The Early Onset Dystonia Protein TorsinA Interacts with Kinesin Light Chain 1*‡‡

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Early onset dystonia is a movement disorder caused by loss of a glutamic acid residue (Glu302/303) in the carboxyl-terminal portion of the AAA+ protein, torsinA. We identified the light chain subunit (KLC1) of kinesin-I as an interacting partner for torsinA, with binding occurring between the tetra-ripeptide repeat domain of KLC1 and the carboxyl-terminal region of torsinA. Co-immunoprecipitation analysis demonstrated that wild-type torsinA and kinesin-I form a complex in vivo. In cultured cortical neurons, both proteins co-localized along processes with enrichment at growth cones. Wild-type torsinA expressed in CAD cells co-localized with endogenous KLC1 at the distal end of processes, whereas mutant torsinA remained confined to the cell body. Subcellular fractionation of adult rat brain revealed torsinA and KLC associated with cofractionating membranes, and both proteins were co-immunoprecipitated after cross-linking cytoplasmically oriented proteins on isolated rat brain membranes. These studies suggest that wild-type torsinA undergoes anterograde transport along microtubules mediated by kinesin and may act as a molecular chaperone regulating kinesin activity and/or cargo binding.

The dystonias represent a varied group of movement disorders characterized by twisted and contractive movements and postures throughout the body (1), with early onset dystonia being the most common and severe form of the inherited dystonias. Onset of symptoms occurs in a limb between 5 and 20 years of age, and symptoms generalize by spreading to other limbs, physically incapacitating most patients within approximately 5 years (2, 3). The disease follows an autosomal dominant mode of inheritance with reduced penetrance of 30–40% (4). There are no signs of neurodegeneration in the brains of these patients (5, 6), suggesting that neuronal dysfunction, rather than loss of neurons, underlies disease symptoms. Most cases of early onset dystonia are caused by the deletion of 3 base pairs (ΔGAG) in the DYTI gene that encodes a single glutamic acid residue, Glu302 or Glu303, near the C-terminal region of torsinA (7). This protein, which shares conserved domains with the AAA+ (HSP/Clp-ATPase-AAA) superfamily of chaperone-like proteins (8), is expressed in a broad range of tissues in mammals. Within the human brain, expression levels are highest in dopaminergic neurons of the substantia nigra (9). The DYTI gene is a member of a gene family that includes three other related genes: TOR1B, adjacent to DYTI on chromosome 9q34, encoding torsinB (70% identity); and TOR2A and TOR3A on chromosomes 9q34 and 1q24, respectively, encoding TORP1 and TORP2 (ADIR) (10), sharing 40% identity with torsinA (11). Following transient transfection in mammalian cells, wild-type torsinA localizes predominantly to the endoplasmic reticulum (ER), as does endogenous torsinA (12), whereas mutant (ΔGAG) torsinA accumulates in the perinuclear region (13, 14) and in large membranous structures in the cytoplasm (15–16).

Functions of the torsin gene family have yet to be elucidated. In general, AAA+ proteins are critical to the assembly, disassembly, and operation of protein complexes and often form homo- or oligomeric ring structures with substrate proteins or nucleic acids “threaded” through the hole in the ring (17). They participate in a wide variety of cellular processes, including membrane fusion, organelle biogenesis, movement along microtubule tracks, proteolysis and DNA replication. The C-terminal helical subdomain of AAA+ proteins in particular has been shown to be important for substrate interaction, and the contacts that this region forms in crystal structures of these proteins suggest a role in oligomerization (18). For some AAA+ proteases, mutations in this domain disrupt interaction with partner proteins in a dominant negative manner (19, 20). Both dystonia-associated mutations identified in the DYTI gene are in-frame deletions within the C-terminal region, including the common GAG deletion and an 18-bp deletion (Ph e323–Tyr328del) in one patient with early onset dystonia with myoclonic and tic-like features (21). These deletions could have dominant negative effects on the function of a torsin AAA+ oligomer (22).

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To identify proteins that interact with torsinA, we undertook a yeast two-hybrid screen using the predicted C-terminal α-helical subdomain of human wild-type torsinA as a bait. Two isoforms of kinesin light chain (KLC) 1, KLC1-B and KLC1-C, were identified as specifically interacting proteins, and their interaction was confirmed by affinity precipitation assays, co-immunoprecipitation, and cross-linking experiments. KLC1 is part of the heterotrimeric motor protein kinesin-1 (23) and is thought to be involved in cargo binding and/or regulation of kinesin-1 activity (24, 25). We show that KLC1 is a physiologically relevant binding partner for torsinA, with both proteins co-localizing in cultured primary cortical neurons and both concentrated in neuronal growth cones. Our results provide the first report of a direct interaction between torsinA and another protein.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—TorsinA coding sequences, amino acids 251–332 of wild-type and mutant (ΔGAG, mut) human torsinA (7), were amplified by PCR using mammalian expression constructs for wild-type or ΔGAG torsinA (15) and subcloned into the yeast two-hybrid vector pEG202 (26), generating the constructs pEG202-torAwt-(251–332) and pEG202-torAΔG-(251–332), respectively. The cDNA sequences for both KLC1, amino acids 112–556 (isoform C), or amino acids 229–547 (isoform B), isolated from the yeast two-hybrid brain cDNA library (MoBiTec), were subcloned into the mammalian expression vector pcDNA3/5 (Invitrogen), containing a FLAG tag in-frame at the 5′-end of the multicloning site (27). KLC1 coding sequences were amplified by PCR from isolated library plasmids and cloned into pcDNA3/5/F generating the constructs FLAGKLC1-B-(229–547) and FLAGKLC1-C-(112–556). Likewise, the same KLC1 coding sequences were subcloned into the bacterial expression vector pGEX4T1 (Amersham Biosciences), generating the constructs GST-KLC1-B-(229–547) and GST-KLC1-C-(112–556), which were used to produce GST-KLC1 fusion proteins. The integrity of all inserts was confirmed by sequencing.

**Cell Lines**—HEK293T/17 human embryonic kidney cells (Dr. D. Baltimore, MIT), the mouse central nervous system catecholaminergic cell line CIT (28) (kindly provided by Dr. J. Wang, Tufts University), the human neuroblastoma cell line SH-SY5Y (ATCC) (29), and a rat cell line CAD (28) (kindly provided by Dr. J. Wang, Tufts University) were used in this study: polyclonal antibody TAB1, generated (12, 15).

**Antibodies**—The following, previously characterized torsinA antibodies were used in this study: polyclonal antibody TAB1, generated against a C-terminal region (amino acids 299–312) of human torsinA, which recognizes both torsinA and torsinB; mouse monoclonal antibody (mAb) D-MG10, generated against exon 4 (amino acids 208–249) of human torsinA, which recognizes torsinA, but not torsinB; mAb D-M2A8, raised against a maltose-binding protein-torsinA fusion protein (amino acids 51–332 of torsinA), which recognizes only torsinA (12, 15). Other mAbs used include 63-90 and KLC-All (31), which recognize both KLC1 and KLC2 (kindly provided by Dr. Scott Brady, University of Texas); M2 anti-FLAG (Sigma); H2 against kinesin heavy chain (KHC) (Chemicon); 6E9 against tuberin; and DM1A against α-tubulin (Sigma). Other polyclonal antibodies used included SPA-860 against the D-M2A8, raised against a maltose-binding protein-torsinA fusion protein which recognizes both torsinA and torsinB; mouse monoclonal antibody (mAb) D-MG10, generated against exon 4 (amino acids 208–249) of human torsinA, which recognizes torsinA, but not torsinB; mAb D-M2A8, raised against a maltose-binding protein-torsinA fusion protein (amino acids 51–332 of torsinA), which recognizes only torsinA (12, 15).

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GST in Vitro Pull-down Assays—HEK 293T/17 cells, transfected with mammalian expression constructs encoding full-length wild-type or ΔGAG torsinA, were lysed in 0.1% Nonidet P-40 and 0.5% sodium deoxycholate. Culture supernatants were precipitated with GST affinity resin and washed extensively with PBS. After an additional wash with 0.1% Nonidet P-40 buffer, bound proteins were eluted from the beads by resuspension in SDS-PAGE loading buffer and resolved by SDS-PAGE followed by immunoblotting using standard methods. For pull-downs of endogenous torsinA, PC12 cells were lysed in RIPA buffer.

Transfection and Immunofluorescence of CAD Cells—CAD and HEK293T/17 cells were transiently transfected as described (15). 12 h after transfection, cells were replated onto glass coverslips and differentiated to single cell identity (serum-free Dulbecco's modified Eagle's medium/F12). 48–72 h later, cells were fixed in 4% paraformaldehyde at room temperature for 20 min. Immunofluorescence of fixed CAD cells was carried out as described (15), except that Alexa 594-conjugated goat anti-mouse and Alexa 488-conjugated goat anti-rabbit were used as secondary antibodies. Confocal images were captured using LSM 5 Pascal software coupled to a Zeiss LSM Pascal Vario 2 RGB confocal system. For quantification of co-localization of endogenous torsinA immunoreactive inclusions, cells transiently transfected with ΔGAG torsinA, 192 transfected cells from two independent experiments showing aggregates were scored visually for co-localization of both proteins by overlapping fluorescence.

**Immunoprecipitations**—For immunoprecipitations (IPs), PC12 cells, SH-SY5Y cells, or primary cortical neurons were lysed by resuspension in ice-cold RIPA buffer, as above. Lysates were subjected to IP overnight at 4 °C. Subsequently, antibody-antigen complexes were precipitated by the addition of Protein A-agarose (for polyclonal antibodies) or Protein G-agarose (for monoclonal antibodies; Roche Applied Science). Bound proteins were eluted from the beads by resuspension in SDS-PAGE loading buffer and resolved by SDS-PAGE followed by immunoblotting. Control human brain (hippocampal tissue from an anonymous donor, no diagnostic abnormality reported) was obtained with IRB approval from Dr. Jean-Paul Vonsattel (Massachusetts General Hospital). Human and embryonic (E16) or adult rat brain tissue were lysed in RIPA buffer and sonicated twice for 45 s at a pulse intensity of 6 using a 550 sonic dissector (Fisher), and insoluble material was removed by centrifugation at 16,000 × g for 30 min. IPs were carried out as above.

Fractionation of Rat Brain—For each fractionation, whole fresh brain from one adult rat (>12 weeks) was homogenized in HB buffer (20 mM HEPES, pH 7.5, 40 mM potassium chloride, 5 mM EGTA, 5 mM magnesium chloride with protease inhibitors). Vesicle-enriched membrane fractions (P3) were separated by sequential centrifugation at 900 × g for 5 min (P1), 9,000 × g for 10 min (P2), and 120,000 × g for 90 min (P3), slightly modified from Ref. 36. The P3 pellet was resuspended in HB buffer containing 0.32 M sucrose and loaded on top of 11 ml of a 0.32–1.9 M continuous sucrose gradient in HB buffer. Gradients were centrifuged at 100,000 × g in a SW41 rotor for 16–18 h, and 1.9 M sucrose fractions were removed from the top of the gradient. Beads were resuspended with chloroform-methanol (37) and resuspended in equal volumes of 1× SDS sample buffer before resolution by SDS-PAGE, followed by immunoblotting.

**Cross-linking of Rat Brain Membrane Surface Proteins Followed by Immunoprecipitation**—For cross-linking of cytoplasmically oriented proteins, 5 ml P3 vesicle-enriched fraction from rat brain membrane was resuspended in HB buffer containing 0.32 M sucrose. The water-soluble cross-linker bisulfosucinimidylbuvate (Pierce) was added to 5 mM final concentration, samples were incubated for 30 min at room temperature, and the reaction was quenched by the addition of 50 mM

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**V. Ramesh, unpublished data.**
Tris, pH 7.5, and incubation for 15 min at room temperature. Subsequently, NaCl, Nonidet P-40, deoxycholate, SDS, and protease inhibitors were added to the same concentrations as in RIPA buffer (see above), the indicated antibodies were added, and immunoprecipitations were carried out as described above, except that beads were washed three times with 0.1% Nonidet P-40.

**Biotinylation of Rat Brain Membrane Surface Proteins Followed by Precipitation with Immobilized Streptavidin—**Total rat brain was homogenized in HB buffer, as above, including 0.32 M sucrose. Membranes were fractionated by sequential centrifugation at 900 × g for 5 min (P1), 9,000 × g for 10 min (P2), and 120,000 × g centrifugation for 90 min (P3), as above. Pellets were resuspended in HB buffer including 0.32 M sucrose, and biotinylation with 0.5 mg/ml Sulfo-NHS-LC-biotin (Pierce) for 30 min at room temperature was carried out essentially as described (38). Reactions were quenched by the addition of 50 mM Tris, pH 9.0, and incubation at room temperature for 15 min. Subsequently, NaCl, Nonidet P-40, deoxycholate, SDS, and protease inhibitors were added to the same concentrations as in RIPA buffer (see above), and samples were incubated with immobilized streptavidin (Pierce) for 1 h. Except for incubation periods during biotinylation and quenching reactions, samples were kept at 4 °C throughout. Beads were washed three times in 1% Nonidet P-40 buffer, and samples were resolved by SDS-PAGE, followed by immunoblotting. To evaluate glycosylation, endoglycosidase H or peptide-N-glycosidase F digestion of rat brain samples was performed as recommended by the manufacturer (New England Biolabs) prior to SDS-PAGE.

**Primary Culture of Rat Cortical Neurons—**Brains were obtained from day 17–18 Sprague-Dawley rat embryos, and primary cultures were prepared essentially as described (39) with the following modifications: coverslips were coated overnight in 0.1 mg/ml poly-D-lysine (Sigma); embryonic cortices were minced and dissociated in 2.5 units/ml dispase (Sigma); and dissociated neurons were plated in minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. After 8 days in culture, coverslips were fixed with 4% paraformaldehyde in PBS for 20 min and subsequently processed for immunocytochemistry.

**Immunofluorescence of Rat Cortical Neurons—**For double labeling with two mAbs, fixed neurons were permeabilized with 0.1% Nonidet P-40 in PBS for 30 min, blocked for 1 h in 10% normal goat serum, and incubated with the first mAb in 1% bovine serum albumin in PBS for 1 h at room temperature, followed by three washes in PBS. Cells were then incubated with Alexa 594-conjugated goat anti-mouse antibody (1:1000) for 1 h, washed as above, incubated with the second mAb in bovine serum albumin/PBS for 1 h at room temperature, and washed as above, followed by incubation with Alexa 488-conjugated goat antimouse (1:2500) for 45 min, and again washed as above. Coverslips were mounted as above, and confocal images were captured using either a Nikon TE 300 microscope and a Bio-Rad MRC 1024 laser confocal imaging system (see Fig. 4, A–O) or a Zeiss confocal imaging system, as above (see Fig. 4, P–R).

### RESULTS

**The KLC1 TPR Domain Binds Directly to the C Terminus of TorsinA—**To identify proteins that interact directly with wild-type human torsinA, amino acids 251–332 of the protein were used as a bait (Fig. 1A) to screen an adult human brain cDNA library using the LexA-based yeast two-hybrid system (26). This sequence contains the site of the GAG deletion and is predicted by alignment to comprise the small C-terminal helical subdomain of the AAA-fold (18). In a screen of 1 × 10^6 independent transformants, 15 positive clones were identified, representing three groups. The 10 clones of one group all encoded overlapping fragments of human kinesin light chain 1 (KLC1), including two differentially spliced isoforms, KLC1-B and KLC1-C (Fig. 1, B and C). The three clones of another group encoded the nonmotor domain of an unconventional myosin, whereas a third group encoded two overlapping cDNA fragments of an uncharacterized protein.

The two human KLC1 isoforms were identical to the alternatively spliced KLC1 isoforms B and C recently identified in a human keratinocyte cDNA library (40). Compared with isoform B, isoform C contains an additional nine amino acids (YSMSVEVNG) close to the C terminus (Fig. 1C). Of the 10 KLC1 clones, one encoded KLC1-B (229–547), and nine clones encoded KLC1-C (112–256). Neither interacted with any of four different control bait plasmids (Table I), demonstrating a specific interaction between torsinA (251–332) and both KLC1 isoforms. Since the KLC1-B cDNA fragment encoded only the major part of the TPR domain, but not the N-terminal heptad repeat (Fig. 1B), we conclude that the TPR domain and C-terminal region of KLC1 (amino acids 209–547) are sufficient to mediate the interaction with the C-terminal part of torsinA (amino acids 251–332).

In yeast colony filter assays (Table I), we observed a difference in the strength of lacZ reporter gene activation and therefore of the interaction between KLC1 isoforms and wild-type or mutant (ΔGAG) forms of torsinA. This apparent difference in binding affinity was supported by yeast liquid culture β-galactosidase assays (Supplemental Fig. 1), but not by affinity precipitation assays (see below).

The interaction between endogenous torsinA and KLC1 was
Further confirmed by in vitro affinity precipitation (GST pull-down) assays (Fig. 2). In order to examine whether endogenous torsinA interacts with KLC1, GST-KLC1 fusion proteins were incubated with lysates of rat pheochromocytoma PC12 cells, which express torsinA at high levels (12). GST-KLC1-B-(229–547) and GST-KLC1-C-(112–556) both bound endogenous torsinA (Fig. 2A), with no significant binding of torsinA to the GST control or beads alone. GST-KLC1 fusion proteins were also incubated with protein extracts from HEK 293T/17 cells transfected with full-length wild-type torsinA cDNAs. Wild-type torsinA bound to both GST-KLC1 isoforms but only negligibly to GST alone, with apparently somewhat more wild-type torsinA being retained by GST-KLC1-C-(112–556) than by GST-KLC1-B-(229–547) (Fig. 2B).

In contrast to the yeast liquid culture β-galactosidase assays, however, we did not observe a difference in binding affinity of wild-type versus mutant torsinA to KLC1 in these affinity precipitation assays (Supplemental Fig. 2) using protein extracts from HEK 293T/17 cells transfected with full-length wild-type or mutant torsinA cDNAs.

**Endogenous TorsinA and Kinesin-I Exist in a Complex in Vivo**—To evaluate the association of endogenous torsinA and KLC1, we performed immunoprecipitations from a variety of tissue sources (Fig. 3). In human brain (hippocampus), a polyclonal antibody against torsinA, TAB1, specifically precipitated torsinA, KLC1, and kinesin heavy chain (KHC) but not KLC2 (Fig. 3A), indicating that these three proteins interact in vivo. Preimmune serum did not precipitate any of these proteins. We also observed co-precipitation of torsinA and KHC in adult rat brain (Fig. 3B), rat primary cortical neurons (Fig. 3C), embryonic (E16) rat brain (Supplemental Fig. 3A), and PC12 cells (Supplemental Fig. 3B). In the converse experiment, immunoprecipitation of kinesin in SH-SY5Y cells co-precipitated torsinA (Fig. 3D). Together, these results indicate that kinesin-I, which in mammals is a heterotrimer consisting of two heavy chains (KHCs) and two light chains (KLCs), is a physiologically relevant binding partner for wild-type torsinA. The fact that we were able to detect co-precipitated KLC only in human brain (Fig. 3A) and not in other tissue sources (Fig. 3, B–D), whereas KHC co-precipitated with torsinA in all tissues and cells, is probably due to decreased efficiency in detection of KLC in Western blotting procedures (31, 41).

**Table I**

| Binding domain | Activation domain | lacZ reporter gene activation<sup>a</sup> |
|----------------|------------------|----------------------------------------|
|                |                  | Without galactose | With galactose |
| torA WT-(251–332) | KLC1-C-(112–556) | – | ++|
| torA WT-(251–332) | KLC1-B-(229–547) | – | ++|
| torA ΔGAG-(251–332) | KLC1-C-(112–556) | – | ++|
| torA ΔGAG-(251–332) | KLC1-B-(229–547) | – | ++|
| TubSH3 + X25 | KLC1-C-(112–556) | – | – |
| TubSH3 + X25 | KLC1-B-(229–547) | – | – |
| Cdc2 | KLC1-C-(112–556) | – | – |
| Cdc2 | KLC1-B-(229–547) | – | – |
| Fus3 | KLC1-C-(112–556) | – | – |
| Fus3 | KLC1-B-(229–547) | – | – |
| Bicoid | KLC1-C-(112–556) | – | – |
| Bicoid | KLC1-B-(229–547) | – | – |

<sup>a</sup> Symbols indicate degree of lacZ reporter gene activation, as judged by intensity of blue color in yeast colony filter assays. —, white; (+), weak blue; +, moderately intense blue; ++, intense blue; +++, very intense blue.

Further investigation of endogenous torsinA and KLC1 proteins in tissue sources (Fig. 3). In human brain (hippocampus), a polyclonal antibody against torsinA, TAB1, specifically precipitated torsinA, KLC1, and kinesin heavy chain (KHC) but not KLC2 (Fig. 3A), indicating that these three proteins interact in vivo. Preimmune serum did not precipitate any of these proteins. We also observed co-precipitation of torsinA and KHC in adult rat brain (Fig. 3B), rat primary cortical neurons (Fig. 3C), embryonic (E16) rat brain (Supplemental Fig. 3A), and PC12 cells (Supplemental Fig. 3B). In the converse experiment, immunoprecipitation of kinesin in SH-SY5Y cells co-precipitated torsinA (Fig. 3D). Together, these results indicate that kinesin-I, which in mammals is a heterotrimer consisting of two heavy chains (KHCs) and two light chains (KLCs), is a physiologically relevant binding partner for wild-type torsinA. The fact that we were able to detect co-precipitated KLC only in human brain (Fig. 3A) and not in other tissue sources (Fig. 3, B–D), whereas KHC co-precipitated with torsinA in all tissues and cells, is probably due to decreased efficiency in detection of KLC in Western blotting procedures (31, 41).

**TorsinA, KLC, and KHC Co-localize in Primary Neurons and Are Enriched at Neuronal Growth Cones**—To investigate whether torsinA and kinesin-I co-localize to the same subcellular compartments in neurons, we performed immunocytochemistry on rat primary cortical neuronal cultures (Fig. 4). TorsinA and KLC showed a high extent of co-localization (Fig. 4, C and F) in cell bodies (Fig. 4, A–C) and neurites (Fig. 4, D–F) with a punctate, apparently vesicular pattern. TorsinA and KHC also co-localized with a similar distribution (Fig. 4, J–L). Because two monoclonal antibodies and sequential incubation was used for double-labeling (see “Experimental Procedures”), we performed control labelings using only one primary monoclonal antibody (KLC-All (Fig. 4, G–I) or D-MG10 (Fig. 4, M–O)) with both anti-mouse secondary antibodies. No significant background signal was observed in these controls. We observed an enrichment of both torsinA and KLC in the proximal to central growth cone region (Fig. 4, P–R) relative to other segments of neuronal processes. TorsinA showed partial co-localization with GAP-43/neuromodulin in these neuronal cultures (data not shown). GAP-43 is known to be enriched in growth cones (42) and a cargo of kinesin-I (43, 44).

**Wild-type TorsinA Co-localizes with Endogenous Kinesin at the Tips of Processes, whereas Mutant TorsinA Remains Confined to the Cell Body**—Next, we analyzed the subcellular localization of torsinA and **FLAG-KLC1-C-(112–256)** when both proteins were overexpressed simultaneously in the mammalian neuronal-like cell line CAD (28), which has low levels of endogenous torsin (15). Transient co-transfection with both wild-type torsinA and **FLAG-KLC1-C-(112–256)** cDNAs (Fig. 5, A–C) showed partial co-localization of both proteins in the cell body (arrowhead in C), with extensive co-localization in the processes, especially at the distal ends (arrows in C). In contrast, co-transfection with mutant torsinA and **FLAG-KLC1-C-(112–256)** cDNAs (Fig. 5, D–F) yielded large inclusions, which were intensely labeled for torsinA, but not **FLAG** (Fig. 5, D and E). TorsinA immunoreactivity was concentrated in the cell body with a reduced amount in the processes. Overexpressed **FLAG-tagged KLC1-C-(112–256)** was found throughout the cytoplasm in a fine punctate pattern (Fig. 5F), typical of the distribution of endogenous KLC (45).

In CAD cells transiently transfected only with wild-type torsinA cDNA, torsinA and endogenous KLC also co-localized in the cell body and at the distal end of processes (Fig. 5, G–I), as above. In contrast, transient transfection with mutant torsinA alone again yielded torsinA-positive inclusions confined to the cell body, with loss of torsin enrichment at the distal end of processes (Fig. 5, J–L; arrow in L). In a majority of cells transfected with mutant torsinA (~57% on average), a significant portion of endogenous KLC co-localized with torsinA in these inclusions (Fig. 5, M–O), as did endogenous KHC (data not shown), whereas in other transfected cells, this was not apparent (Fig. 5, J–L). The distribution of microtubules in CAD cells transfected with either wild-type or mutant torsinA, as assessed with an antibody against β-tubulin, was not apparently altered (data not shown), arguing against a nonspecific disruption of cytoskeletal elements.

**TorsinA and KLC Co-fractionate in Sucrose Gradients of Rat Brain Vesicle-enriched Membranes and Can Be Cross-linked and Biotinylated on the Cytoplasmic Surface of Isolated Membranes**—To directly identify a membrane compartment containing both torsinA and KLC, subcellular fractionation of adult rat brain was carried out by differential centrifugation. Both torsinA and KLC were enriched in a P3 vesicle-enriched membrane fraction, although both were also present in the P1 fraction (Fig. 6A). This distribution of torsin was similar to that observed for another ER protein, BiP/Grp78. The P3 fraction
was further resolved on a continuous 0.32–1.9 M sucrose gradient, which revealed significant overlap in the distributions of torsinA, KLC, and the ER marker calnexin (Fig. 6B).

Kinesin is located at the cytoplasmic surface of vesicles (24), whereas torsinA when derived from cultured cells behaves as a luminal ER protein in protease protection assays (12, 16). To determine whether a fraction of torsinA in mammalian brain exists on the cytoplasmic surface of vesicle-enriched membranes, we cross-linked cytoplasmically oriented proteins of rat brain P3 membranes using the water-soluble cross-linker bis(sulfosuccinimidyl)suberate, which does not cross membranes, followed by immunoprecipitation under stringent conditions (RIPA buffer). Antibodies against torsinA specifically precipitated higher molecular weight bands, which were recognized by antibodies against KHC, KLC, and torsinA (Fig. 7A), but not by a control antibody against protein-disulfide isomerase, an ER-resident protein (data not shown). The apparent molecular mass of the predominant band, ~220–240 kDa, is consistent with a multiprotein complex containing torsinA (~37 kDa), KLC1 (~64 kDa), and KHC (~130 kDa). Negative controls, which included mouse IgG (Fig. 7A), an unrelated antibody raised against tuberin (6E9), and an antibody against protein-disulfide isomerase (data not shown) did not precipitate these higher molecular weight bands.

To further confirm these findings by an independent method, subcellular fractionation of rat brain by differential centrifugation was carried out essentially as above (except that homogenization was carried out in HB buffer containing 0.32 M sucrose), and P1, P2, and P3 fractions were biotinylated using a water-soluble biotinylation reagent (Sulfo-NHS-LC-Biotin). Subsequent precipitation of biotinylated membrane surface proteins with immobilized streptavidin under stringent conditions precipitated KHC, as expected, and two lower molecular mass variants of immunoreactive torsinA (~30 and 33 kDa), but not the 37-kDa form, from the P1 fraction, which contains a mixture of different types of organelles (Fig. 7B). These two torsinA variants were immunoreactive with two different antibodies against torsinA, D-M2A8 and TAB1. No precipitation of the entirely luminal protein BiP/Grp78 was observed, suggesting that membranes remained largely intact during fractionation. TorsinA (apparent mass 37 kDa) possesses two N-linked glycosylation sites, matching the consensus sequence

![Fig. 2. TorsinA and KLC1 interact in affinity precipitation (GST binding, “pull-down”) assays.](image-url)

A, endogenous wild-type torsin, derived from PC12 cells and solubilized in RIPA buffer, binds both KLC1 isoforms. GST and glutathione-Sepharose beads were used as negative controls. Western blot was probed with anti-torsin TAB1 antibody. TCL, total cell lysate. B, wild-type torsinA binds different KLC isotypes. A Western blot shows GST-KLC1-C-(112–556) and GST-KLC1-B-(229–547) both binding to full-length wild-type torsinA derived from transiently transfected HEK293T cells, solubilized in 0.1% Nonidet P-40 buffer. GST and glutathione-Sepharose beads were used as negative controls. D-MG10 mAb was used to detect torsinA. Approximately equal loading of GST or GST-KLC1 fusion proteins was confirmed by Ponceau-S staining (bottom panels).

![Fig. 3. TorsinA exists in a complex with kinesin-I in vivo.](image-url)

A, co-IP of endogenous torsinA, KLC and KHC from human brain (hippocampus) using TAB1 anti-torsin polyclonal antibody (IP TAB1). THL, total hippocampal lysate; IP con, control IP with preimmune serum. Western blot was probed with the following mAbs: H2 against KHC, 63-90 against KLC, or D-M2A8 against torsinA. B–D, co-IP of endogenous torsinA and KHC from a rat primary cortical neuron sample (C), or SH-SY5Y human neuroblastoma cell line (D). TAB1 and appropriate controls were used for immunoprecipitations, as in A. In SH-SY5Y cells (D), additional reciprocal co-immunoprecipitation was performed using H2 anti-KHC mAb (IP H2). TL, total lysate; IP con M2, control IP with an unrelated monoclonal antibody, M2. Westerns blots were probed with H2, D-MG10, or D-M2A8 mAbs.
NX(T/S), Asn143 and Asn158, which have been demonstrated to be glycosylated in cultured cells (16). The two biotinylated torsinA variants identified in this study correspond in apparent mass to nonglycosylated forms of torsinA, with the 30-kDa variant presumably undergoing further proteolytic processing or possibly representing an alternative splice form. Consistent with this hypothesis, treatment with endoglycosidase H or peptide-N-glucosaminyl asparagine amidase F reduced the 37-kDa immunoreactive form to a predominant band of ~33 kDa, corresponding in apparent mass to the biotinylated 33-kDa variant (Fig. 7C), whereas the 30- and 33-kDa variants were not further reduced in size. Taken together, these data indicate that some portion of torsinA in rat brain is exposed on the cytoplasmic surface of vesicle-enriched membranes and thereby capable of interacting with kinesin-I.

**DISCUSSION**

Our results demonstrate that the TPR domain of the KLC1 subunit of kinesin-I interacts directly with the carboxyl-terminal region of torsinA, a novel protein responsible for early onset torsion dystonia. This is the first reported binding partner for torsinA and hence provides critical insights into the function of this protein. KLC1 was initially identified as a binding partner for torsinA in a yeast two-hybrid screen, and the interaction between the two proteins was verified by affinity precipitation assays, co-immunoprecipitation, and cross-linking experiments. The association of these proteins was further confirmed by similar patterns of subcellular fractionation from rat brain tissue and co-localization in primary rat cortical neurons in cell bodies, neurites, and growth cones. Wild type torsinA and KLC1 were distributed together throughout the cytoplasm and processes of neural cells with enrichment at distal ends, whereas mutant torsinA was retained in the cell body in inclusions, frequently altering the distribution of endogenous kinesin-I by incorporation into these inclusions. These findings support the hypothesis that torsin participates in intracellular trafficking mediated by kinesin microtubule motors and predict a possible molecular etiology of early onset dystonia by interference with intracellular trafficking.

**Direct Interaction of TorsinA and KLC—**KLC1 is a subunit of kinesin-I, also named conventional kinesin, a plus-end-directed molecular motor protein involved in transport of membranous organelles along microtubules within cells (23). KLC is composed of two notable domains: an N-terminal heptad repeat region that mediates binding to the stalk and tail domains of KHC and a C-terminal domain of six imperfect TPR motifs (25). Two highly conserved domains in different subunits of kinesin-I have been proposed as cargo binding sites: the tail region of KHC (46) and the TPR domain of KLC. Recently, two different classes of proteins have been reported to bind directly to the TPR domain of KLC: the amyloid precursor protein (44) and the c-Jun N-terminal kinase-interacting proteins JIP-1, -2, and -3 (47, 48). Here we demonstrate that the KLC1 TPR domain interacts directly with the C terminus of torsinA. TPR motifs occur in proteins as tandem repeat arrays, and TPR-containing proteins often form scaffolds that mediate protein-protein in-
Wild-type, but not mutant, torsinA displays polarized distribution and co-localizes with FLAG-KLC-C-(112–256) and endogenous kinesin in transfected CAD cells. CAD cells were transiently transfected with wild-type torsinA and FLAG-KLC-C-(112–256) (A–C), mutant torsinA and FLAG-KLC-C-(112–256) (D–F), wild-type torsinA (G–I), or mutant torsinA (J–O) expression constructs. A–C, torsinA was detected with TAB1 polyclonal antibody (green; similar to the pattern detected with D-MG10), and FLAG-KLC-C-(112–256) was detected with M2 anti-FLAG mAb (red). The merged image is shown in C. Arrowhead, overlapping localization in the cell body; arrows, co-localization at the distal end of processes. Scale bar, 10 μm. D–F, the same antibodies as in A and B were used to detect mutant torsinA (green) and FLAG-KLC-C-(112–256) (red). The merged image (F) shows no notable co-localization of these proteins. Scale bar, 10 μm. G–I, torsinA was detected with TAB1 polyclonal antibody (green), and endogenous KLC was detected with 63-90 mAb (red). The merged image (I) shows co-localization of both proteins in the cell body and at the distal end of processes (arrows in I). Scale bar, 10 μm. J–L, mutant torsinA (green) was detected with the same antibody as in G; endogenous KHC was detected with H2 mAb (red). This antibody detected an identical pattern of kinesin distribution as 63-90, as previously described (45). The merged image (L) shows confinement of mutant torsinA aggregates to the cell body, whereas KHC is enriched at the distal end of processes (arrow in L). Scale bar, 10 μm. M–O, the same antibodies as in G and H were used to detect mutant torsinA (green) and endogenous KLC (red). The merged image (O) shows co-localization of both proteins in large inclusions. Scale bar, 10 μm.
teractions and assemble multiprotein complexes (49) by binding short peptides at the C terminus of their target proteins (50, 51). The KLC TPR domain interacts with JIP-1 and JIP-2 through their extreme C termini (47), and its interaction with torsinA also involves the C-terminal region. In our two-hybrid screen from human brain, we isolated two distinct isoforms of KLC1. These KLC1 isoforms are identical to two alternatively spliced isoforms recently identified in a human keratinocyte cDNA library (40). The existence of multiple, differentially spliced KLC isoforms could reflect cell type-specific expression or targeting of kinesin to different classes of organelles.

Mutant Torsins and Mutant Kinesin Interfere with Intracellular Trafficking—Wild-type torsinA expressed in CAD cells co-localized with endogenous KLC at the distal end of processes, whereas CAD cells transfected with mutant torsinA cDNA formed large torsin-positive inclusions confined to the cell body, as previously described (15). These results are consistent with plus-end-directed transport of wild-type, but not mutant, torsinA along microtubules via kinesin and suggest that the binding of torsinA to KLC1 as a cargo is required for proper trafficking of torsinA within the cell. It remains to be determined whether mutant torsinA affects microtubule binding, motor activity, or cargo binding of kinesin.

If torsin binds kinesin, mutations in either might produce similar phenotypes. A torsin homolog in nematodes, OOC-5, is critical for nuclear-centrosome rotation preceding the establishment of spindle orientation and polarity in early cell division in Caenorhabditis elegans (52). Mutations in OOC-5 are maternal effect-lethal and prevent proper localization of the partitioning-defective proteins specifically at the two-cell stage. Mutants in UNC-116, the only KHC gene in C. elegans, also show maternal effect defects in both the first cell division and neuromuscular development (53, 54). These overlapping phenotypes support the concept of torsins and kinesins participating in common molecular pathways.

Topographic Distribution of TorsinA—The orientation of torsinA with respect to different membrane compartments in cells remains an enigma. Experiments using cultured cell lines indicate that torsinA is membrane-associated (12, 16, 55, 56) and resides primarily in the lumen of the ER but with little to none of the protein being exposed to the cytoplasmic surface, as assessed by protease protection assays (12, 16). In contrast, the present study shows that in homogenates prepared from rat brain, a significant fraction of membrane-associated torsinA is exposed to the cytoplasm, as determined by cross-linking and biotinylation assays. It may be that torsinA exists in two states in nervous tissue, one within the lumen of the ER and one exposed on the cytoplasmic surface of the ER or vesicles derived from it. In the latter case, one would expect torsinA to be nonglycosylated, which is consistent with the size of immunoreactive torsinA observed to be exposed to the cytoplasm at its C-terminal domain. One potential hypothesis for insertion of this nonglycosylated form of torsinA into the ER membrane would be that the amino-terminal signal sequence initiates translocation and is cleaved, but that the following hydrophobic domain (i.e. amino acids 21–40) serves as a stop-transfer se-
quence and transmembrane anchor for a traditional “type I” membrane protein, with the C-terminal domain exposed to the cytoplasm and thus accessible for binding to kinesin. In these and other studies, it is apparent that torsinA undergoes anterograde transport along neuronal processes to terminals, presumably in association with the ER and/or vesicles (12, 57–59), apparently mediated by kinesin. Ultrastructural studies in primate brain demonstrated torsinA immunoreactivity associated with small clear vesicles in symmetric synapses in the striatum (59). Further, subcellular fractionation of human and monkey brain found enrichment of torsinA in particulate fractions including the P2 (crude synaptosomal membranes), P3 (light vesicles), and LP1 (synaptosomal membranes) fractions (59), the last of which does not contain detectable levels of the ER marker calnexin (60). Thus, whereas a large portion of torsinA is located in the ER lumen in cultured cells, at least some portion of it appears to be exposed on the cytoplasmic surface of other membranes in mammalian brain.

Does TorsinA Act as a Chaperone to Regulate the Activity of Kinesin-I or the Association of Kinesin-I with Cargo?—There is mounting evidence that the majority of kinesin in the cell is kept in an inactive ground state by binding of the KHC tail domain to the motor domain, which maintains kinesin in a folded conformation (61, 62). Factors that have been proposed to regulate kinesin’s ability to bind to and move along microtubules include conformational changes induced by the binding of cargo itself, changes in local pH, phosphorylation state, and modulation by chaperone proteins. At least one unidentified membrane-associated chaperone-type protein is required for release of kinesin from membranous cargo (63), and torsinA appears to be a likely candidate, since it can act as a chaperone protein (64, 65). Further, blocking of a highly conserved epitope within the TPR domain releases kinesin from membranes and inhibits fast axonal transport (31), and our data indicate that torsinA binds to KLC1 through its TPR domain. Thus, our results raise the possibility that torsinA may act as a molecular chaperone for KLC1, potentially regulating the association of kinesin with cargo and/or activating kinesin by changing its conformation from a folded to an unfolded state.

The ER of most vertebrate cells is spread throughout the cell,
including its processes, by kinesin-dependent transport along microtubules (66, 67). Given the association of both torsin and kinesin with the ER and their direct interaction with each other, torsin could also be involved in kinesin-mediated anterograde spreading of tubulovesicular membranes within axons.

Significance of TorsinA Interaction with KLC1 in Early Onset Dystonia—Other neurological diseases caused by loss-of-function mutations in kinesin include Charcot-Marie-Tooth disease type 2A, a hereditary neuropathy caused by a mutation in KIF1B (68), and one form of autosomal dominant hereditary spastic paraplegia, caused by a mutation in the neuronal KHC gene KIF5A (69). Further, amyloid precursor protein, which has been implicated in the pathogenesis of Alzheimer’s disease, interacts directly with the KLC TPR domain (44) and is transported in the same axonal membrane compartment as kinesin-I, β-secretase, and presenilin-1 (36).

Although torsinA is most highly expressed in dopaminergic nigrostriatal neurons, it is widely expressed elsewhere in brain and other tissues (7, 9). However, abnormal movements and postures are the only clinical phenotype reported for patients carrying the DYT1 GAG deletion. This could result from the length of nigrostriatal fibers, which may make them especially vulnerable to deficits in cellular transport systems.

Carriers of the DYT1 GAG deletion who do not experience onset of symptoms by age 28 are very unlikely to develop the disease state. 

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