PRINTOR, A NOVEL TORSINA-INTERACTING PROTEIN IMPLICATED IN DYSTONIA PATHOGENESIS
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Early-onset generalized dystonia (DYT1) is an autosomal dominant neurological disorder caused by deletion of a single glutamate residue (torsinA ΔE) in the C-terminal region of the AAA+ protein torsinA. The pathogenic mechanism by which torsinA ΔE mutation leads to dystonia remains unknown. Here we report the identification and characterization of a 628-amino-acid novel protein, printor, that interacts with torsinA. Printor co-distributes with torsinA in multiple brain regions and co-localizes with torsinA in the endoplasmic reticulum. Interestingly, printor selectively binds to the ATP-free form but not to the ATP-bound form of torsinA, supporting a role for printor as a cofactor rather than a substrate of torsinA. The interaction of printor with torsinA is completely abolished by the dystonia-associated torsinA ΔE mutation. Our findings suggest that printor is a new component of the DYT1 pathogenic pathway and provide a potential molecular target for therapeutic intervention in dystonia.

Early-onset generalized torsion dystonia (DYT1) is the most common and severe form of hereditary dystonia, a movement disorder characterized by involuntary movements and sustained muscle spasms (1). This autosomal dominant disease has childhood onset and its dystonic symptoms are thought to result from neuronal dysfunction rather than neurodegeneration (2, 3). Most DYT1 cases are caused by deletion of a single glutamate residue at position 302 or 303 (torsinA ΔE) of the 332-amino-acid protein torsinA (4). In addition, a different torsinA mutation that deletes amino acids F323-Y328 (torsinA Δ323-8) was identified in a single family with dystonia (5), although the pathogenic significance of this torsinA mutation is unclear because these patients contain a concomitant mutation in another dystonia-related protein, epsilon-sarcoglycan (6). Recently, genetic association studies have implicated polymorphisms in the torsinA gene as a genetic risk factor in the development of adult-onset idiopathic dystonia (7, 8).

TorsinA contains an N-terminal endoplasmic reticulum (ER) signal sequence and a 20-amino-acid hydrophobic region followed by a conserved AAA+ (ATPases associated with a variety of cellular activities) domain (9, 10). Because members of the AAA+ family are known to facilitate conformational changes in target proteins (11, 12), it has been proposed that torsinA may function as a molecular chaperone (13, 14). TorsinA is widely expressed in brain and multiple other tissues (15) and is primarily associated with the endoplasmic reticulum (ER) and nuclear envelope (NE) compartments in cells (16-20). TorsinA is believed to mainly reside in the lumen of the ER and NE (17-19) and has been shown to bind lamina-associated polypeptide 1 (LAP1) (21), luminal domain like LAP1 (LULL1) (21), and nesprins (22). In addition, recent evidence indicates that a significant pool of torsinA exhibits a topology in which the AAA+ domain faces the cytoplasm (20). In support of this topology, torsinA is found in the cytoplasm, neuronal processes, and synaptic terminals (2, 3, 15, 23-26) and has been shown to bind cytosolic proteins snapin (27) and kinesin light chain 1 (20). TorsinA has been proposed to play a role in several cellular processes, including dopaminergic neurotransmission (28-31), NE organization and dynamics (17, 22, 32), and protein trafficking (27, 33). However, the precise biological function of torsinA and its regulation remain unknown.

To gain insights into torsinA function, we performed yeast two-hybrid screens to search for
torsinA-interacting proteins in the brain. We report here the isolation and characterization of a novel protein named printor (protein interactor of torsinA) that interacts selectively with wild-type (WT) torsinA but not the dystonia-associated torsinA ΔE mutant. Our data suggest that printor may serve as a cofactor of torsinA and provide a new molecular target for understanding and treating dystonia.

EXPERIMENTAL PROCEDURES

Expression Constructs and Antibodies—Full-length human printor cDNA (KIAA1384, GenBank™ accession number AB037805) was obtained from Kazusa DNA institute, Japan. Conventional molecular biological techniques (34) were used to subclone the printor cDNA into mammalian vectors expressing N-terminal HA, Myc or FLAG tags for transfection into cells. DNA fragments encoding torsinA WT, WTΔ40, ΔE, Δ323-8, K108A, and E171Q were subcloned into mammalian vectors expressing C-terminal HA, Myc, or FLAG tags. A rabbit polyclonal anti-printor antibody was raised against a synthetic peptide encoding amino acids 1-18 of human printor and affinity purified using the immunogen peptide coupled to a Pierce column as we described previously (35-37). Other antibodies used in this study are as follows: anti-torsinA (16); anti-EEA1 and anti-Tim23 (BD transduction); anti-LAMP2 (H4B4; DSHB, University of Iowa); anti-FLAG (M2; Sigma); anti-calnexin; anti-KDEL (Stressgen); anti-β-actin (Sigma); mouse monoclonal anti-HA (12CA5); and anti-Myc (9E10) (35). Horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, Inc.) were used for immunoblotting. Flourescein isothiocyanate (FITC)-, Texas Red (TR)-, and Cy-5-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, Inc.) were used for immunofluorescence microscopy.

Yeast Two-hybrid Screen—The bait plasmids were constructed by subcloning the full-length human torsinA (WT) or N-terminally truncated torsinA (WTΔ40) into the vector pPC97 (35, 37, 38). For yeast two-hybrid screens, the yeast strain CG-1945 (Clontech) was sequentially transformed with the WT or WTΔ40 bait plasmid and a rat hippocampal/cortical two-hybrid cDNA library (35, 36, 39). Positive clones were selected on 3-amino-triazole-containing medium lacking leucine, tryptophan, and histidine, and confirmed by filter assay for β-galactosidase activity. Prey plasmids from positive clones were rescued and re-transformed into fresh yeast cells with the torsinA bait(s) to confirm the interaction.

Immunohistochemistry—Mouse whole brains were fixed in 4% paraformaldehyde and cut coronally into 1- to 2-mm slices. Brain slices were dehydrated in a graded series of alcohols and xylenes, infiltrated with paraffin using an automated tissue processor (Shandon Hypercenter XP), and then embedded in paraffin blocks. Eight-micrometer sections were cut using a Shandon AS325 microtome and subjected to immunohistochemical analysis as described (40). Briefly, sections were deparaffinized, blocked with normal serum, and then incubated with purified anti-printor antibody and biotinylated secondary antibody, followed by detection using an avidin–biotin–peroxidase complex method (ABC Elite Kit; Vector Laboratories, Burlingame, CA). The chromagen used for color development was 3,3′-diaminobenzidine (DAB), and sections were counterstained with hematoxylin. Omission of the primary antibody was used as a negative control.

Cell Transfections and Co-immunoprecipitation—Transfections of HeLa and SH-SY5Y cells were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Cell lysates were prepared from transfected cells and immunoprecipitations were carried out as described previously (16, 35, 41-43) using indicated antibodies, followed by the recovery of immunocomplexes with protein G-sepharose beads (Upstate). For co-immunoprecipitations of endogenous proteins, Seize Primary Mammalian Immunoprecipitation Kit (Pierce) was used according to manufacturer’s instructions. Anti-printor antibody or pre-immune serum was conjugated to the Amino-Link column (Pierce) and used to purify endogenous protein complexes from mouse cerebellum extracts prepared as described (44). After eluting from column or beads, immunocomplexes were analyzed by SDS-PAGE and immunoblotted with appropriate antibodies and horseradish peroxidase-conjugated...
Results were visualized using enhanced chemiluminescence (ECL).

Subcellular Fractionation—Subcellular fractionation was performed as previously described (36, 44, 45). SH-SY5Y cells were collected by centrifugation and the pellet was homogenized in 1 ml homogenization buffer (250 mM Sucrose, 10 mM HEPES/KOH pH 7.4, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA) containing protease inhibitors and DTT. After a 10-minute centrifugation at 1,000 x g to remove unbroken cells and nuclear material, the supernatant was subjected to a 30-minute centrifugation at 100,000 x g to separate into the cytosolic (supernatant) and membrane (pellet) fractions. Aliquots representing an equal percentage of each fraction were analyzed by SDS-PAGE and immunoblotting. Protein bands on the immunoblots were quantified using NIH Scion Image Software.

Opti-prep Gradient Fractionation—Opti-prep gradient fractionation was performed as previously described (46). SH-SY5Y cells were homogenized in 1 ml of fractionation buffer (10 mM HEPES/KOH, pH 7.4, 1 mM EDTA) containing 250 mM sucrose. After a 10-minute centrifugation at 1,000 X g, the supernatant was layered on a 10-30% Opti-Prep (Nycomed) gradient formed in the fractionation buffer containing 42 mM sucrose, and centrifuged at 4°C for 5 hours at 100,000 X g. Following centrifugation, the gradient was harvested into 250-μl fractions using an Auto Densi-Flow gradient harvester (Labconco). Equal volumes of each fraction were analyzed by SDS-PAGE and by sequential immunoblotting for torsinA, printer, and the ER marker calnexin. Protein bands on the immunoblots were quantified using NIH Scion Image Software.

Identification of Printer, a Novel TorsinA-interacting Protein—To identify proteins that interact with torsinA in the brain, we first screened a rat hippocampal/cortical cDNA library by yeast two-hybrid selection using the full-length torsinA WT as bait. This screen did not result in isolation of any positive clones, which could be due to the possibility that the purported signal sequence and hydrophobic domain of torsinA interfered with the ability of the bait to translocate into the nucleus for yeast two-hybrid interaction. To overcome this problem, we generated a bait construct encoding an N-terminally truncated torsinA (torsinA WTΔ40) lacking these hydrophobic regions (Fig. 1A) and used it to screen the cDNA library. Out of 15 million yeast transformants screened, we isolated three positive clones, all of which encoded part of a novel protein that we named printer (Fig. 1B) because it is a protein interactor of torsinA. Database searches revealed that the printer is the rat homologue of human KIAA1384 [also known as Kelch-like protein 14 (KLHL14), GenBank™ accession number NP_065856], an
uncharacterized protein discovered by the Kazusa DNA Research Institute human cDNA sequencing project (48).

Printor is 628 amino acids in length, with a calculated molecular mass of 70.7 kDa and a theoretical isoelectric point (pI) of 6.68. Sequence analysis revealed that printer contains no signal sequence. Two potential transmembrane domains (residues 562-582 and residues 609-626) were identified at the C-terminus of printer (Fig. 1B) by the TMPred program, which predicted a topology model with an N-terminal ER luminal orientation (Fig. 1D). In support of this model, two putative N-linked glycosylation sites (N146 and N433) were identified by the NetNGlyc 1.0 Server in the region of printer that is N-terminal to the first transmembrane domain (Fig. 1D). Printer also contains an N-terminal region with homology to the BTB (broad complex, tramtrack and bric a brac) domain [also known as POZ (poxvirus and zinc finger) domain], a protein-protein interaction domain that can mediate dimerization (49) or binding to cullin3, a component of a multi-subunit E3 ubiquitin-protein ligase complex (50). It is unclear whether this region is a functional BTB domain because it is interrupted by the insertion of a 48-amino-acid proline/glutamine (P/Q)-rich sequence (Fig. 1B), which is not found in any other BTB-containing protein. The BTB homology region of printer is followed by a BACK (BTB and C-terminal Kelch) domain of unknown function (51) and six kelch repeats (Fig. 1B). The kelch repeat is a motif of ~50 amino acids originally identified in the Drosophila ovarian ring canal protein Kelch (52). Kelch repeats form a β-propeller structure with multiple protein-protein interaction sites (53) and are best characterized as an actin-interacting domain (52, 54), although a number of kelch-containing proteins have no association with actin (53).

Database searches revealed the presence of printer orthologues as uncharacterized or predicted proteins from genome projects in a number of organisms, including human, dog, cow, rat, mouse, chicken, and zebrafish. Printer appears to be highly conserved amongst euteleostomi, or bony vertebrates, but is not found in C. elegans or Drosophila. The amino acid sequence of printer is highly conserved, with the human printer sequence sharing 99% overall amino acid identity with rat printer, 95% overall amino acid identity with chicken printer, and 88% overall amino acid identity with zebrafish printer (Fig. 1C). Moreover, the identified two transmembrane domains, two glycosylation sites, BTB domain, BACK domain, and Kelch repeats are highly conserved among printer orthologues across different species, indicating that printer is an evolutionarily conserved protein among vertebrates.

Printor Co-distributes with TorsinA in Brain as well as Other Tissues—In order to characterize printer protein and its interaction with torsinA, we generated a rabbit anti-printer antibody against an N-terminal printer peptide (aa 1-18) which has 100% amino acid identity across species. Immunoblot analysis showed that the anti-printer antibody, but not the pre-immune serum, specifically recognized ~70 kDa recombinant His-tagged printer protein purified from E. coli as well as Myc-tagged printer protein expressed in transfected SH-SY5Y cells, which was also detected by anti-Myc antibody (Fig. 2A). In addition, the anti-printer antibody also recognized the 70 kDa endogenous printer protein in human non-neuronal HeLa cells, human dopaminergic SH-SY5Y cells, and rat dopaminergic PC12 cells (Fig. 2B), demonstrating that the antibody was capable of recognizing endogenous printer in different cell types and across species. We also observed a number of printer-immunoreactive bands with lower and higher molecular weight (Fig. 2, A and B). Preabsorption with recombinant His-tagged printer protein virtually abolished the immunoreactivity of the anti-printer antibody to both recombinant (Fig. 2A) and endogenous (Fig. 2B) printer proteins, further confirming the specificity of our anti-printer antibody. The preabsorption result indicates that the lower and higher molecular weight bands are specific (Fig. 2A). The lower bands probably represent printer degradation products, while the upper bands may represent post-translationally modified forms of printer (e.g., glycosylated or ubiquitinated printer).

To characterize the tissue distribution of printer, western blot analysis of multiple rat tissue samples was performed using the anti-printer antibody. The result showed that printer is expressed in many tissues, including brain, heart, lung, liver, spleen, kidney and pancreas (Fig. 2C), which agrees with the mRNA expression profile.
found in the HUGE database (Human Unidentified Gene Encoded; http://www.kazusa.or.jp/huge/gf-page/KIAA1384/). The tissue distribution pattern of printor is similar to that of torsinA (Fig. 2C). Western blot analysis of protein extracts from various rat brain regions revealed that printor is widely expressed throughout the brain with high protein expression levels in hippocampus, medulla oblongata, and cerebellum, similar to the expression pattern of torsinA (Fig. 2D).

To determine whether our anti-printor antibody could be used for immunocytochemistry, we performed double label immunofluorescence confocal microscopic analysis of the intracellular distribution of endogenous and recombinant printor protein in SH-SY5Y cells. We observed immunoreactivity to anti-printor antibody in untransfected SH-SY5Y cells and the printor immunoreactivity was significantly increased by expression of Myc-tagged printor (Fig. 2E, top). Furthermore, the staining pattern detected by anti-printor antibody in transfected SH-SY5Y cells expressing Myc-tagged printor showed substantial overlap with the staining by anti-Myc antibody (Fig. 2E, top). The immunoreactivity to the anti-printor antibody was completely eliminated by preabsorption with recombinant His-tagged printor protein (data not shown). Moreover, no immunoreactivity was observed when pre-immune serum was used (data not shown). Together, these results indicate that anti-printor antibody is able to specifically recognize printor protein by immunostaining. Next, we performed double label immunofluorescence confocal microscopic analysis of mouse primary cortical neurons using anti-printor antibody and an antibody to MAP2, a neuron-specific microtubule-associated protein (Fig. 2E, bottom). As expected, the morphology of primary cortical neurons looks very different from that of SH-SY5Y cells (Fig. 2E, top), with multiple MAP2-positive neurites extending from the cell body (Fig. 2E, bottom). We found that printor is localized in the cell body as well as in the MAP2-containing neurites (Fig. 2E, bottom).

We then used our anti-printor antibody to determine printor protein distribution in mouse brain by immunohistochemistry. Printor immunoreactivity was observed in many neurons throughout the brain (Figs. 3B, D, and F). The specificity of printor immunoreactivity was confirmed in controls where anti-printor antibody was omitted (Figs. 3A, C, and E). In the cerebral cortex, printor staining appeared limited to layer 2/3 (Fig. 3G), where neuronal cell bodies and processes were labeled (Fig. 3H). Very little printor immunoreactivity was observed in other cortical layers. Printor was also observed in select neurons of the striatum (Figs. 3I and J). Consistent with the results of Western blot analysis (Fig. 2D), intense printor immunostaining was observed in the hippocampus (Figs. 3K-N) and the cerebellum (Figs. 3O-Q). Pyramidal cell neurons of the CA1 (Figs. 3K and L) and CA3 (Figs. 3M and N) contained printor immunoreactivity in their cell bodies, although staining appeared stronger in the CA3 region compared to the CA1 region. Purkinje cells of the cerebellum displayed strong immunoreactivity in their cell bodies and processes (Figs. 3P and Q). Granular cells of the cerebellum also displayed printor immunoreactivity, but to a lesser degree than Purkinje cells (Figs. 3P and Q). Printor immunoreactivity was also observed in the medial septum, ventral pallidum, thalamus, hypothalamus, amygdala, inferior colliculi, Locus Coeruleus, peripyridal nucleus, raphé nucleus, reticular formation, spinal trigeminal nucleus, and vestibular nuclei (data not shown). Very few glial cells were labeled and no immunoreactivity was observed in the corpus callosum (data not shown). Together, our results indicate that printor is primarily expressed in neurons but not glia.

Printor associates with torsinA in cells and in the brain. To verify that the interaction between torsinA and printor detected in the yeast-two hybrid screen occurs in vivo, co-immunoprecipitation assays were performed. Because the interaction was identified using torsinA WTΔ40 bait, HeLa cells transfected with Myc-tagged printor and either HA-tagged torsinA WTΔ40 or HA vector were subjected to immunoprecipitation using anti-HA antibody as previously described (39). The result showed that printor co-immunoprecipitated with HA-tagged torsinA WTΔ40, but not with HA alone (Fig. 4A), indicating a specific interaction. We next examined the interaction between full-length torsinA WT and printor and found that torsinA WT, but not the vector control, was able to co-immunoprecipitate printor protein (Fig. 4B), confirming a specific association between torsinA and printor in transfected cells. We then performed
additional co-immunoprecipitation experiments to examine the association of endogenous printor and torsinA in mouse cerebellum homogenates (Fig. 4C). Anti-printor antibody, but not the preimmune serum, was able to co-immunoprecipitate printor and torsinA from the homogenates, indicating the existence of an endogenous torsinA-printor complex.

**Printor Exists in Both Cytosolic and Membrane-associated Pools and Associates with the ER**—To investigate the intracellular distribution of endogenous printor protein, we performed subcellular fractionation experiments to separate the postnuclear supernatant from SH-SY5Y cells into cytosol and membrane fractions. Western blot analysis of these fractions revealed that printor was present in both the cytosol and membrane fractions (Fig. 5A). Quantification analysis revealed that 16.2 ± 1.4% of printor is in the cytosolic fraction and 83.8 ± 1.4% of printor in the membrane-associated pool (Fig. 5B). This is in contrast to torsinA or calnexin, which are exclusively found in the membrane fraction (Fig. 5A). To determine the membrane compartment(s) which printor is associated with, we performed double-labeling immunofluorescence confocal microscopic analysis to compare the intracellular distribution of printor with various markers of intracellular organelles in SH-SY5Y cells (Fig. 5C). We found that the distribution of printor exhibited a significant overlap with that of the ER marker KDEL, but not the early endosomal marker EEA1, lysosomal marker LAMP2, or mitochondrial marker TIM23, suggesting that printor is associated with the ER compartment.

**Printor Co-localizes with TorsinA Mainly at the ER Rather than the NE**—To determine if printor and torsinA co-localized in the ER, we first performed triple-labeling immunofluorescence confocal microscopic analysis in SH-SY5Y cells expressing C-terminally HA-tagged torsinA and Myc-tagged printor with anti-HA, anti-Myc, and anti-KDEL antibodies. We observed a significant overlap in the three immunostaining patterns (Fig. 6A), suggesting that printor and torsinA co-localize at the ER. We then performed triple-labeling analysis with anti-printor, anti-torsinA, and anti-KDEL antibodies and found that endogenous printor and torsinA co-localize at the ER (Fig. 6A). To complement our immunocytochemistry data, we performed density gradient fractionation experiments to determine if endogenous torsinA and printor associate with the ER membrane compartment. Upon fractionation of SH-SY5Y post-nuclear supernatant on a 10-30% linear Opti-prep gradient, a clear co-fractionation of printor with torsinA and the ER integral membrane protein calnexin was observed in fractions 4-12, providing additional evidence supporting the association of printor with torsinA at the ER membrane compartment (Fig. 6B). Quantification analysis revealed that these three proteins co-fractionate in two membrane pools which peaked in fraction 7 and fraction 11 (Fig. 6C), which may represent smooth ER and rough ER membranes, respectively.

Given that the ER membrane is in structural continuity with the NE membrane (55), we next performed quantitative double-labeling analysis as described (16) to compare the relative distribution of printor and the ER marker KDEL between the ER and NE subdomains in the non-neuronal cell line HeLa (Fig. 7A) and the dopaminergic neuronal cell line SH-SY5Y (Fig. 7A). The results revealed that in both cell types, the relative NE/ER distribution of printor is significantly lower than that of KDEL, indicating that printor is preferentially localized to the ER compared to KDEL (Fig. 7B). To control for cell-type variation in the relative NE/ER distribution of KDEL, we determined the NE preference of printor by normalizing the NE/ER ratio of printor to the corresponding NE/ER ratio of KDEL in the same cells (Fig. 7C). We found that the NE preference of printor in both cell types was less than 1, consistent with an enhanced ER preference. This result is in direct contrast to our previous finding that torsinA exhibits neuronal cell-type-specific NE preference (16) and suggests that the main site of co-localization between printor and torsinA is at the ER rather than the NE.

**Printor Shows Reduced Co-localization with ATP-bound Form of TorsinA**—Since torsinA contains a AAA + ATPase domain and is predicted to be a chaperone protein that binds a substrate(s) in an ATPase cycle-dependent manner (11, 12), we examined the co-localization of printor with the ATP-binding-deficient mutant torsinA K108A, which would be unable to bind the substrate(s), and the “substrate trap” ATP-hydrolysis-deficient mutant torsinA E171Q, which would bind tightly to the substrate(s). Double-labeling
immunofluorescence confocal microscopic analysis of SH-SY5Y cells co-expressing Myc-tagged printor and C-terminally HA-tagged torsinA WT, torsinA K108A or torsinA E171Q revealed that the ATP-free form of torsinA (K108A), like torsinA WT, visibly co-localized with printor, but the ATP-bound form of torsinA (E171Q) appeared to have reduced co-localization with printor (Fig. 8A). Quantification analysis showed that 51.5 ± 1.3% of printor co-localizes with torsinA K108A, which is similar to the extent of printor co-localization with torsinA WT (53.7 ± 2.1%). In contrast, only 36.1 ± 2.1% of printor co-localized with torsinA E171Q, indicating a significant (p < 0.005) reduction (Fig. 8B).

Reciprocal quantification analysis showed that 62.4 ± 1.4% of torsinA K108A co-localizes with printor, which is similar to the extent of torsinA WT co-localization with printor (63.7 ± 1.7%). In contrast, torsinA E171Q exhibited a significantly (p < 0.005) decreased level of co-localization with printor (52.9 ± 2.1%) compared to torsinA WT.

To determine whether the observed reduction in the co-localization between printor and the ATP-bound form of torsinA (E171Q) was due to a change in torsinA localization, we performed additional double-labeling immunofluorescence microscopic analysis to compare the intracellular distribution of C-terminally HA-tagged torsinA WT, torsinA K108A, and torsinA E171Q in SH-SY5Y cells (Fig. 9A). Consistent with our previous report (16), we found the NE/ER ratio of torsinA WT was significantly greater than the NE/ER ratio of KDEL in SH-SY5Y cells (Fig. 9B), indicating a preferential localization of torsinA WT to the NE compared to the ER marker KDEL. The NE/ER ratio of torsinA K108A was similar to that of torsinA WT, but torsinA E171Q showed a significantly higher NE/ER ratio compared to torsinA WT (Fig. 9B). After normalization to the NE/ER ratio of KDEL in the same cells, torsinA E171Q had an NE preference of 2.27 ± 0.07 compared to 1.92 ± 0.08 for torsinA WT and 1.91 ± 0.07 for torsinA K108A (Fig. 9C). Together, these results suggest that ATP-binding induces translocation of torsinA from the ER to the NE, leading to reduced co-localization of torsinA with printor.

Dystonia-associated Mutations Reduce the Co-localization Between Printor and TorsinA—To determine the effects of dystonia-associated mutations on torsinA co-localization with printor, we performed double-labeling immunofluorescence confocal microscopic analysis to examine the co-localization of C-terminally HA-tagged torsinA WT, torsinA ΔE or torsinA Δ323-8 with Myc-tagged printor in SH-SY5Y cells. We found that torsinA WT visibly co-localized with printor, but torsinA ΔE and Δ323-8 mutants appeared to have reduced co-localization with printor (Fig. 8A). Quantification analysis revealed that, while 53.7 ± 2.1% of printor co-localized with torsinA WT, only 42.0 ± 1.6% and 43.7 ± 1.5% of printor co-localized with torsinA ΔE and torsinA Δ323-8 mutant, respectively (Fig. 8B), indicating that both dystonia-associated mutations cause a significant (p < 0.005) reduction in the co-localization of printor with torsinA. Reciprocal quantification analysis showed that 56.4 ± 2.0% of torsinA ΔE and 58.0 ± 1.9% of torsinA Δ323-8 mutant proteins co-localized with printor, both of which represent a significant (p < 0.05) decrease compared to the extent of torsinA WT co-localization with printor (63.7 ± 1.7%). As we have shown previously (16), both torsinA ΔE and Δ323-8 mutations promoted translocation of torsinA from the ER to the NE (Fig. 9). Together, these data suggest that the reduced co-localization of printor with torsinA ΔE and torsinA Δ323-8 mutants may result from the mislocalization of dystonia-associated mutants to the NE.

TorsinA ΔE and E171Q Mutations Disrupt the Interaction of TorsinA with Printor—To determine the effects of various torsinA mutations on the ability of torsinA to bind printor, we performed co-immunoprecipitation analysis in SH-SY5Y cells co-expressing HA-tagged printor and either C-terminally Myc-tagged torsinA WT, torsinA K108A, torsinA E171Q, torsinA ΔE, torsinA Δ323-8, or the Myc vector control. We found that, like torsinA WT, torsinA K108A, an ATP-binding-deficient mutant, which cannot bind the substrate, was able to interact with printor. In contrast, the “substrate trap” ATP-hydrolysis-deficient mutant torsinA E171Q was incapable of interacting with printor (Fig. 8C). These data argue against the possibility of printor being a torsinA substrate and suggest that printor may be a cofactor of torsinA. Our co-immunoprecipitation analysis revealed that the rare dystonia mutation...
torsinA Δ323-8 had no significant effect on the interaction of torsinA with printor. In contrast, DYT1 dystonia-associated mutation torsinA ΔE completely abolished the ability of torsinA to bind printor (Fig. 8C).

DISCUSSION

TorsinA ΔE mutation is the major cause for DYT1 dystonia, but little is known about torsinA function and its regulation in cells and how torsinA ΔE mutation leads to the disease pathogenesis. In the present study, we identified and characterized a novel protein, printor, that interacts and co-localizes with torsinA. Printor is a novel member of the BTB-BACK-Kelch (BBK) protein family (51, 56, 57) and contains an N-terminal BTB homology region, a BACK domain, and six kelch repeats. Several BBK proteins have been implicated in neuronal development (58-60) and protein ubiquitination (50, 57, 61-63), and mutations in at least one BBK protein, gigaxonin, lead to neuronal dysfunction (64). A major difference between printor and other BBK proteins is that the printor BTB homology region contains a 48-amino-acid P/Q-rich intervening sequence, making it difficult to predict whether this region of printor represents a functional BTB domain. The six kelch repeats of printor are predicted to form a β-propeller structure with multiple protein binding sites (53, 56). Thus, printor could potentially interact with multiple proteins or participate in the formation of multi-protein complexes. The finding that printor orthologues are encoded by the genomes of chordate animals, together with our biochemical data, indicates that the importance of the printor-torsinA association is widespread but previously unrecognized.

Our immunohistochemical and immunoblot analyses reveal that, like torsinA, printor is expressed in the brain, where it is enriched in neurons but not glia. The presence of printor in neuronal cell bodies and processes suggests that printor may participate in neuronal and synaptic function. The abundant expression of printor and its co-distribution with torsinA in brain regions thought to be affected in dystonia (e.g., cerebellum) supports a role for printor in the pathogenesis of dystonia. However, similar to the expression pattern of torsinA, printor is not confined to the dystonia-affected brain regions, but rather, it is widely distributed throughout the brain and is also expressed in many other tissues, suggesting that printor, like torsinA, may have a functional role important to many cell types, including neurons.

TorsinA contains a well conserved AAA+ ATPase domain and is believed to have a chaperone-like function in promoting conformational remodeling of substrate proteins (13, 14, 65). Several binding partners of torsinA have been reported, including LAP1 and its homologous protein LULL1 (21), nesprins (22), snapin (27), and dopamine transporter (33). These torsinA binding partners have been proposed to be substrates of torsinA (20-22, 27, 33), and some of these proteins have been shown to preferentially bind to the ATP-bound form of torsinA (21, 22, 33). In contrast, we found that printor binds to torsinA WT and the ATP-binding-deficient mutant torsinA K108A, but not to the “substrate trap” ATP-hydrolysis-deficient mutant torsinA E171Q. Our results support a role for printor as a cofactor rather than a substrate of torsinA. Increasing evidence indicate that AAA+ proteins utilize cofactors to extend and regulate their binding repertoire and achieve functional specificity (66-68). For example, the AAA+ protein NSF cofactor, α-SNAP, recruits NSF to the SNARE complex and stimulates the ATPase activity of NSF for disassembly of the SNARE complex (11, 12). Cdc48/p97 AAA+ ATPase regulates membrane fusion through the interaction with its cofactor p47, and participates in the ER-associated degradation (ERAD) process by forming a complex with its cofactors, Ufd1 and Npl4 (68). Given that torsinA is a 332-amino-acid protein with a 220-amino-acid AAA+ ATPase homology domain and 40-amino-acid hydrophobic regions (69), it seems particularly plausible that torsinA ATPase would use co-factor(s) to extend its binding repertoire for substrate recognition and function regulation. Printor, with its multiple protein-protein interaction domains, could either directly recruit torsinA substrates or act as a scaffold for organizing a multi-component torsinA chaperone complex. Alternatively, printor could act as a regulator of torsinA, either modulating torsinA ATPase activity or regulating torsinA protein expression level or localization. Further
studies are needed to determine the exact role of the printor-torsinA interaction.

Our finding of an endogenous printor-torsinA complex and the co-localization of printor with torsinA mainly at the ER rather than the NE suggests that printor may act as a cofactor for mediating the ER function of torsinA, participating in substrate recognition and processing. Printor contains two putative transmembrane domains at its C-terminus and two potential N-linked glycosylation sites in the N-terminal region (Fig. 1). This supports a luminal orientation for the N-terminus of printor (Fig. 1D) and suggests that the lumen of the ER is the likely location of the torsinA-printor interaction. Consistent with this possibility, our subcellular fractionations studies reveal that a majority of endogenous printor are membrane-associated and co-fractionate with endogenous torsinA and the ER membrane on the Opti-prep density gradient. Together, our results raise the possibility that the torsinA and printor may function together as a chaperone complex for regulating protein folding in the ER lumen or protein trafficking through the ER.

In addition to the membrane-associated pool, our subcellular fractionations analysis indicates that there is a small cytosolic pool of endogenous printor in SH-SY5Y cells. Although torsinA is generally thought to be a ER luminal protein, a small population of torsinA has been observed in the cytosol (10, 70) and a significant pool of torsinA has been shown to have the AAA'-domain facing the cytoplasm (20). Moreover, torsinA has been reported to bind several cytosolic proteins (20, 27, 71). Thus, it is possible that a small pool of printor may interact with torsinA in the cytosol and regulate cytosolic protein folding.

Although torsinA ΔE mutation accounts for most cases of DYT1 dystonia, the pathological mechanism by which torsinA mutation causes dystonia remains elusive. To understand torsinA function in normal physiology and its dysfunction in dystonia, a major research effort in the field has been directed towards the search for binding partners of torsinA. Thus far, this effort has resulted in the isolation of several torsinA binding partners (20-22, 27). However, none of these interactions is impaired by the dystonia-associated torsinA ΔE mutation. In contrast, our study reveals, for the first time, an interaction between torsinA and its putative cofactor printor that is completely disrupted by torsinA ΔE mutation. Our data supports a loss-of-function pathogenic mechanism for the torsinA ΔE mutation in dystonia and underscores the importance of the printor-torsinA interaction in normal physiology.

In addition to torsinA ΔE mutation, torsinA Δ323-8 mutation has also been associated with dystonia, although it is unclear whether torsinA Δ323-8 is pathogenic or not owing to its co-occurrence with a mutation in another dystonia-related protein, epsilon-sarcoglycan (6). The structural and functional consequences of torsinA Δ323-8 mutation remain mostly unknown. Our results reveal that torsinA Δ323-8 mutation, like torsinA ΔE mutation, promotes translocation of torsinA from the ER to the NE, leading to reduced co-localization of torsinA with printor. However, unlike torsinA ΔE mutation, torsinA Δ323-8 mutation does not affect the interaction of torsinA with printor. These data suggest that the printor binding site involves the second α-helix in the torsinA C-terminal region where glutamate residues 302 and 303 reside, but not the extreme α-helix in the torsinA C-terminal tail where amino acids F323-Y328 reside (72). Thus, deletion of a single glutamate residue at position 302 or 303 (torsinA ΔE) would alter the helical register and disrupt the ability of torsin A to bind printor, whereas deletion of amino acids F323-Y328 (torsinA Δ323-8) would not affect the ability of torsin A to bind printor.

In conclusion, our study identifies a novel protein, printor, which interacts and co-localizes with torsinA, and provides evidence supporting a role for printor as a cofactor rather than a substrate of torsinA. Our findings suggest that disruption of the printor-torsinA interaction by the torsinA ΔE mutation could contribute to the pathophysiology of DYT1 dystonia.
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**FOOTNOTES**

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The abbreviations used are: ER, Endoplasmic Reticulum; NE, Nuclear envelope; WT, Wild-type; FITC, Flourescein isothiocyanate; TR, Texas Red; HA, Hemaglutinin; ECL, Enhanced chemiluminescence; AAA+, ATPases associated with a variety of cellular activities.

**FIGURE LEGENDS**

FIGURE 1. Isolation of printor as a torsinA-interacting protein from yeast-two hybrid screen. A. Domain structure of torsinA. S, ER signal sequence; H, hydrophobic region; AAA+, ATPases associated with a variety of cellular activities. The locations of dystonia-associated torsinA mutations are indicated on the domain structure. Baits used for yeast-two hybrid screens are indicated below the domain structure. *Gal4 DBD*, Gal4 DNA-binding domain. B. Domain structure of printor (top) and the torsinA-interacting clone isolated from the yeast-two hybrid screen (bottom). *BTB*, broad complex, tramtrack and bric-a-brac domain; *P/Q*, proline/glutamine-rich region; *BACK*, BTB and C-terminal Kelch domain; *K*, kelch repeat; TM, transmembrane domain. C. The amino acid sequence homology between human printor and its orthologues. *Hs*, Homo sapiens; *Rn*, Rattus norvegicus; *Gg*, Gallus gallus; *Dr*, Danio rerio. *D.*
Model of membrane topology of torsinA and printor at the ER. Potential glycosylation sites for both torsinA (N143 and N158) and printor (N146 and N433) are indicated.

FIGURE 2. Printor co-distributes with torsinA in multiple tissues and brain regions. A. Lysates from transfected SH-SY5Y cells expressing Myc-tagged printor or purified His-tagged printor protein were analyzed by immunoblotting with pre-immune serum, anti-printor antibody, anti-printor antibody preabsorbed with recombinant printor protein, or anti-Myc antibody. Recomb., recombinant. B. Equal amounts of lysates (50 μg) from the indicated cells were analyzed by immunoblotting using anti-printor, preabsorbed anti-printor, and anti-β-actin antibodies. The asterisks indicate bands that probably represent printor degradation products. C. Equal amounts of homogenates (100 μg) from the indicated rat tissues were analyzed by immunoblotting using anti-printor, anti-torsinA and anti-β-actin antibodies. Sk., skeletal. D. Equal amounts of homogenates (100 μg) from the indicated rat brain regions were analyzed by immunoblotting using anti-printor, anti-torsinA, and anti-β-actin antibodies. Sup., superior; Inf., inferior. E. SH-SY5Y cells overexpressing Myc-tagged printor (top) were immunostained with primary antibodies against printor and Myc tag, followed by detection with secondary antibodies conjugated to TR (Myc, red) or FITC (printor, green). Primary cortical neurons (bottom) were immunostained with primary antibodies against printor and MAP2, followed by detection with secondary antibodies conjugated to TR (printor, red) or FITC (MAP2, green). Hoechst stain was used to visualize the nucleus. Scale bar, 10 μm. All data are representative of at least three independent experiments.

FIGURE 3. Immunohistochemical analysis of printor protein distribution in mouse brain. Coronal sections through A & B. striatum, C & D. hippocampus, and E & F. cerebellum were immunostained with either B, D, & F. anti-printor antibody or A, C, & E. no primary antibody and counterstained with hematoxylin. G & H. Printor immunostaining in the cortex (Ctx). Numbers indicate cortical layers. Neurons in the layer 2/3 were strongly stained. I & J. Printor immunostaining in the striatum (Str). K & L. Printor immunostaining in the hippocampal CA1 region. Printor immunoreactivity was seen in the pyramidal layer (PL) but not in the molecular layer (ML) or granular layer (GL). M & N. Printor immunostaining in the hippocampal CA3 region. Printor immunoreactivity was found in the PL but not in the ML. O, P, & Q. Printor immunostaining in the cerebellum (CB). Purkinje cell layer (PCL) neurons display intense immunostaining compared to the granular layer (GL). Purkinje cell projections in the molecular layer (ML) were also labeled. Arrow indicates cell body and arrowhead indicates neuronal projection. Scale bar is 1.25 mm in A-F; 125 μm in G; 250 μm in I, K, M, and O; 60 μm in H, J, L, N, and P; 30 μm in Q.

FIGURE 4. Printor and torsinA interact in vivo. A. Co-immunoprecipitation of printor with torsinA WTA40. Lysates from HeLa cells expressing either HA-tagged torsinA WTA40 or HA vector and Myc-tagged printor were subjected to immunoprecipitation with anti-HA antibody (12CA5). Immunoprecipitates were analyzed by immunoblotting (IB) with anti-HA and anti-Myc antibodies. The asterisks indicate potential degradation products of printor. B. Co-immunoprecipitation of printor with full-length torsinA. Lysates from HeLa cells expressing HA-tagged printor or HA vector and C-terminally FLAG-tagged torsinA or FLAG vector were subjected to immunoprecipitation with anti-FLAG antibody. The immunoprecipitates were analyzed by immunoblotting with anti-FLAG and anti-HA antibodies. C. Association of endogenous torsinA and printor in the mouse brain. Homogenates from mouse cerebellum were subjected to immunoprecipitation using anti-printor antibody or the preimmune serum, followed by immunoblot analysis with anti-printor and anti-torsinA antibodies.

FIGURE 5. Printor is found in both cytosolic and membrane-associated fractions. A. Post-nuclear supernatant (PN) from SH-SY5Y cells was separated into cytosol (C) and membrane (M) fractions. Aliquots representing an equal percentage of each fraction were analyzed by immunoblotting with anti-printor, anti-torsinA and anti-calnexin antibodies. B. The level of indicated proteins in each fraction was
quantified using NIH Scion Image and shown as a percentage of the total level of the indicated protein. Data represent mean ± S.E. from at least three independent experiments. C. SH-SY5Y cells expressing Myc-tagged printor were immunostained with anti-Myc and either anti-KDEL, anti-EEA1, anti-LAMP2, or anti-TIM23 primary antibodies followed by detection with secondary antibodies conjugated to TR (marker proteins, red), or FITC (printor, green). Hoechst stain was used to visualize the nucleus. Scale bars, 10 μm.

**FIGURE 6. Co-localization of printor and torsinA in the ER.** A. SH-SY5Y cells expressing Myc-tagged printor and C-terminally HA-tagged torsinA (top) were immunostained with primary antibodies against Myc, HA, and KDEL, followed by detection with secondary antibodies conjugated to TR (torsinA, red), FITC (printor, green), or Cy-5 (KDEL, blue). Untransfected SH-SY5Y cells (bottom) were immunostained with primary antibodies against printor, torsinA, and KDEL, followed by detection with secondary antibodies conjugated to TR (torsinA, red), FITC (printor, green), or Cy-5 (KDEL, blue). Scale bars, 10 μm. B. Post-nuclear supernatant from SH-SY5Y cells was fractionated on a 10-30% Opti-prep gradient into 18 fractions, with fraction 1 corresponding to the top of the gradient. Equal volumes of each fraction were analyzed by SDS-PAGE followed by immunoblotting using anti-printor, anti-torsinA and anti-calnexin antibodies. C. The level of indicated protein in each fraction was quantified using NIH Scion Image and shown as a percentage of the total level of the indicated protein. Data are representative of at least three independent experiments.

**FIGURE 7. Printor displays ER preference in both HeLa and SH-SY5Y cells.** A. HeLa (top) or SH-SY5Y (bottom) cells expressing Myc-tagged printor were stained with primary antibodies against Myc and ER marker KDEL, followed by detection with secondary antibodies conjugated to TR (KDEL, red) or FITC (printor, green). Hoechst stain was used to visualize the nucleus. Scale bars, 10 μm. B. Quantification shows the relative distribution of printor and KDEL in the NE versus the ER. Data represent mean ± S.E. from at least three independent experiments. *Significantly different from the NE/ER ratio of KDEL (p < 0.05). C. NE preference of printor was determined by normalizing the NE/ER ratio of printor in HeLa or SH-SY5Y cells to the corresponding NE/ER ratio of KDEL in the same cells. Data represent mean ± S.E. from at least three independent experiments.

**FIGURE 8. Printor interaction and co-localization with torsinA is disrupted by torsinA ΔE and E171Q mutations.** A. Co-localization between printor and WT or mutant torsinA. SH-SY5Y cells co-expressing Myc-tagged printor and C-terminally HA-tagged torsinA WT, torsinA ΔE, torsinA Δ323-8, torsinA K108A or torsinA E171Q were stained with primary antibodies against HA and Myc, followed by detection with secondary antibodies conjugated to TR (torsinA, red) or FITC (printor, green). Hoechst stain was used to visualize the nucleus. Scale bars, 10 μm. B. Quantification shows the percentage of printor protein that co-localizes with WT or mutant torsinA. Data represent mean ± S.E. from at least three independent experiments. *Significantly different from the percentage of printor co-localization with torsinA WT (p < 0.005). C. Extracts from SH-SY5Y cells expressing Myc-tagged printor and C-terminally HA-tagged torsinA WT, torsinA ΔE, torsinA Δ323-8, torsinA K108A or torsinA E171Q were subjected to immunoprecipitation with anti-HA antibody followed by immunoblotting with anti-HA and anti-Myc antibodies.

**FIGURE 9. TorsinA E171Q mutation and dystonia-associated mutations promote translocation of torsinA from the ER to NE.** A. SH-SY5Y cells expressing C-terminally HA-tagged torsinA WT, torsinA ΔE, torsinA Δ323-8, torsinA K108A, or torsinA E171Q were stained with primary antibodies against HA and ER marker KDEL, followed by detection with secondary antibodies conjugated to TR (KDEL, red) or FITC (torsinA, green). Hoechst stain was used to visualize the nucleus. Scale bars, 10 μm. B. Quantification shows the relative distribution of torsinA and KDEL in the NE versus the ER. Data represent mean ± S.E. from at least three independent experiments. *Significantly different from the
NE/ER ratio of torsinA WT ($p < 0.05$). C. NE preference of torsinA was determined by normalizing the NE/ER ratio of torsinA to the corresponding NE/ER ratio of KDEL in the same cells. Data represent mean ± S.E. from at least three independent experiments. *Significantly different from torsinA WT ($p < 0.05$).
Figure 4

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A

|          | Input | IP: α-HA |
|----------|-------|----------|
| HA-TorsinA WTΔ40 | -     | +        |
| Myc-Printer    | +     | +        |
| HA vector      | +     | -        |

IB: α-Myc
- 82
- 64

* Printor

IB: α-HA
- 25
- 19

- TorsinA WTΔ40
- IgG Light Chain

B

|          | Input | IP: α-FLAG |
|----------|-------|------------|
| TorsinA WT-FLAG | +     | -          |
| HA-Printer    | -     | +          |
| FLAG Vector   | +     | -          |
| HA Vector     | +     | -          |

IB: α-HA
- 82
- 64

- Printor

IB: α-FLAG
- 37

- TorsinA

C

|          | Pre-immune | α-printer |
|----------|------------|-----------|
| TorsinA  | 37         | -         |
| Printor  | 64         | -         |

- TorsinA
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A

B

C

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Figure 8

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Figure 9
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