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Microtubule-associated protein 1B: a neuronal binding partner for gigaxonin

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Giant axonal neuropathy (GAN), an autosomal recessive disorder caused by mutations in *GAN*, is characterized cytopathologically by cytoskeletal abnormality. Based on its sequence, gigaxonin contains an NH_2-terminal BTB domain followed by six kelch repeats, which are believed to be important for protein–protein interactions (Adams, J., R. Kelso, and L. Cooley. 2000. *Trends Cell Biol.* 10:17–24.). Here, we report the identification of a neuronal binding partner of gigaxonin. Results obtained from yeast two-hybrid screening, cotransfections, and coimmunoprecipitations demonstrate that gigaxonin binds directly to microtubule-associated protein (MAP)1B light chain (LC; MAP1B-LC), a protein involved in maintaining the integrity of cytoskeletal structures and promoting neuronal stability. Studies using double immunofluorescent microscopy and ultrastructural analysis revealed physiological colocalization of gigaxonin with MAP1B in neurons. Furthermore, in transfected cells the specific interaction of gigaxonin with MAP1B is shown to enhance the microtubule stability required for axonal transport over long distance. At least two different mutations identified in GAN patients (Bomont, P., L. Cavalier, F. Blondeau, C. Ben Hamida, S. Belal, M. Tazir, E. Demir, H. Topaloglu, R. Korinthenberg, B. Tuysuz, et al. 2000. *Nat. Genet.* 26:370–374.) lead to loss of gigaxonin–MAP1B-LC interaction. The devastating axonal degeneration and neuronal death found in GAN patients point to the importance of gigaxonin for neuronal survival. Our findings may provide important insights into the pathogenesis of neurodegenerative disorders related to cytoskeletal abnormalities.

Introduction

The cytoskeleton network, which is responsible for cell architecture, intracellular transport, mitosis, cell motility, and differentiation, is composed of microtubules, actin microfilaments (MFs), and intermediate filaments (IFs).* Several lines of evidence have demonstrated that not only do microtubules, actin microfilaments, and IFs interconnect physically through cross-linking proteins, but the properties of these networks can also be modulated by different associated proteins (Houseweart and Cleveland, 1999; Fuchs and Karakesisoglou, 2001). Cytoskeletal organization and dynamics depend on protein self-associations and interactions with a variety of binding partners such as microtubule-associated proteins (MAPs) (Sanchez et al., 2000). MAP1B is one of the major MAPs expressed in juvenile neurons. Since its initial identification, the biological roles of MAP1B have been of great interest. Acting as a complex of an NH_2-terminal heavy chain (HC; MAP1B-HC) and COOH-terminal light chain (LC; MAP1B-LC), MAP1B is involved in many cellular processes including morphogenesis, differentiation, and maintenance of cytoskeletal integrity (for review see Tucker, 1990; Hirokawa, 1994). Several attempts including gene-targeting studies have been made to determine MAP1B's role in brain development and functions (Edelmann et al., 1996; Takei et al., 1997, 2000; Gonzalez-Billault et al., 2000; Meixner et al., 2000; Teng et al., 2001). However, the functional significance of interactions between MAP1B and other proteins remains largely unclear. Intriguingly, a growing body of evidence indicates that several human and mouse diseases can be attributed to aberrant interactions between cytoskeletons and their associated proteins (Garcia and Cleveland, 2001).

Giant axonal neuropathy (GAN) is a severe recessive motor and sensory neuropathy affecting both peripheral nerves and the central nervous system (Berg et al., 1972). A prominent pathological feature of GAN is the presence of giant axonal swellings densely packed with aberrant IFs, abnormal micro-

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*Abbreviations used in this paper: DRG, dorsal root ganglia; GAN, giant axonal neuropathy; IF, intermediate filament; MAP, microtubule-associated protein.

Key words: gigaxonin; MAP1B; cytoskeletal organization; giant axonal neuropathy; protein–protein interaction

tubule network, and accumulation of other membranous organelles (Prineas et al., 1976; Bousquet, et al., 1996). The onset of disease symptoms usually occurs by age four and death commonly follows in adolescence (Quvrier, 1989). The disease locus has been assigned to chromosome 16q24.1 (Ben Hamída et al., 1997). Recently, Bomont et al. (2000) identified a novel gene (*GAN*) encoding gigaxonin. Out of 15 families, 14 distinct *GAN* mutations were identified. The mutations were distributed evenly throughout the coding sequence, including one truncating mutation, four nonsense mutations in the kelch repeat domain, and nine missense mutations over the entire eleven coding exons. Although the discovery of the *GAN* gene represents an important step toward elucidating the pathogenesis of GAN, the molecular mechanisms underlying the abnormal cytoskeletons remains poorly understood. Because alterations in the cytoskeletal network are a general feature of several neurodegenerative disorders, including ALS (Hirano, 1991; Williamson et al., 1996), understanding the functions of gigaxonin may provide insights into the pathogenesis of more prevalent degenerative disorders.

The presence of aberrant cytoskeletal networks in GAN suggests that gigaxonin may function as a novel and distinct cytoskeletal protein. In this report, we present in vivo and in vitro evidence that identifies a neuronal binding partner for gigaxonin. We found that through its kelch repeat domain gigaxonin binds directly to the COOH end of MAP1B-LC, an interaction that may regulate the activity of MAP1B complex and enhances microtubule stability against depolymerizing reagents.

Results and discussion

Gigaxonin protein is expressed in brain and other tissues

The coding sequence of gigaxonin was amplified by PCR. Northern blot analysis revealed a specific band of 4.6 kb present in all tissues examined and prominent in brain, heart, and muscle (unpublished data), demonstrating ubiquitous expression of the gene. This result is consistent with observations of multiple tissue pathologies in GAN patients. To characterize the function(s) of gigaxonin and determine its protein expression, we raised a peptide-specific antibody with an epitope at the COOH end. After confirming the specificity of the antibody, we analyzed gigaxonin protein expression in mouse tissues by immunoblot assay. A single protein band of the expected size of 70 kD was observed in brain, heart, and muscle tissues and in transfected COS-7 cells, verifying the protein expression of gigaxonin (Fig. 1).

Gigaxonin interacts directly with MAP1B-LC in vitro

Abnormal aggregation of multiple tissue-specific IFs in the affected cells of GAN implicates a generalized disorder of cytoskeleton organization. We tested whether gigaxonin might have the capacity to associate directly with cytoskeletal networks. To facilitate the assays, a HA epitope tag was engineered at the NH_2-terminal of the gigaxonin coding region. The construct was transiently transfected into COS-7 cells, and the protein was found to diffusely distribute throughout the cytoplasm (Fig. 2 D), suggesting that the protein may lack a direct cytoskeleton-binding site.

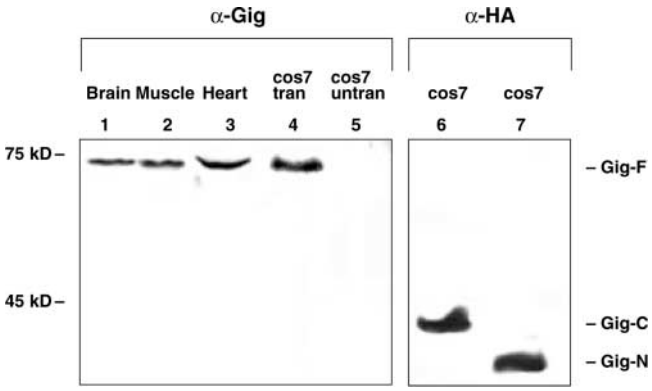

Figure 1. **Protein expression of gigaxonin.** Proteins isolated from mouse tissues were analyzed by immunoblot with rabbit antigigaxonin (lanes 1–5) or mouse anti-HA (Covance) (lanes 6 and 7). The single band of ~70 kD (lanes 1–4), which is absent in the untransfected COS-7 cells (lane 5), indicates the full-length gigaxonin protein. The 40- and 28-kD bands from transfected COS-7 cells represent the COOH-terminal domain (Gig-C, lane 6) and NH_2-terminal domain (Gig-N, lane 7), respectively. Migration of protein standard (Amersham Biosciences) is indicated at left.

We set out to understand gigaxonin's biological role by using the yeast two-hybrid system to identify proteins with which gigaxonin interacts. The full-length gigaxonin was fused to a GAL4 DNA-binding domain and used as the bait to screen a human brain cDNA library. Identification of multiple positive clones suggested that microtubule-associated protein 1B light chain (MAP1B-LC) is a neuronal binding partner for gigaxonin. Further assays were performed to confirm this interaction. When full-length HA-gigaxonin (HA-Gig-full) was cotransfected with flag-MAP1B-LC, the gigaxonin staining pattern changed to a filamentous array (Fig. 2 A) and the protein colocalized with MAP1B-LC along the microtubule network (Fig. 2, B and C). Additional double staining against tubulin and MAP1B-LC on gigaxonin–MAP1B-LC–cotransfected cells confirmed that MAP1B-LC aligns with microtubules (unpublished data). The direct interaction between these two proteins was assessed by cotransfection followed by coimmunoprecipitation. A specific band of full-length gigaxonin that manifested exclusively in the cotransfection lane and positive control lane (Fig. 2 E, lanes 1 and 5) confirmed the direct association between MAP1B-LC and gigaxonin.

Gigaxonin physically colocalizes with MAP1B in neurons

The observed binding of gigaxonin to MAP1B-LC in vitro suggested that the interaction might represent a biologically significant association. We sought to define MAP1B as a physiological binding partner for gigaxonin in neurons in vivo by carrying out double immunofluorescent microscopy on cultured DRG neurons to test whether the two proteins colocalize. A gigaxonin-specific antibody showed strong colabeling with MAP1B (Fig. 3, A–C). The physical colocalization of the two proteins in cultured neurons provided the first evidence of a corresponding interaction in vivo. We then conducted coIP assays using mouse brain tissue. The specific detection of gigaxonin from the protein complex coimmunoprecipitated by anti–MAP1B-LC antibody further confirmed the direct physiological association between MAP1B-LC and gigaxonin (unpublished data).

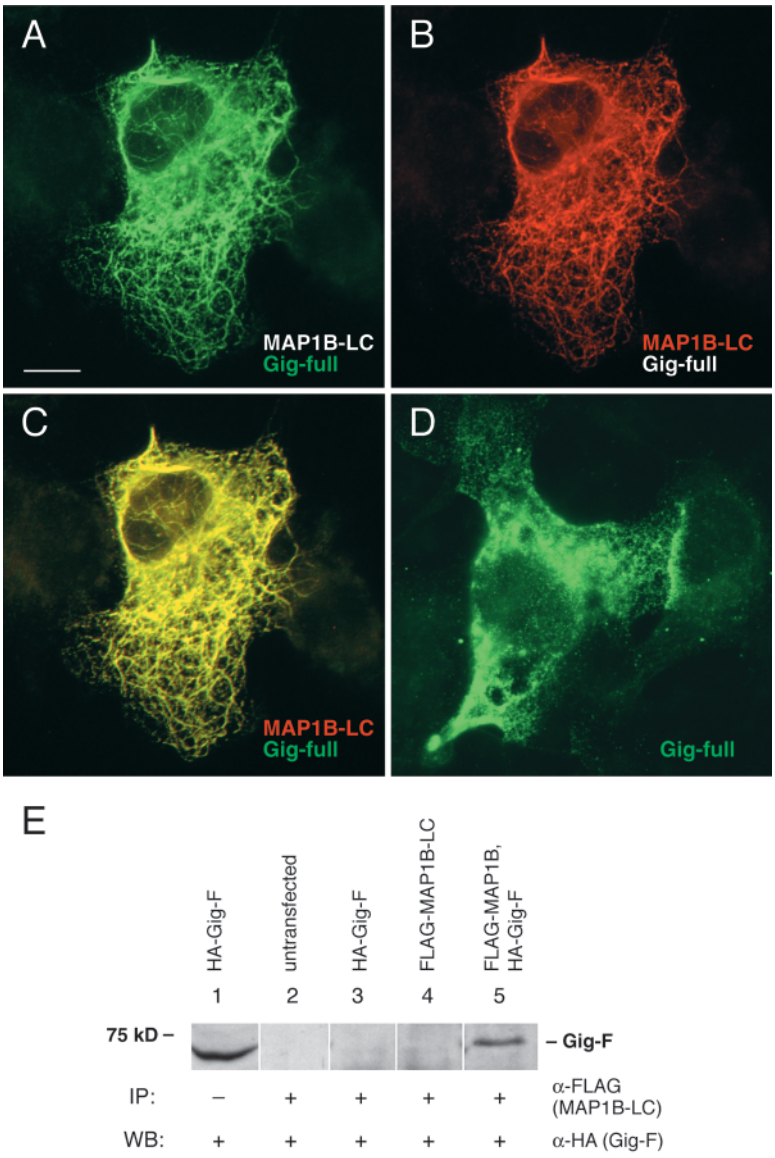

Figure 2. **Gigaxonin associates with MAP1B-LC on microtubules.** The expression constructs of HA-Gig-full and flag-MAP1B-LC were cotransfected into COS-7 cells (A–D). The cells were subjected to double immunofluorescence as described previously (Yang et al., 1996). Antibodies are indicated in each panel: (A) Gig-full, (mouse anti-HA); (B) MAP1B-LC (rabbit anti-flag; Sigma-Aldrich). Note that gigaxonin displayed a network array that coaligned with MAP1B-LC in cotransfected cells (C), and a diffuse accumulation in cytoplasm in single transfected cells (D). Bar, 12 μm. (E) The cotransfected cells were processed for coIP using anti-flag (MAP1B-LC) and immunoblotted with anti-HA (gigaxonin, Gig-F). Note the specific band of Gig-F present in cotransfection lane (lane 5) but absent in the single transfections of MAP1B-LC (lane 4) or Gig-F (lane 3). The total cell lysates from Gig-F single transfection without IP (lane 1) and untransfected COS-7 (lane 2) served as controls in this assay.

To obtain in vivo evidence for this association at the ultrastructural level, we employed double immuno-EM labeling using two different sizes of gold-conjugated particles and three separate trials. Indeed, gigaxonin colocalized with MAP1B in mouse sciatic nerves (Fig. 3 D). Approximately 43 ± 7% of gigaxonin labeling colocalizes with MAP1B, while 41 ± 6% of the MAP1B-associated gold particles were within 15 nm of gigaxonin labeling. In contrast, no significant labeling of gigaxonin and MAP1B was detected in negative control samples in which only secondary antibodies were applied (Fig. 3 E). It is interesting to note that the golden particles appeared to be associated not only with filamentous cytoskeleton but also with spherical structures. This implies that gigaxonin may also play a role in connecting vesicles with cytoskeleton by interacting with both MAP1B and certain vesicular-associated protein. However, this implication awaits further investigations.

Gigaxonin competes with MAP1B-HC for binding to MAP1B-LC

To define more precisely the domains responsible for the association of the two proteins, we first mapped gigaxonin's binding site for MAP1B-LC. HA epitope–tagged domains of both NH_2-terminal BTB and COOH-terminal kelch repeats of gigaxonin were examined. Immunoblot analysis confirmed that both domains were stably expressed and were of the expected size (Fig. 1, lanes 6 and 7). The COOH kelch repeat domain of gigaxonin (Gig-C) coaligned with MAP1B-LC in cotransfected cells (Fig. 4, A and B), supporting the notion that the kelch repeat motif is important for protein–protein interactions. In comparison, the BTB domain was found to distribute diffusely in the cytoplasm and failed to display specific associations (unpbulished data). We then examined the NH_2-terminal microtubule-binding domain (MAP1B-LC-MTBD) and COOH terminus of MAP1B-LC (MAP1B-LC-CT) in transfected COS-7 cells to identify the target for gigaxonin association. We found that the MAP1B-LC-CT displayed a staining pattern of short actin stress filaments (Fig. 4 C), which is consistent with published reports by others (Togel et al., 1998). Indeed, gigaxonin's kelch repeat domain colocalized with MAP1B-LC-CT (Fig. 4 D). The results also revealed that MAP1B-LC could bind to gigaxonin's kelch repeat domain

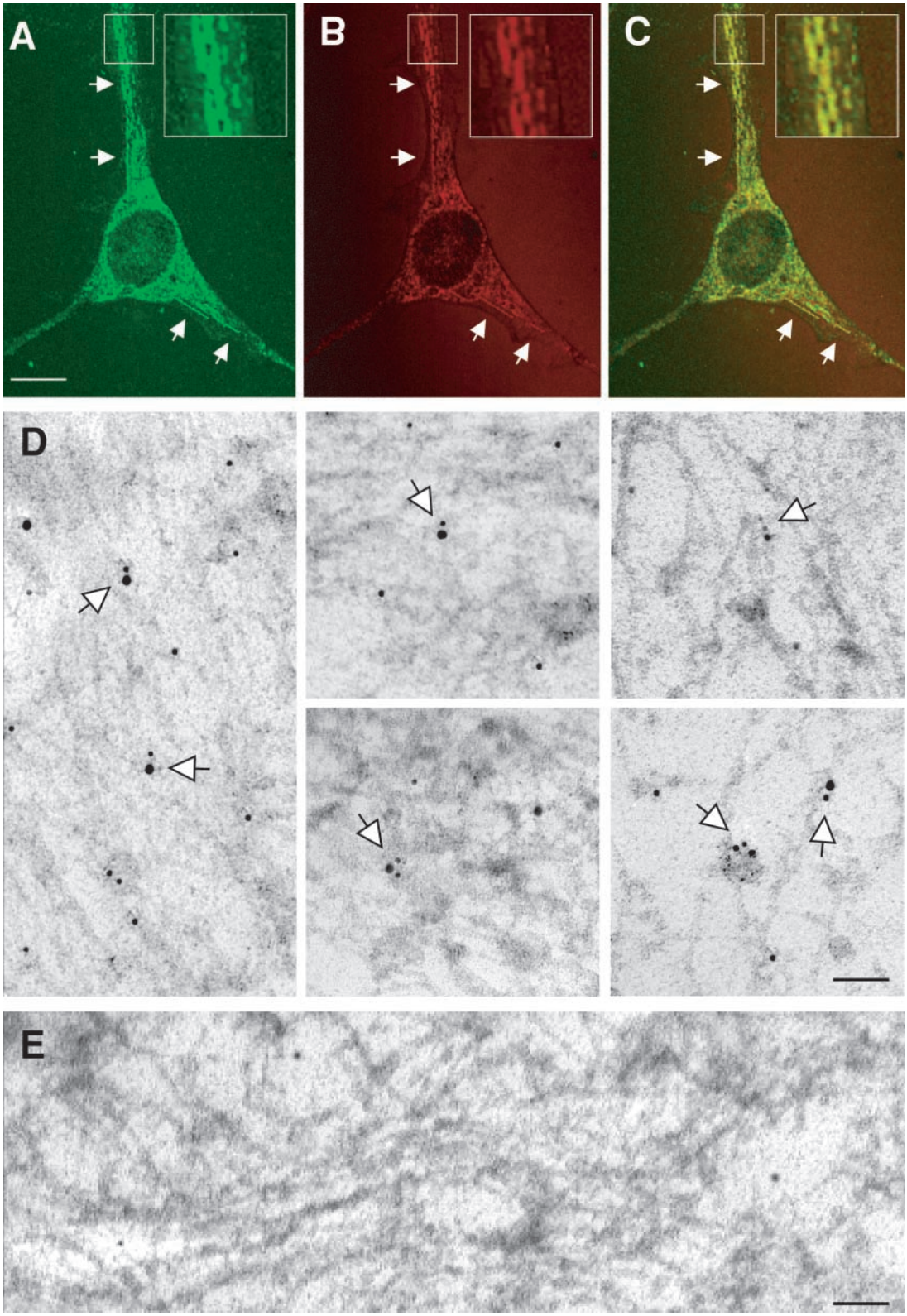

Figure 3. **Gigaxonin physiologically colocalizes with MAP1B in neurons.** (A–C) The cultured mouse DRG neurons were subjected to double immunofluorescence using anti–mouse MAP1B-LC (Sigma-Aldrich) (A, green) and rabbit antigigaxonin (B, red). Arrows denote colocalizations on cytoskeletal structures. Insets in A, B, and C show higher magnifications of the colocalization areas in the white boxes. (D) For double immuno EM, sciatic nerve samples were colabeled with rabbit antigigaxonin and mouse anti–MAP1B-LC followed by gold-conjugated secondary antibodies against mouse (small particles) and rabbit (large particles). The large particles represent gigaxonin, and the small particles identify MAP1B. Arrows identify colocalizations. The samples labeled with only secondary antibodies were used as negative control (E). Bar: (A–C) 20 μm; (D and E) 200 nm.

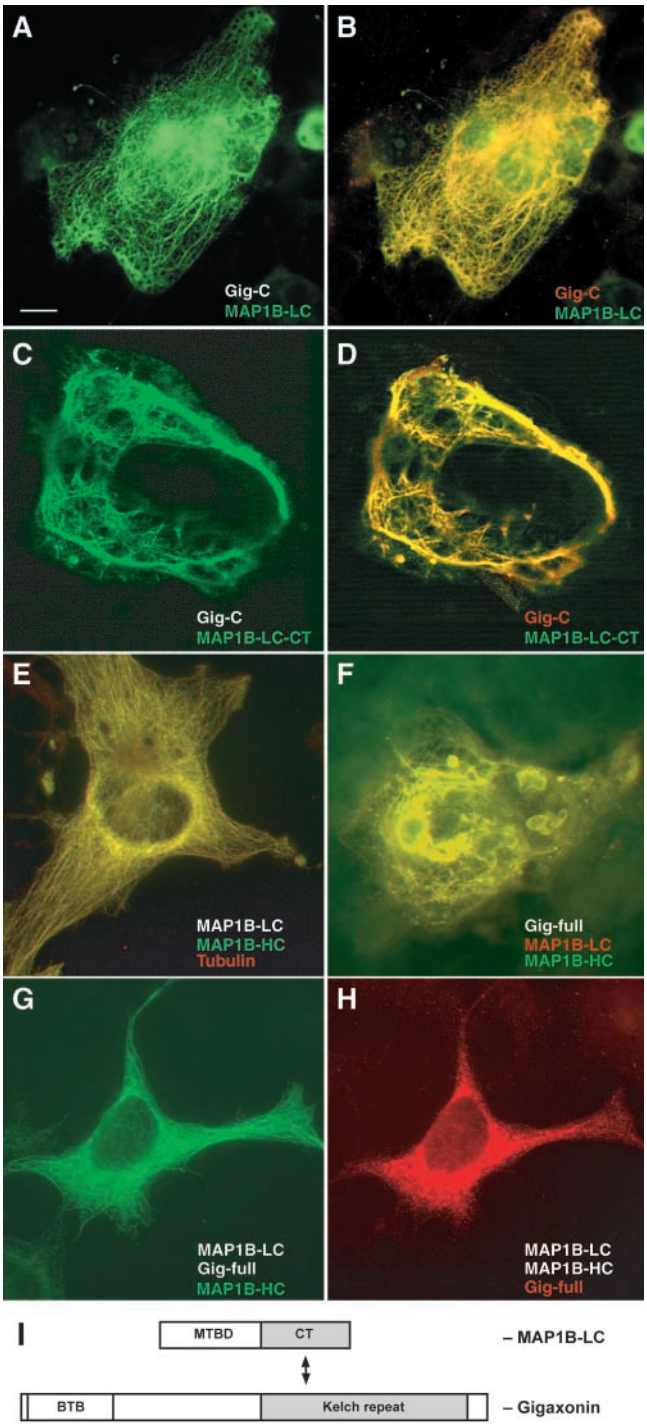

Figure 4. **The kelch repeat domain of gigaxonin binds to the COOH terminus of MAP1B-LC.** The cells were cotransfected for 30 h with flag-MAP1B-LC and HA-Gig-C (A and B), or flag-MAP1B-LC-CT and HA-Gig-C (C and D), or flag-MAP1B-LC and MAP1B-HC-myc (E), or flag-MAP1B-LC, MAP1B-HC-myc, and HA-Gig-full (F–H). (A–D) Mouse anti-HA (Gig-C; B and D, red) and rabbit anti-flag (MAP1B-LC or MAP1B-LC-CT; A and C, green). Note that the Gig-C colocalized with MAP1B-LC-CT. (E and F) Sheep antitubulin (Cytoskeleton Inc.; E, red); mouse anti-myc (CLONTECH Laboratories, Inc.; E and F, green); rabbit anti-flag (F, red) and rabbit anti-myc (G, green); mouse anti-HA (H, red). Bar, 12 μm. The diagram in I indicates that the kelch repeat domain of gigaxonin interacts with the COOH terminus of MAP1B-LC.

without losing its ability to bind microtubules and actins. Thus, through its kelch repeat domain gigaxonin interacts directly with the COOH terminus of MAP1B-LC and associates indirectly with cytoskeletal filaments.

MAP1B-LC harbors multiple binding sites for protein–protein interactions at its COOH terminus including those for gigaxonin and HC of MAP1B. We investigated whether the association of MAP1B-LC with gigaxonin affects its interaction with MAP1B-HC. When myc epitope–tagged MAP1B-HC (MAP1B-HC-myc) was expressed alone in COS-7 cells, the HC protein diffusely accumulated in the cytoplasm without filamentous appearance (unpublished data). This finding is in agreement with reported findings (Togel et al., 1998). However, when the HC was cotransfected with MAP1B-LC into COS-7 cells, HC colocalized with LC on microtubules (Fig. 4 E). Intriguingly, when the MAP1B-HC-myc, flag-MAP1B-LC, and HA-gigaxonin were coexpressed in triple transfected COS-7 cells, MAP1B-HC appeared in five separate trials to compete with gigaxonin for binding to MAP1B-LC. Although the LC remained bound to microtubules, only one of the two proteins, either HC or gigaxonin, could colocalize with LC on microtubules. In ~69% of the triple transfected cells, the HC retained its association with LC on microtubules (Fig. 4, F and G), whereas gigaxonin diffusely distributed in cells (Fig. 4 H). The association of MAP1B-LC with gigaxonin but not with HC could be only observed in ~10% of the triple transfected cells (unpublished data), suggesting that MAP1B-LC has a preferential association with MAP1B-HC over gigaxonin. It has been suggested that the HC might function as the regulatory subunit of the MAP1B complex to control LC activity (Togel et al., 1998). Our findings raise the possibility that through the competitive binding to the LC with the HC, gigaxonin may play an important role in regulating functions of the MAP1B complex. A regulatory interaction between LC and HC of MAP1B might be critical for normal functions of neurons.

The interaction of gigaxonin with MAP1B significantly enhances microtubule stability

To assess the functional significance of the interaction between MAP1B-LC and gigaxonin, we examined microtubule stability in cells transfected either with MAP1B-LC alone or MAP1B and gigaxonin together. The transfected cells were treated with colchicine, a depolymerizing agent. In untransfected control cells, all microtubules depolymerized within 15 min of colchicine treatment (Fig. 5 A). In agreement with outside published reports, MAP1B had only a modest effect on microtubule stabilization (Takemura et al., 1992; Togel et al., 1998). After treatment for 30 min at concentrations up to 4.5 μM, a significant number of microtubule arrays (62%) in 82% of MAP1B-LC single transfected cells began to disappear; within 60 min, no network was visible (Fig. 5 B). An extraordinary feature of cells cotransfected with MAP1B-LC and gigaxonin was that gigaxonin increased the ability of MAP1B-LC to render the endogenous microtubule network resistant to microtubule-destabilizing reagents. In >70% of MAP1B/gigaxonin double transfected cells, the microtubule network remained intact even after a 90-min treatment with colchicine. In a sizable number of double transfected cells

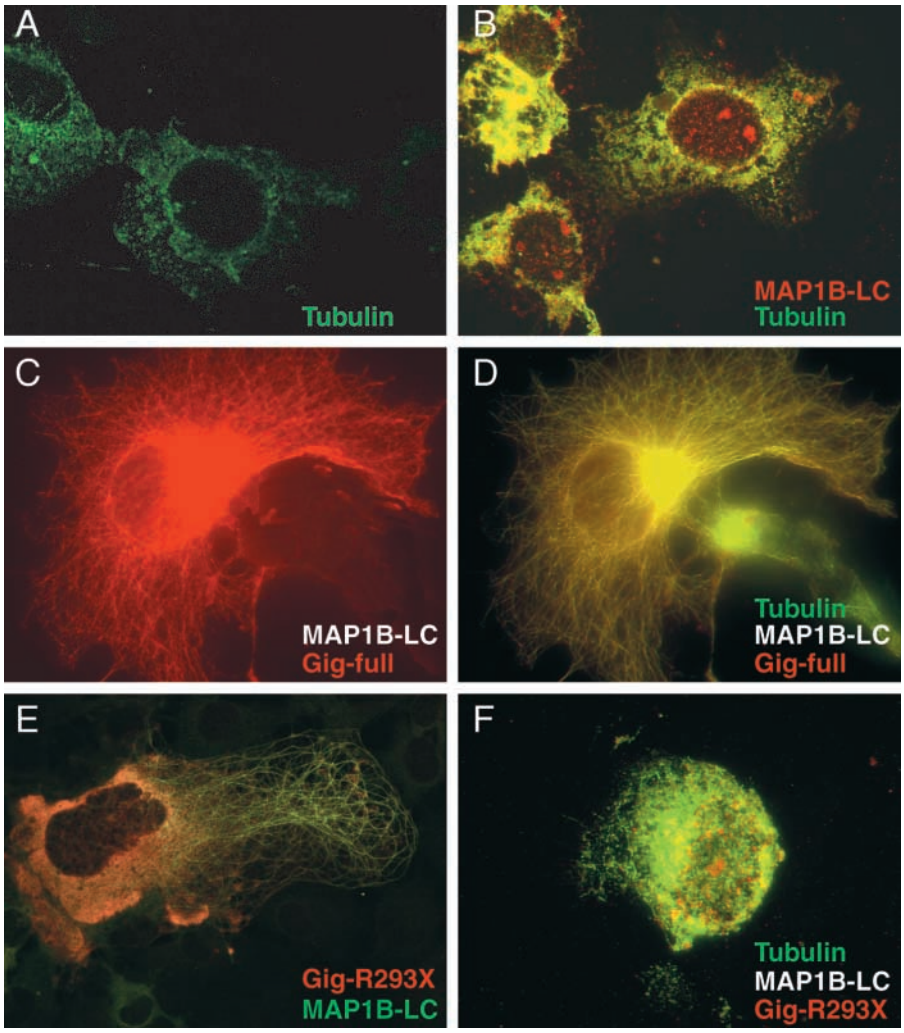

Figure 5. **The gigaxonin–MAP1B-LC interaction enhances microtubule stability.** Both transfected and untransfected cells were double stained using antibodies indicated in each frame. After treatment with colchicine, the microtubules were seen to be depolymerized within 15 min in untransfected (A) and within 60 min in flag-MAP1B-LC single transfected cells (B). In contrast, the intact network could still be found in 20% of HA-Gig-full/flag-MAP1B-LC cotransfected cells after 2 h treatment (C and D). Double immunostaining reveals that the HA-Gig-G293X (mutant gigaxonin, red) lost association with MAP1B-LC (E, green) and HA-Gig-G293X/flag-MAP1B-LC displayed no enhancement of microtubule stability (F). Bar, 12 μm.

(37%), the association of gigaxonin with MAP1B-LC was seen to protect microtubules against drug-induced disassembly for 2 h (Fig. 5, C and D). All of the analyses on microtubule stability were conducted on three independent experiments. These findings reveal an important correlation between gigaxonin–MAP1B interaction and microtubule stability in cultured cells. An integrated and well-stabilized neuronal cytoskeleton is essential to neuronal survival.

Mutations in GAN disrupt the interaction of gigaxonin with MAP1B

To assess whether the mutations associated with GAN disrupt the interaction of gigaxonin with MAP1B-LC, we used PCRs to generate mutations in full-length gigaxonin. From the 14 mutations identified in GAN patients, we analyzed two mutations for a possible loss of the specific gigaxonin–MAP1B-LC interaction: the truncating mutation at the amino acid eight and one nonsense mutation in the kelch repeat domain (R293X). As judged by immunoblot analysis and immunofluorescence microscopy of transfected cells, both mutations resulted in unstable expression of the mutant proteins, undetectable level in the case of truncating mutation, and a greatly decreased level in the nonsense mutation (unpublished data). Of the few detectable cells transfected with HA epitope–tagged R293X construct (HA-Gig-R293X), the R293X mutant protein was diffusely accumulated and had completely lost its association with MAB1B-LC (Fig. 5 E). Our results reveal that through disruption of gigaxonin's kelch repeat domain at least 2 out of the 14 distinct mutations can cause loss of gigaxonin–MAP1B interaction. Moreover, the microtubules in cells cotransfected with HA-Gig-R293X and flag-MAP1B-LC failed to display the extraordinary stability seen in cells cotransfected with wild-type gigaxonin and MAP1B-LC (Fig. 5 F).

Together, our data demonstrates that gigaxonin directly associates with MAP1B-LC in the nervous system, an interaction that is disrupted by mutations identified in GAN patients. Gigaxonin–MAP1B-LC interaction renders microtubules resistant to destabilizing reagents. We speculate that through competing with MAP1B-HC for binding to the MAP1B-LC, gigaxonin–MAP1B-LC interaction may regulate the association between LC and HC of MAP1B. The identification of a neuronal binding partner for gigaxonin serves as a prelude for future studies investigating the pathogenesis of this disorder in vivo.

Materials and methods

Coimmunoprecipitation

The lysates of cells cotransfected with gigaxonin, or MAP1B-LC, or both were incubated with anti-Flag beads (F7425; Sigma-Aldrich) at 4°C for 4 h to capture the flag-MAP1B-LC and its associated protein. After washing,

the bound proteins were eluted with SDS sample buffer, analyzed via SDS-PAGE, and immunoblotted with anti-HA antibody (Covance) to detect the HA-gigaxonin. The proteins were visualized using ECL chemiluminescence (Amersham Biosciences).

Yeast two-hybrid screening

Full-length gigaxonin was subcloned in frame at the 3′ end of a GAL4 DNA-binding domain (DNA-BD) into pGBKT7 vector. The DNA–BD–gigaxonin construct was transformed into the yeast strain, AH109, according to the manufacturer's instructions (CLONTECH Laboratories, Inc.). Immunoblots from the transformants were conducted to verify stable expression of fusion protein. A concentrated overnight culture of AH109/DNA–BD–gigaxonin was combined with a yeast culture of pretransformed MATCHMAKER human brain cDNA library. This mating mixture was incubated and plated on selection plates. Transformant colonies growing on the plates were then sorted and further analyzed for their putative positives.

Double immuno EM

Animals were killed by intravenous perfusion with 2% paraformaldehyde and 0.05% glutaraldehyde. The dissected samples of sciatic nerves were processed and embedded for EM (Coulombe et al., 1989). The antibody incorporations of mouse anti–MAP1B-LC (Sigma-Aldrich) and rabbit antigigaxonin on ultrathin sections were visualized with 12 nm anti–rabbit and 6 nm anti–mouse gold-conjugated particles. After staining with uranyl acetate, followed by lead citrate, the sections were analyzed under a Philips CM10 microscope.

Neuron culture

The mice DRGs were microdissected and enzymatically dissociated by incubating the tissues with 0.5% trypsin (Sigma-Aldrich) for 15–30 min at 37°C and then plated on coverslips coated with 0.02% collagen. Cells were cultured in MEM medium containing NGF for 7 d. The cells were then fixed and subjected to immunofluorescence staining as described (Yang et al., 1996).

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