The AAA+ protein torsinA interacts with a conserved domain present in LAP1 and a novel ER protein

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A glutamic acid deletion (ΔE) in the AAA+ protein torsinA causes DYT1 dystonia. Although the majority of torsinA resides within the endoplasmic reticulum (ER), torsinA binds a substrate in the lumen of the nuclear envelope (NE), and the ΔE mutation enhances this interaction. Using a novel cell-based screen, we identify lamina-associated polypeptide 1 (LAP1) as a torsinA-interacting protein. LAP1 may be a torsinA substrate, as expression of the isolated luminal domain of LAP1 inhibits the NE localization of “substrate trap” EQ-torsinA and EQ-torsinA coimmunoprecipitates with LAP1 to a greater extent than wild-type torsinA. Furthermore, we identify a novel transmembrane protein, luminal domain like LAP1 (LULL1), which also appears to interact with torsinA. Interestingly, LULL1 resides in the main ER. Consequently, torsinA interacts directly or indirectly with a novel class of transmembrane proteins that are localized in different subdomains of the ER system, either or both of which may play a role in the pathogenesis of DYT1 dystonia.

Introduction

DYT1 dystonia is an autosomal dominant childhood-onset neurological disease characterized by prolonged involuntary twisting movements that reflect neuronal dysfunction rather than neurodegeneration (Fahn et al., 1987; Berardelli et al., 1998). The mechanism by which the pathogenic mutation in the AAA+ protein torsinA produces DYT1 dystonia is unknown (Ozelius et al., 1997). Because AAA+ proteins are chaperones that alter the conformation of substrates, the identity of substrate determines the biological pathway modulated by AAA+ protein function (Vale, 2000). For example, the role of the AAA protein NSF in neuronal function is best appreciated when one considers that it acts upon SNARE complexes.

TorsinA resides in the ER lumen, but several observations indicate that it interacts with a nuclear envelope (NE) substrate (for review see Gerace, 2004). In addition, disease-associated ΔE-torsinA accumulates abnormally in the NE, suggesting that NE dysfunction may contribute to disease pathogenesis (Goodchild and Dauer, 2004). Consequently, identifying a NE substrate of torsinA is likely to further our understanding of the molecular pathogenesis of DYT1 dystonia.

Because torsinA is expected to alter the conformation of a NE luminal protein, characterizing this interaction may also provide insight into the functional organization of the NE and the poorly understood roles of NE resident proteins and their associated genetic diseases.

Results and discussion

We have previously shown that, although wild-type (WT) torsinA is predominantly localized in the main ER, pathogenic ΔE-torsinA and a predicted “substrate trap” ATP hydrolysis-deficient EQ-torsinA concentrate in the NE (Fig. 1 A; Vale, 2000; Goodchild and Dauer, 2004). NE resident proteins typically concentrate in the nuclear membrane through a selective retention mechanism mediated by binding to the nuclear lamina (Burke and Stewart, 2002). Consequently, NE proteins are less mobile in the NE than in the ER membrane (Ellenberg et al., 1997). If torsinA interacts with a NE protein, it should therefore display similarly reduced mobility in the NE. We tested this concept by examining the mobility of torsinA using FRAP analysis of BHK21 cells transiently overexpressing GFPWT-, GFPΔE-, and GFPΔE-torsinA. At moderate expression levels, both GFPΔE- and GFPΔE-torsinA selectively localize in the NE (Fig. 1 B); these cells were used for NE FRAP measurements. Cells expressing higher levels of these proteins also contain fluorescence in the main ER (Fig. 1 D), allowing us to perform ER FRAP measurements. In the ER, all three forms of GFP-torsinA displayed a similar time course of fluorescence recovery (~65% after 210 s; Fig. 1 E). In contrast, the NE fluorescence recovery of GFPΔE- and GFPΔE-torsinA was markedly slower than GFPWT-torsinA (Fig. 1 C). In the
NE, only 50% of GFPΔE-torsinA and 40% of GFPEQ-torsinA fluorescence recovered within 330 s (Fig. 1 C), at which time 75% of GFPWT-torsinA fluorescence had returned. However, it is possible that contaminating fluorescence from ER GFPWT-torsinA may contribute to an overestimate of NE GFPWT-torsinA recovery. The rate of GFPEQ-torsinA FRAP is slower than that of some well characterized transmembrane NE proteins (such as emerin), but is comparable to others (Ellenberg et al., 1997; Östlund et al., 1999; Daigle et al., 2001; Shimi et al., 2004). Because torsinA is restricted to the ER lumen/perinuclear space, it cannot bind to nuclear lamins. Therefore, these findings are consistent with the hypothesis that the NE accumulation of ΔE-torsinA is caused by an abnormal interaction with an immobilized transmembrane substrate. The rate of GFPΔE- and GFPEQ-torsinA fluorescence recovery is likely to be a function of (a) the degree to which its NE binding partner is immobilized and (b) the rate at which torsinA cycles on and off this partner. A higher rate of cycling might explain the faster recovery of GFPΔE-torsinA compared with GFPEQ-torsinA.

Lamina-associated polypeptide 1 (LAP1) is a torsinA binding protein

Based on the behavior of WT and mutant torsinA, we next sought to identify a torsinA NE binding partner. We developed a screening procedure based on the assumption that over-expressing a NE-localized torsinA substrate would increase the amount of torsinA in the NE, which is normally quite low. We selected candidate proteins that normally reside in the NE and contain a predicted luminal domain that is conserved between mammalian species because these features indicate a potential functional role within the NE lumen. Cells stably expressing GFPWT-torsinA (BHK-GFPWT; Fig. 1 A) were transfected with 18 candidate protein cDNAs in a reporter plasmid that coexpresses β-galactosidase (Table I and Fig. 2 A). Of all tested NE candidate proteins, only LAP1 recruited GFPWT-torsinA to the NE in a uniform perinuclear distribution reminiscent of substrate trap GFPEQ-torsinA (Table I and Fig. 2 A; compare transfected and untransfected cells). Occasionally, cells expressing high levels of lamin B receptor, LUMA, and Sun2 contained bright puncta of GFPWT-torsinA. These puncta were considered to be a nonspecific effect of gross
overexpression because they were randomly located in the NE and ER. We further examined the LAP1 recruitment of GFPWT-torsinA by expressing myc-tagged LAP1 (myc-LAP1) in BHK_{GFPWT} cells, which were transfected with different NE proteins and transfected cells were identified by colabeling with antibodies against reporter proteins (see Fig. 2 A). The nuclear envelope transmembrane proteins (NET) are described by Schirmer et al. (2003) and UNCL by Fitzgerald et al. (2000). See Burke and Stewart (2002) for descriptions of other candidate proteins.

Table 1. Effect of NE candidate proteins on GFPWT-torsinA

| Candidate protein | Change in GFPWT-torsinA distribution |
|-------------------|--------------------------------------|
| Emerin            | None                                 |
| gp210             | None                                 |
| LAP1              | Increased NE labeling                |
| LAP2β             | None                                 |
| Lamin B receptor  | Puncta in ER/NE                      |
| LUMA              | Puncta in ER/NE                      |
| MAN1              | None                                 |
| Nesprin-1         | None                                 |
| Nurim             | None                                 |
| NET3              | None                                 |
| NET4              | None                                 |
| NET26             | None                                 |
| NET31             | None                                 |
| NET39             | None                                 |
| NET51             | None                                 |
| Sun1 (Unc84A homologue) | None                             |
| Sun2 (Unc84B homologue) | Puncta in ER/NE                    |
| UNCL (Unc50 homologue) | None                             |

*BHK_{GFPWT} cells were transfected with NE resident proteins and transfected cells were identified by colabeling with antibodies against reporter proteins (see Fig. 2 A). The nuclear envelope transmembrane proteins (NET) are described by Schirmer et al. (2003) and UNCL by Fitzgerald et al. (2000). See Burke and Stewart (2002) for descriptions of other candidate proteins.*
citations from lysates of BHK<sub>GFPEQ</sub> cells transfected with myc-LAP1 demonstrated that GFPEQ-torsinA coimmunoprecipitates with myc-LAP1 (Fig. 2 F), further supporting the idea that LAP1 and torsinA interact in the NE.

Next, we examined whether or not the luminal domain of LAP1 is responsible for its interaction with torsinA, as predicted by our model. To explore this question, we tested if the isolated luminal domain of LAP1 is capable of altering the perinuclear subcellular distribution of EQ-torsinA. We generated myc-tagged constructs containing the LAP1 luminal domain with (myc-210LAP1) or without (myc-240LAP1) the transmembrane domain (Fig. 3 A; Kondo et al., 2002). As expected, these fragments fail to concentrate in the NE and instead localize in the main ER (Fig. 3 B, left). Expression of either LAP1 luminal fragment produced a clear redistribution of GFPEQ-torsinA from the NE to the ER (Fig. 3 B). Myc-210LAP1 causes a similar redistribution of disease-associated GFP<sub>/H9004</sub>E-torsinA (Fig. 3 C), and in all instances we observed strong colocalization between labeling for GFP and myc (Fig. 3, B and C). The effect of the LAP1 luminal domain was specific, as the luminal domain of the nucleoporin gp210 (Wozniak and Blobel, 1992) did not alter the subcellular distribution of GFPEQ-torsinA (Fig. 3 B, bottom).

These data indicate that LAP1 may be a NE-localized torsinA substrate. LAP1 was originally identified as the antigen recognized by a monoclonal antibody generated against purified rat liver nuclear envelopes (RL13). Three RL13 immunoreactive NE proteins were designated LAP1A, B, and C (with molecular masses of 75, 68, and 55 kD, respectively; Senior and Gerace, 1988). A single exon encodes the entire transmembrane and luminal domains of LAP1 in rat, mouse, and human, suggesting that LAP1 isoforms vary only in their nucleoplasmic portion. Interestingly, the luminal domain of human LAP1 is 86% identical to mouse LAP1, whereas the nucleoplasmic domains exhibit only 46% sequence identity. This comparison suggests that torsinA interacts with a domain of LAP1 that has a conserved role in the lumen of the NE.

Lumenal domain like LAP1 (LULL1) is a novel ER-localized LAP1 homologue

Because the LAP1 luminal domain appears to be a torsinA-interacting motif, we searched for other proteins containing this domain by performing a BLAST search of the NCBI database. This search identified a single novel human cDNA (GenBank/EMBL/DDBJ accession no. NM_145034) encoding a protein with a luminal domain like LAP1, which we named LULL1 (Fig. 4, A and B). The LULL1 gene encodes a protein containing a single predicted transmembrane domain and appears to have arisen from a gene duplication event because it is located adjacent to the LAP1 gene on human chromosome 1q24. cDNA clones also exist for rat and mouse forms of LULL1, and the LAP1 and LULL1 genes are also adjacent within these genomes. In contrast to the conserved luminal domains of LAP1 and LULL1, there is significant divergence between the NH<sub>2</sub>-terminal regions of these proteins that extend outside of the secretory pathway (Fig. 4, A and B).

To explore whether or not LULL1 interacts with torsinA, we isolated a human cDNA that matched the sequence of NM_145034. Transient transfection of BHK21 cells with myc-tagged LULL1 generates a protein of ~75 kD that is insoluble in the absence of detergent but is solubilized by 1% Triton X-100, suggesting the presence of a membrane spanning domain (Fig. 4 C). When NH<sub>2</sub>- (myc-LULL1) or COOH-terminal (LULL1-myc)–tagged LULL1 were transfected into BHK21 or HeLa cells, they colocalized with PDI (Fig. 4 D and not depicted for HeLa cells), including in low expressing cells. Like torsinA, both LAP1 and LULL1 proteins are PNGaseF- and endoglycosidase H–sensitive glycoproteins, indicating that they are retained within the ER system (Fig. 4 E).

We transfected myc-LULL1 into BHK<sub>GFPEQ</sub> cells to determine if this ER-localized LAP1 homologue also interacts with torsinA. Consistent with this notion, myc-LULL1 produced a clear redistribution of GFPEQ-torsinA from the NE to the ER and there was strong colocalization between GFP and myc labeling in transfected cells (Fig. 5 A).
lar results with a LULL1 fragment containing only the transmembrane and lumenal domains (208LULL1; Fig. 5 A), confirming that this domain is responsible for the effects observed with full-length LULL1. In addition, GFPEQ-torsinA coimmunoprecipitates with myc-LULL1 from lysates of myc-LULL1–transfected BHK cells (Fig. 5 B). Together, these results suggest that LULL1 interacts with torsinA in the main ER.

Like torsinA, LAP1 and LULL1 mRNAs are widely expressed in both neural and nonneural tissue (Fig. 5 C), which is consistent with the hypothesis that these proteins may be physiologically relevant interactors of torsinA.

Next, we sought to understand why, if torsinA interactors exist in both the NE and ER, substrate trap EQ-torsinA appears to localize exclusively to the NE. One important technical consideration is that the much smaller volume of the NE, compared with the ER, makes torsinA far easier to detect in the NE when subcellular localization is assessed by fluorescence microscopy. In addition, the relative steady-state levels of torsinA and its interactors will influence the subcellular localization of torsinA. To assess the relative steady-state levels of these proteins, we used rabbit polyclonal antibodies raised against the mouse forms of LAP1, LULL1, or torsinA that similarly detect their respective antigens (Fig. 5 D). In NIH-3T3 lysate, these antibodies recognize proteins of the appropriate molecular masses, including the three previously described isoforms of LAP1 (Fig. 5 C), which is consistent with the hypothesis that these proteins may be physiologically relevant interactors of torsinA.

To examine whether or not LAP1 and LULL1 may be torsinA substrates, we compared the interaction of these proteins with WT- and EQ-torsinA. Because AAA+ proteins typically form high affinity interactions with substrate when bound to ATP (Vale, 2000), substrates of torsinA will bind more tightly to EQ-torsinA than the WT protein. We tested if this was the case for LAP1 and LULL1 by performing immunoprecipitations on lysates from LAP1- or LULL1-transfected BHK cells (Fig. 5 G, WCL). GFPEQ-torsinA readily immunoprecipitated with either LAP1 or LULL1. However, to detect the association of GFPWT-torsinA with LAP1 or LULL1, it was necessary to perform immunoprecipitations from a much greater amount of protein lysate (Fig. 5 G, WCL). These data suggest strongly, but do not prove, that LAP1 and LULL1 are substrates of torsinA; an adaptor protein could mediate the interaction between torsinA and LAP1 or LULL1.

Several lines of evidence indicate that torsinA has a role in the NE (Gerace, 2004). We demonstrate that this function of torsinA may involve an interaction with LAP1, and that the behavior of LAP1 is consistent with that of a torsinA substrate (i.e., it is more tightly associated with EQ- than WT-torsinA). Although the functional role of LAP1 is poorly understood, it is known to bind A- and B-type lamins (Senior and Gerace, 1988; Foisner and Gerace, 1993; Martin et al., 1995). This suggests that alterations in torsinA function may affect the nuclear lamina, raising the possibility that DYT1 dystonia shares molecular abnormalities with diseases that result from laminA mutations (Burke and Stewart, 2002; De Sandre-Giovannoli et al., 2003). The fact that alterations in both lamin A and torsinA function lead to NE morphologic abnormalities is consistent with this notion (Sullivan et al., 1999; Naismith et al., 2004).
We also identify a novel ER protein, LULL1, that interacts with torsinA through a region conserved with the LAP1 lumenal domain; this protein also behaves like a torsinA substrate. The striking homology between the LAP1 and LULL1 lumenal domains suggests that they are similarly modified by the AAA+/H11001 chaperone activity of torsinA. In addition, LAP1 and LULL1 share other features. They both contain a single membrane-spanning domain and their nucleoplasmic (LAP1) and cytoplasmic (LULL1) regions are similarly sized. Consequently, these proteins may be engaged in similar roles in the NE and ER and contribute to a biological process that is common to both compartments. In light of the mechanism of AAA+/H11001 protein function, our data suggest that alterations in LAP1 or LULL1 activity may therefore participate in the pathogenesis of DYT1 dystonia.

Materials and methods

Cell culture
BHK21, NIH-3T3, and HeLa cell lines were cultured using standard conditions [American Type Culture Collection]. The generation and characterization of BHKGFPEQ, BHKGFPEQ, and BHKGFPEQ Cells has been described previously (Goodchild and Dauer, 2004). All cell transfections were performed using Lipofectamine Plus (Invitrogen) according to the manufacturer’s instructions.

FRAP
The day after transfection, cells were trypsinized and replated at 10–20% confluence in collagen-coated chambered coverglasses (LabTekII) in Dulbecco’s minimum essential medium containing 1% FBS. Immediately before imaging, this media was replaced with media containing 10 mM Hepes buffer, pH 7.5. Imaging and photobleaching were performed using a Plan NEOFLUAR 100×/1.30 oil objective on an inverted confocal microscope (model LSM510 Meta; Carl Zeiss MicroImaging, Inc.). Cells trans-
fected with GFP fusion proteins were imaged with 488-nm light. DsRed with 516-nm light, using 2% laser power and a pin hole of 1 airy unit. After two imaging scans, a selected area of the ER or NE (region of interest [ROI]) was bleached using maximal laser power for 20 iterations, and then the photobleached cell was imaged at 15-s intervals for 3–6 min. Collected images were analyzed in Adobe Photoshop to calculate the mean fluorescence intensity in the ROI as a function of time after photobleaching. To correct for whole cell photobleaching caused by the bleaching pulse and image capture, fluorescence intensity was also measured in an unbleached area (UA) at all time points and a fractional correction calculated as UA/UA0. ROI fluorescence intensity at each time point was corrected by these values and then normalized so that the prebleach fluorescence level equaled 100 and immediate postbleach level was zero.

**Immunolabeling**

Immunofluorescence labeling was performed on cells 48 h after transfection using methanol-fixed cells grown on collagen-coated glass coverslips (Carolina Scientific). Coverslips were blocked for 1 h at RT in block solution (PBS, 0.25% Triton X-100 and 10% normal donkey serum), incubated overnight at 4°C in primary antibodies diluted in block solution. The next day coverslips were washed, incubated with secondary antibodies (diluted in block solution), and washed in PBS before mounting using Vectashield Mounting Media with DAPI (Vector Laboratories). In double labeling experiments, GFP was detected with FITC- and myc with Texas red–conjugated secondary antibodies to minimize the possibility of “bleed through.” Digital images were acquired using a laser scanning confocal microscope (model LSM510 Meta; Carl Zeiss MicroImaging, Inc.). FITC and Texas red red images were acquired successively and figures were prepared in Adobe Photoshop.

**Antibodies**

Antibodies used were as follows: affinity purified rabbit polyclonal anti-torsinA raised against residues 319–332 of mouse torsinA (a gift from B. Lauring, Columbia University, New York, NY), rabbit polyclonal anti-LAP1 raised against residues 463–478 of mouse LAP1 (Covance Research Products), and rabbit polyclonal anti-LULL1 raised against residues 107–120 of mouse LULL1 (Covance Research Products). Other antibodies were rabbit anti-GFP 1:1,000 (AbCam), mouse anti–β-galactosidase 1:100 (Sigma-Aldrich), mouse anti-myc 1:500 (CLONTECH Laboratories, Inc.), rabbit anti-myc 1:100 (Sigma-Aldrich), and mouse anti-PDI 1:100 (StressGen Biotechnologies). All secondary antibodies were raised in donkey and prepared in Adobe Photoshop.

**Immunoprecipitation**

Immunoprecipitations were performed at 24 h after transfection using mouse monoclonal anti-myc (CLONTECH Laboratories, Inc.) and Agarose Protein G immunoprecipitation kit (Roche) according to the manufacturer's instructions, except that buffers contained only Igepal CA630.

**LULL1 characterization**

The solubility of LULL1 was investigated by lysing myc-tagged LULL1-transfected BHK21 cells in buffer (50 mM Tris-HCl, pH 7.5, and protease inhibitors) with and without 1% Triton X-100. Homogenates were incubated on ice for 20 min and centrifuged at 20,000 g for 15 min to separate supernatant and pellet fractions. Pellets were solubilized by heating to 95°C in 180 μl of 1 × lysis sample buffer. Supernatants were also brought to a 180-μl volume and 1× concentration. Equal volumes of pellet and supernatant fractions were used in SDS-PAGE. The glycosylation state of myc-tagged LAP1 and LULL1 was examined by digesting lysates from transfected BHK21 cells with PNGaseF or Endoglycosidase H (New England Biolabs, Inc.) according to the manufacturer’s instructions.

**RT-PCR**

Total RNA was prepared from mouse tissues using Trizol reagent (Invitrogen), and cDNA was generated from 1 μg of total RNA with oligo dT primers and SuperScript III (Invitrogen), all according to the manufacturer’s instructions. Normalization of samples was performed by increasing or decreasing the amount of template cDNA dependent on amplification efficiency determined using GAPDH primers. Primers were designed to amplify cDNA regions that spanned at least one intron boundary, along with the lumenal domain of LAP1 and LULL1, to prevent amplification from genomic DNA.

**Online supplemental material**

Details of plasmid construction for the candidate cDNA screen, primer details, and the generation of LAP1 and LULL1 fusion and truncated constructs is contained in the online supplemental material. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200411026/DC1.

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