR-amplified from pEFP-C1 (Clontech) using primers containing a 5’ NheI site (5’-AATGCTAGGCTGCGAACCCCATGTGTAACCA-3’) and HindIII site (5’-CAGAAGCTGCTGCTGCTGCTGCT-3’).

Vectors were produced by co-transfection of 293T cells with the lentivirus packaging plasmid (pCMV89.1), envelope-coding plasmid (pVSVG) and vector construct, yielding typical titers of 1×10^9 transducing units/ml (Sena-Esteves et al., 2004).

For GST-KASH, the GST domain (amino acids 910-975) of nesprin-3z was amplified by PCR using Phusion Hot Start and primers containing 5’ BamHI (5’-GGGATCCATCATCCTCCAAATGGATG-3’) and 3’ XhoI (5’-CATAAGCT- TATGACCTGAGCCACCCAGAG-3’) sites. After digestion with BamHI and XhoI, the sequence was inserted into the PGEX-4T1 vector in-frame with GST. GFP-KASH derived from nesprin-1 and -2 were kindly provided by Brian Burke, University of Florida.

Antibodies

Antibodies included mouse monoclonals specific to torsinA (D-M2A8 and D-M10 (Hewett et al., 2003) and MBP (New England Biolabs) and rabbit polyclonal antibodies to torsinA and torsinB [TABI (Hewett et al., 2003)] and specific to torsinB [TB2 (Hewett et al., 2004)]. human plectin (BD Biosciences or Epitomics), mouse nesprin-3z (Wilhelmsen et al., 2005), protein disulfide isomerase (PDI; SFA-891, Stressgen), pericentrin (Covance), rabbit GFP (Molecular Probes), goat GFP (Abcam), α-tubulin (Sigma-Aldrich), vimentin (Lab Vision), GAPDH (MAB374, Chemicon), pericentrin (PRB-432C, Covance), GST (Abgent), β-actin (clone AC-15, Sigma) and Alexa Fluor 488 phalloidin (Molecular Probes).

Immunocytochemistry

Immunocytochemistry was carried out as described (Hewett et al., 2006). DNA was stained with 0.1% 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Antibody dilutions were as follows: D-M2A8 (1:100), α-tubulin (1:1000), GFP (1:2000), human plectin (BD Biosciences) (1:50), nesprin-3z (1:1000), PDI (1:200), pericentrin
(1:1000), vimentin (1:2000), β-actin (1:500) and Alexa Fluor 488 phalloidin (1:1000) was used to stain actin.

Immunoprecipitation, pull-downs and western blots
For immunoprecipitation, total cell lysates (1×10^6 cells) were resuspended in 1 ml RIPA lysis buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 1% NP40, 0.5% deoxycholate, 0.1% SDS (Hewett et al., 2006)]. The lysates were immunoprecipitated overnight at 4°C using specific antibodies: 10 μl D-M2A8 and D-M10 (1:1) or 10 μl goat anti-mouse IgG (Abcam) per 500 μg lysate in 1 ml on a 361-nm UV laser, including a tunable two-photon source for reduced photodynamic damage. This microscope also has DIC optics and a photomultiplier as well as a 361-nm UV laser, including a tunable two-photon source for reduced photodynamic damage. We also used an Olympus FluoView 1000 confocal microscope with filters, respectively. The different fluorochromes were scanned sequentially using the HeNe (543 nm) and HeNe (633 nm) lasers were used for excitation. Green, red and interference contrast (DIC) objective on an inverted microscope (Axiovert 200M, Carl Zeiss LSM 5 Pascal laser-scanning confocal microscope was used. Images were acquired using a 10×, 40× or 63× numerical aperture 1.4 PlanApo differential interference contrast (DIC) objective on an inverted microscope (Axiovert 200M, Zeiss) equipped with an LSM 510META scan head (Zeiss). Argon ion (488 nm), HeNe (543 nm) and HeNe (633 nm) lasers were used for excitation. Green, red and blue fluorescence emissions were detected through BP 505-530, 560-615 and 650 filters, respectively. The different fluorochromes were scanned sequentively using the multitracking function to avoid any bleed through among these fluorescent dyes. Brightfield images were obtained using the argon 488-nm laser. The resolution was 2048, the emission data were acquired with a 1024 × 1024 pixel FuegoView 1000 confocal microscope with independent photostimulation and imaging channels, with the same lasers as above as well as a 361-nm UV laser, including a tunable two-photon source for reduced photodynamic damage. This microscope also has DIC optics and a photomultiplier collector for transmitted light imaging.

Electron microscopy
MEFs were grown as confluent monolayers on plastic coverslips, fixed in situ in 2% glutaraldehyde in 30 mM HEPES (pH 7.3), 150 mM NaCl, 2 mM CaCl₂, 10 mM MgCl₂. Coverslips were then washed in the same buffer without glutaraldehyde, incubated for 30 minutes in 0.5% KMnO₄ in 150 mM NaCl, then dehydrated, embedded in araldite resin, sectioned and stained with uranyl acetate and lead citrate according to standard procedures. Images were viewed in a JEOI electron microscope operating at 100 kV and photographed with a charge-coupled device camera system (Advanced Microscopy Techniques).

Statistical analysis
One-way analysis was performed using Prism software (version 3.0). Results are expressed as mean ± s.e.m. Multiple comparison analysis of variance of the groups was performed by a two-way ANOVA test.

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References

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