Giant axonal neuropathy–associated gigaxonin mutations impair intermediate filament protein degradation

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Giant axonal neuropathy (GAN) is an early-onset neurological disorder caused by mutations in the GAN gene (encoding for gigaxonin), which is predicted to be an E3 ligase adaptor. In GAN, aggregates of intermediate filaments (IFs) represent the main pathological feature detected in neurons and other cell types, including patients’ dermal fibroblasts. The molecular mechanism by which these mutations cause IFs to aggregate is unknown. Using fibroblasts from patients and normal individuals, as well as Ganc−/− mice, we demonstrated that gigaxonin was responsible for the degradation of vimentin IFs. Gigaxonin was similarly involved in the degradation of peripherin and neurofilament IF proteins in neurons. Furthermore, proteasome inhibition by MG-132 reversed the clearance of IF proteins in cells overexpressing gigaxonin, demonstrating the involvement of the proteasomal degradation pathway. Together, these findings identify gigaxonin as a major factor in the degradation of cytoskeletal IFs and provide an explanation for IF aggregate accumulation, the subcellular hallmark of this devastating human disease.

Introduction
Abnormal aggregates of intermediate filaments (IFs) are pathological hallmarks of tissue-specific diseases, including keratin aggregates in Mallory bodies in diseased hepatocytes (1); Rosenthal fibers in the glial cells of Alexander disease patients, which contain glial fibrillary acidic protein (GFAP) (2); and neuronal IF aggregates in the neurons of patients with amyotrophic lateral sclerosis (ALS), Alzheimer’s disease (3), Parkinson’s disease, dementia with Lewy bodies, neuronal IF inclusion disease (NIFID), giant axonal neuropathy (GAN) (4–6), some forms of Charcot-Marie-Tooth disease (CMT), diabetic neuropathy, and spinal muscular atrophy (7, 8). Although numerous mutant genes have been identified that cause these diseases, few have provided insights into the mechanisms responsible for IF aggregate formation. GAN is unique with respect to these diseases, as it affects numerous classes of cytoskeletal IFs (4–6, 9–15). We therefore reasoned that a detailed analysis of this disease might provide insights into common mechanisms responsible for the formation of IF aggregates in different tissues of GAN patients and into the regulation of the turnover of this major class of cytoskeletal proteins.

GAN is a recessive disease (4, 5, 16, 17) caused by mutations in the GAN gene (encoding for gigaxonin), which is located on chromosome 16q24. Over 30 distinct mutations distributed throughout the GAN gene have been identified (9, 11, 16). GAN patients are born apparently normal, but typically by 3–5 years of age, they display progressive muscle weakness, diminished tendon reflexes, and pronounced gait disturbances. Subsequently, they become wheelchair bound, experience dysmetria and seizures, and ultimately require feeding and breathing tubes. Patients typically die during the second or third decade of life. Histopathology reveals the presence of large axonal swellings (“giant axons”) filled with type IV neuronal IFs — the neurofilament (NF) triplet proteins NF light, medium, and heavy chain (NF-L, NF-M, and NF-H, respectively) — in the CNS and peripheral nervous system (PNS) (4, 5, 18). In addition to the NF triplet proteins, the type III IF protein peripherin is also a component of the endogenous IF networks in neurons of the PNS (19). Thus, it is likely that the aggregates detected in the giant axons of PNS neurons in GAN patients contain both the triplet proteins and peripherin. Most patients also have characteristic kinky hair, which suggests that type I and II keratin IFs are affected (12, 13). Moreover, as first shown more than 30 years ago by Pena (6, 15) and subsequently verified by others (10, 20–22), cultured skin fibroblasts from patients contain large IF aggregates of type III vimentin (encoded by VIM) (6, 10, 15, 20–22), and similar IF aggregates have been detected in Schwann cells, melanocytes, endothelial cells, muscle cells, and Langerhans cells (14, 23). Taken together, these observations suggest that gigaxonin mutations affect many members of the cytoskeletal IF protein family.

Based on sequence homology, gigaxonin appears to be an E3 ubiquitin ligase adaptor and thus is likely involved in degrading protein substrates via the ubiquitin-proteasome system (UPS). Gigaxonin belongs to the BTB-KELCH domain family of adaptor proteins. The KELCH domain interacts with the protein targeted for degradation, whereas the BTB domain interacts with the Cullin-3 component of the ubiquitin ligase complex (24–27). In support of gigaxonin’s role in regulating the turnover of IF proteins, histological examination of Ganc−/− mice has revealed pathological features similar to those found in GAN patients. These include accumulation of IF proteins, formation of aggregates in the ner-

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 2013;123(5):1964–1975. doi:10.1172/JCI66387.
gigaxonin in mediating the degradation of vimentin. Because gigaxonin is a putative E3 ligase adaptor, its expression is likely to cause IF clearance via a degradative pathway. However, in principle, it is also possible that the decrease in vimentin protein is caused by a reduction in transcription of VIM mRNA. To test this, VIM mRNA levels were determined by RT-PCR after expression of WT or SS2G gigaxonin in BJ5ta cells. Neither WT nor mutant gigaxonin had any detectable effect on VIM mRNA levels (Figure 2C). This provides further evidence in support of a role for gigaxonin in mediating the degradation of vimentin.

Time course of vimentin clearance due to gigaxonin expression. To define the time course of the loss of the VIF network and clearance of vimentin from cells. Given our observations that mutations in or deletions of gigaxonin caused IF aggregation, we sought to test the possibility that restoration of normal VIF networks might be achieved by introducing WT gigaxonin into GAN cells or Gan−/− MEFS. However, we found that expressing WT gigaxonin did not just clear the VIF aggregates, but in fact had the more dramatic effect of causing almost complete loss of vimentin in both control and GAN cells (Figure 2, A, D, and E). Although VIF disappeared by 72 hours, MTs and MFs appeared normal (Figure 2, D–F, and refs. 15, 20), and no changes in the levels of tubulin and actin were detected by immunoblotting (Figure 2A). We also tested whether expression of WT gigaxonin resulted in a loss of TBCB, as previously reported (32). Immunoblotting revealed no changes in TBCB levels in control or GAN cells under the same experimental conditions (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI66387DS1). This latter finding confirmed the results of other studies (20, 29). To further confirm the role of gigaxonin in regulating IF protein levels, a mutant form, S52G gigaxonin (9), was expressed in BJ5ta cells. Under these conditions, no change in vimentin levels were detected by immunoblotting (Figure 2B and Supplemental Figure 2). Thus, this mutant form of gigaxonin, unlike WT gigaxonin, could not clear vimentin. Overall, these results demonstrated that gigaxonin has a substantial effect on VIFs, but not on the other major cytoskeletal proteins, tubulin and actin.

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Expression of WT gigaxonin causes loss of VIF networks and clearance of vimentin from cells. Given our observations that mutations in or deletions of gigaxonin caused IF aggregation, we sought to test the possibility that restoration of normal VIF networks might be achieved by introducing WT gigaxonin into GAN cells or Gan−/− MEFS. However, we found that expressing WT gigaxonin did not just clear the VIF aggregates, but in fact had the more dramatic effect of causing almost complete loss of vimentin in both control and GAN cells (Figure 2, A, D, and E). Although VIF disappeared by 72 hours, MTs and MFs appeared normal (Figure 2, D–F, and refs. 15, 20), and no changes in the levels of tubulin and actin were detected by immunoblotting (Figure 2A). We also tested whether expression of WT gigaxonin resulted in a loss of TBCB, as previously reported (32). Immunoblotting revealed no changes in TBCB levels in control or GAN cells under the same experimental conditions (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI66387DS1). This latter finding confirmed the results of other studies (20, 29). To further confirm the role of gigaxonin in regulating IF protein levels, a mutant form, S52G gigaxonin (9), was expressed in BJ5ta cells. Under these conditions, no change in vimentin levels were detected by immunoblotting (Figure 2B and Supplemental Figure 2). Thus, this mutant form of gigaxonin, unlike WT gigaxonin, could not clear vimentin. Overall, these results demonstrated that gigaxonin has a substantial effect on VIFs, but not on the other major cytoskeletal proteins, tubulin and actin.
The disappearance of vimentin was inversely related to the time course and levels of WT gigaxonin expression. Parallel immunofluorescence studies showed that at 24 hours after induction of gigaxonin expression, VIFs were still observed, but by 48 hours, mature IFs disappeared, and only short IFs and nonfilamentous vimentin particles remained (Figure 3B and ref. 34). By 72 hours, there was no detectable vimentin (Figure 3B). These observations suggest that VIFs are aggregated in both GAN cells and Gan−/− MEFs. (A) VIF networks in a control (Con) cell and in 2 different GAN cells, the latter of which were aggregated into bundles and large bodies (arrows). HM, higher magnification. Representative images, 5 preparations of 3 GAN patient lines. (B) TEM showing aggregated VIF in GAN cells. Mitochondrion (M) and elements of the endoplasmic reticulum (arrow) are indicated in the right panel. (C) MTs (top) and actin (bottom) in double-stained GAN cells. Representative images, 5 preparations. (D) Western blot analyses of control fibroblasts (AG08470) and 3 GAN cell lines. Fold changes (± SD) in GAN line vimentin levels relative to control were as follows: F07476 (left), 1.623 ± 0.161; 08F699 (middle), 0.687 ± 0.05; F09133 (right), 1.183 ± 0.064 (P = 0.0039). Representative blots, 3 preparations. (E) VIFs were only aggregated in Gan−/− MEFs, not WT MEFs. Representative images, 5 preparations. (F) Western blotting of WT and Gan−/− MEF lysates. Representative blots, 3 preparations. (G) TEM showing VIF aggregates in Gan−/− MEFs. (H) MT (top) and actin (bottom) organization in Gan−/− MEFs. Representative images, 5 preparations. Scale bars: 10 μm (A, C, E, and H); 5.0 μm (B, left, and G, left); 0.5 μm (B, right, and G, right).
first disassembled into short IFs and nonfilamentous particles that were subsequently further degraded. This series of VIF degradation steps was similar for GAN cells and WT and Gan–/– MEFs (Figure 3, C–E). These observations also demonstrated that in GAN cells and Gan–/– MEFs, the aggregates were the last vimentin structures to disappear after gigaxonin expression (Figure 3, C and E), most likely attributable to the high concentration of VIF in the aggregates relative to the rest of the cell. In contrast, time course analysis after S52G gigaxonin expression in BJ5ta cells revealed no change in vimentin protein levels and no obvious structural changes in VIF (Figure 2B and Supplemental Figure 2, A and B). These results confirmed that disease-causing mutations in gigaxonin result in the loss of its function, insofar as its ability to clear vimentin is concerned.

Interactions between gigaxonin and vimentin. With respect to the localization of gigaxonin, immunofluorescence revealed that it was evenly distributed throughout the cytoplasm in control cells. However, in GAN cells, gigaxonin was concentrated in the VIF aggregates relative to the rest of the cell. In contrast, time course analysis after S52G gigaxonin expression in BJ5ta cells revealed no change in vimentin protein levels and no obvious structural changes in VIF (Figure 2B and Supplemental Figure 2, A and B).
antibodies revealed the presence of vimentin by immunoblotting, and the reciprocal experiment (IP using anti-vimentin) showed the presence of gigaxonin (Figure 4, B and C). Additional evidence for an interaction between gigaxonin and vimentin in cell lysates was obtained using ELISA (Figure 4D). Taken together, these results revealed an interaction between vimentin and gigaxonin. However, further studies are required to determine whether this interaction is direct or mediated by other proteins. In addition, we do not know the assembly state of vimentin involved in the interaction with gigaxonin under normal in vivo physiological conditions. In IP experiments, vimentin was completely solubilized in the RIPA buffer used to optimize conditions for vimentin/gigaxonin interactions.

The central rod domain of vimentin is required for its clearance by gigaxonin. In order to identify which region of vimentin is important for its clearance by gigaxonin, FLAG-tagged deletion constructs of its 3 major domains (Figure 4E) were prepared: the non-α-helical
N-terminal domain (referred to herein as the FLAG-ΔN-VIM construct), the non–α-helical C-terminal domain (FLAG-ΔC-VIM), and the central rod domain, with both the N- and C-termini deleted (FLAG-Rod-VIM). These constructs were used to establish lines of mouse 3T3 fibroblasts. After expression of WT gigaxonin for 72 hours, all 3 deletion constructs of vimentin were cleared, as determined by immunoblotting. As expected, the endogenous full-length vimentin was also cleared within these same cells (Figure 4F). These results suggest that, as the common domain in all 3 deletion constructs, the central rod domain of vimentin is required for its clearance by gigaxonin (Figure 4, E and F).

Additionally, we expressed FLAG-tagged non–α-helical N and C termini alone (referred to herein as Head and Tail, respectively). We found that the FLAG-Head-VIM and FLAG-Tail-VIM constructs could only be expressed at extremely low levels in 3T3 cells, undetectable by immunoblotting; by immunofluorescence, they were mainly associated with the endogenous VIF network (data not shown). Therefore, we prepared FLAG-Head-VIM and FLAG-Tail-VIM deletion construct–expressing lines using Vim−/− MEFs in order to visualize them by immunofluorescence in the absence of an extensive endogenous VIF network (see Methods). As a further control, we also prepared individual lines of Vim−/− MEFs express-
Mechanism responsible for VIF clearance by gigaxonin. (A) Immunoblotting of control and WT gigaxonin expressing BJ5ta cells treated with MG-132 using antibodies to vimentin, actin, or FLAG (gigaxonin). MG-132 treatment caused substantial recovery of vimentin in gigaxonin-expressing cells. Representative blots, 4 experiments. (B) In vitro ubiquitination assay showing ubiquitination ladder for the positive control sic 60–barnase–DHFR, but not for vimentin in the same preparation. Representative autoradiogram, 3 experiments. Lanes were run on the same gel but were noncontiguous (black line). (C) Immunoblotting of lysates of BJ5ta cells that had expressed gigaxonin for 72 hours and subsequently were treated with chloroquine (CQ; lysosome inhibitor), MG-132 (proteasome inhibitor), or 3-MA (autophagy inhibitor) for 12 hours. Blots were probed with antibodies to vimentin, actin, tubulin, or gigaxonin. The untreated cell lysate (−) contained very small amounts of vimentin. Vimentin recovery was seen only upon inhibition of proteasomes, not by inhibition of lysosomes or autophagy. Representative immunoblots, 4 experiments. (D) Longer exposure of the anti-vimentin full-length immunoblot in C, showing absence of a typical ubiquitinated protein ladder for vimentin. Representative blots from 4 experiments.
these cells were then separately treated for 12 hours with inhibitors for autophagy (3-methyl adenine; 3-MA) or lysosomal degradation (chloroquine) or with MG-132 as a control. Immunoblotting revealed the reappearance of vimentin only with MG-132 treatment (Figure 5C). It should also be noted that there was no evidence for the typical ladder of vimentin — which would indicate ubiquitination — when these same samples were immunoblotted with anti-vimentin (Figure 5D).

Discussion

Our results showed that the normal function of gigaxonin, a predicted E3 ligase adaptor protein, was to target certain IF proteins (e.g., vimentin, peripherin, and NF-L) for degradation. They also provided a framework for understanding the molecular basis of the formation of the large IF aggregates seen in different cell types of GAN patients. In both PNS and CNS, large axonal aggregates of IFs are the pathological hallmark of GAN. Although the exact mechanism behind the formation of these aggregates is not known, our results suggest that under normal conditions, gigaxonin regulates the degradation of IF proteins within axons. Therefore, when gigaxonin is defective, as in GAN, it leads to local accumulation of IFs along axons. Support for this possibility stems from the finding that the UPS is present along axons (e.g., ref. 38). Since GAN is a progressive disorder beginning at an early age with no detectable phenotype, it is likely that giant axons form slowly over prolonged periods beginning in early childhood and into the teenage years. The normal slow turnover of IF proteins (39–44) could therefore lead to the gradual accumulation of polymerized IFs into aggregates in the absence of normally functioning gigaxonin. Support for this possibility comes from our observation of GAN patient fibroblasts, which showed a passage-dependent increase in the number of cells containing VIF aggregates. The formation of large IF aggregates within axons could lead to altered axonal transport and progressive neuronal dysfunction, accounting for the hallmark features of the disease, particularly the early-onset neuropathy. In support of defective transport mechanisms, we found accumulation of mitochondria in close proximity to and within the IF aggregates. This most likely reflects the previously reported interactions, binding, and anchorage of mitochondria to both VIFs (45) and NF IFs (46). It is also important to note that the nervous system contains both neuronal and glial cells. Therefore the glial-specific IF protein GFAP, as well as VIFs in astrocytes (47), could also contribute to the GAN phenotype.

The finding that GAN was caused by mutations in a single gene suggests that gene replacement may be a successful therapeutic approach. Indeed, overexpression of WT gigaxonin did lead to the disappearance of VIF aggregates, as well as those containing peripherin and NF IFs. Although we did not test whether GFAP IFs are cleared in glial cells, it is also conceivable that the similar aggregates of this type III IF protein might be cleared by gigaxonin in patients with Alexander disease (2). However, as in any gene rescue therapy, one must carefully consider dosage, since high levels of gigaxonin expression can lead to a complete loss of the normal IF system in numerous cell types. Although the overexpression of gigaxonin has provided us with the most convincing evidence in support of its role in IF protein degradation, it is possible that excess expression of gigaxonin may have side effects that we have been unable to detect. We feel that this is unlikely, as the overexpression of gigaxonin for prolonged periods (up to a month) had no obvious effects on cell growth (data not shown). We also showed that overexpression of the S52G mutant of gigaxonin, at the same level as WT gigaxonin overexpression, had no effect on the organization of VIF networks, and there was no degradation of vimentin. These findings emphasize that the specific targets for gigaxonin are members of the cytoskeletal IF protein family. In further support of the specificity for IF cytoskeletal systems, we could detect no decreases in the level of either tubulin or actin when gigaxonin was expressed in cells. However, we did detect an increase in the amount of tubulin in GAN cells expressing defective gigaxonin, even though the overall organization of MTs appeared normal. Based on the well-established interactions between IFs and MTs, it is conceivable that alterations in IF protein turnover caused by gigaxonin mutations could stimulate tubulin expression by unknown feedback mechanisms (34, 48–50).

The finding that gigaxonin expression degraded IF proteins was consistent with their higher expression levels and IF aggregate formation previously seen in the differentiated tissues of adult Gan–/– mice (28, 29). However, the Gan–/– MEFs analyzed in the present study showed no difference in vimentin levels compared with WT MEFs. This discrepancy may be related to the early developmental stage at which the MEFs were obtained. For example, there could be less accumulation of IFs in embryonic fibroblasts relative to that seen in adult differentiated cells. The fact that human patients appear normal during their first few years of life also suggests that it takes a significant amount of time to accumulate IF aggregates within neurons that can cause clinically detectable neurological phenotypes. Further studies are required to understand the role and regulation of gigaxonin during early development and, in GAN patients, as a function of disease progression.

IFs have been shown to be composed of stable proteins with a half-life ranging from 18 hours to 8 days (39–42). However, very little is known about the pathways involved in their degradation in normal cells. Although our present results demonstrated that gigaxonin targets IF proteins for degradation via the UPS, the precise mechanism for proteasome targeting remains unknown. An insight regarding this mechanism comes from the observation that numerous nonfilamentous vimentin particles and short IFs appeared as the VIF network was being cleared by expressing gigaxonin. This finding suggests that long mature IFs are first disassembled into smaller structures, which have previously been identified as assembly/disassembly intermediates (51), before being degraded. However it is not known whether short IFs, unit length filaments (ULFs), tetratrams, dimers, monomers, or other intermediates are targeted for degradation. The mechanism by which gigaxonin expression leads to VIF disassembly is also unknown.

It is conceivable that interactions between gigaxonin and vimentin participate in VIF disassembly, especially since our evidence suggests that this E3 ligase adaptor interacts with the highly conserved central rod domain, a region critically involved in regulating IF disassembly by unknown feedback mechanisms (34, 48–50). Subsequently, in its role as an E3 ligase adaptor, gigaxonin could target the smallest disassembly units for degradation. It is also possible that phosphorylation of vimentin may be involved in the process of its degradation by proteasomes, since the major known regulator of IF disassembly is phosphorylation (54–56). However, the state of vimentin phosphorylation in the presence of elevated gigaxonin remains unknown. Recent studies involving IF protein degradation in muscle atrophy provide additional insights into a possible relationship between phosphorylation and degradation of IF proteins (57). Specifically, desmin, the protein comprising the IF of skeletal muscle, was found to be phosphorylated, disassem-
bled, and ubiquitinated by the E3 ligase TRIM32 and subsequently degraded (57). In contrast, our inability to detect ubiquitination of vimentin suggests that alternate mechanisms may be involved in targeting it to the proteasome. For example, gigaxonin may be involved in the ubiquitination of other proteins that bind to and target vimentin to the proteasome. In support of this possibility, it has previously been shown that direct target protein ubiquitination is not required for proteasomal degradation (58–61). Rather, an intermediate protein that is ubiquitinated binds to the target protein and escorts it to the proteasome for degradation. One of the best-studied examples of this type of pathway involves the human papilloma virus oncoprotein E7 and the degradation of its binding partner, the tumor-suppressor protein retinoblastoma (Rb) (62, 63). Therefore, Rb is not directly ubiquitinated, but is targeted to the proteasome by binding to ubiquitinated E7. A comparable mechanism may exist for the degradation of IF proteins mediated by gigaxonin. Nonetheless, the fact that gigaxonin is predicted to be an E3 ligase adaptor suggests that vimentin should become ubiquitinated. However, it is well known that ubiquitination of a protein is often difficult to detect for numerous technical reasons, including specific deubiquitinasen activity (64). Therefore, further studies are required to determine whether vimentin is ubiquitinated.

Although it is not known how gigaxonin is regulated in normal cells, it has previously been shown that it is expressed at extremely low levels (~7,500 molecules/cell; ref. 20). In contrast, IF proteins such as vimentin can represent 2%–3% or more of total cell protein (65), yet only a small fraction appears to be in a soluble form (66). These limiting amounts of gigaxonin may therefore only be capable of targeting this small “soluble” pool of vimentin, which can then be degraded by the proteasome. In addition, the levels of gigaxonin in cells may be regulated at the transcriptional level and/or by its turnover. Better understanding of how gigaxonin is regulated is an essential element of understanding its role in the degradation of VIFs and of peripherin and NF IF proteins.

In summary, we identified gigaxonin as the first E3 ligase adaptor that targets cytoskeletal IF proteins for degradation. Our findings provided insight into the mechanisms responsible for the degradation of several major types of IF proteins and provided a framework for understanding the mechanisms responsible for the progressive neurological disorder GAN. Based on these findings, it is possible that the abnormal regulation of gigaxonin may also be involved in the numerous other diseases known to involve accumulations of IF and/or their constituent proteins, such as Alzheimer’s disease and Parkinson’s disease (see Introduction and refs. 3, 7).

Methods

Cell culture. BJ5ta human foreskin fibroblasts and NIH-3T3 mouse fibroblasts (ATCC) were maintained in DMEM (Invitrogen) supplemented with 10% calf serum (HyClone). MEFs from control or Gal−/− mice (28) were cultured in DMEM containing 10% FCS. PC12 cells were differentiated with nerve growth factor (NGF; Invitrogen) as described previously (67–69). SH-SY-5Y cells were differentiated by culturing them in medium supplemented with 10 μM retinoic acid (RA; Sigma-Aldrich) for 1 week and then 10 μM RA plus 50 ng/ml brain-derived neurotrophic factor (BDNF; Sigma-Aldrich) for an additional week. Dorsal root ganglia (DRG) cultures were prepared from E13.5 embryos. DRGs were dissected and dissociated for 15 minutes at 37°C in 0.05% trypsin plus 0.53 mM EDTA (Invitrogen). After trypsinization, DRGs were rinsed with L-15 containing trypsin inhibitor (1 mg/ml; Sigma-Aldrich) and then triturated. Neurons were plated onto 4-well plates (LabTek) precoated with poly-γ-lysine and extracellular matrix (Sigma-Aldrich) and maintained in neurobasal medium supplemented with B-27 supplement plus 0.5 mM glutamine and 15 μg/ml NGF. Culture media were supplemented with penicillin/streptomycin (Sigma-Aldrich), and all cell cultures were maintained at 37°C in a humidified CO2 incubator.

Antibodies and reagents. Mouse anti-vimentin, rat and mouse anti–β-tubulin, mouse anti–β-actin, rabbit anti-gigaxonin, rabbit anti-FLAG, mouse anti–NF-L, rabbit anti-TBCB, and mouse anti-actin were obtained from Sigma-Aldrich. Mouse anti-GAPDH (Biolegend), chicken anti-vimentin (Covance), rabbit anti–vimentin 314, and rabbit anti–peripherin 199 have been described previously (54, 70). Secondary antibodies included Alexa Fluor 488–, Alexa Fluor 568–, FITC–, and rhodamine-conjugated goat anti-mouse, anti-chicken, and anti-rabbit IgG (Invitrogen). Secondary antibodies for immunoblotting included peroxidase-conjugated goat anti-mouse and anti-rabbit (Jackson ImmunoResearch). TRITC-conjugated phalloidin and Hoechst 33342 (Invitrogen) were used to stain actin and nuclei, respectively.

In some experiments, treatment with MG-132 (12 μM; Calbiochem; 10 or 20 mg/ml stock in DMSO) was used to inhibit proteasomes, chloroquine (150 μM; Sigma-Aldrich) was used to inhibit lysosome function, and 3-MA (5 mM; Sigma-Aldrich) was used to inhibit autophagy. Cells were exposed to one of these inhibitors for 6–12 hours. In the MG-132 experiments, control cells were treated with a comparable concentration of DMSO.

DNA constructs. Plasmid vectors for eukaryotic expression of WT gigaxonin (pTL3.1-gigaxonin) or mutant gigaxonin (pTL3.1-293X gigaxonin and pTL3.1-R138H gigaxonin) were previously described (10). The lentiviral vector pLEX-MCS–FLAG-tagged WT gigaxonin was generated as described below. FLAG-tagged gigaxonin was amplified by PCR using primers 5′-CCCCCCCCACCTAGTATGGACTACAAAGACGATGAC-3′ (forward) and 5′-CCCCCCCCGCGCCGCTCAAGGGAAATGAAACGGA-3′ (reverse). The PCR product was then digested with Spel and NotI and cloned into the pLEX-MCS backbone that was previously digested with the same restriction enzymes. The lentiviral vector expressing mutant FLAG-tagged S52G gigaxonin was prepared by introducing the 154A→G point mutation in the WT sequence of gigaxonin using the QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene). Lentiviral vectors coding for nontagged WT gigaxonin (pLEX-MCS gigaxonin) were prepared by digesting pTL3.1 with BamHI and NotI to release WT gigaxonin, then cloning it into the pLEX-MCS vector that had been double digested with BamHI and NotI.

FLAG-AC-VIM (vimentin with its non–α-helical C-terminal domain [aa 1–411] deleted), FLAG-AN-VIM (vimentin with its non–α-helical N-terminal domain [aa 103–466] deleted), and FLAG-Rod-VIM (vimentin rod domain; aa 103–411) were cloned into the lentiviral pLEX-MCS vector between BamHI and NotI sites using the InFusion HD Cloning System (Clontech). Each fragment was amplified using Phusion High Fidelity Polymerase, and the FLAG tag was included in the 5′ primer for each deletion fragment. FLAG-FL-VIM (full-length vimentin), FLAG-Head-VIM (vimentin head domain only [aa 1–102]), and FLAG-Tail-VIM (vimentin tail domain only [aa 411–466]) were recombinant proteins were maintained in their respective culture medium supplemented with 2 μg/ml puromycin (Sigma-Aldrich). Vim−/− MEFs (gift of J.E. Eriksson and E. Torvaldsen, Åbo Akademi University, Turku, Finland) were grown in DMEM containing 10% FCS. PC12 cells were differentiated with nerve growth factor (NGF; Invitrogen) as described previously (67–69). SH-SY-5Y cells were differentiated by culturing them in medium supplemented with 10 μM retinoic acid (RA; Sigma-Aldrich) for 1 week and then 10 μM RA plus 50 ng/ml brain-derived neurotrophic factor (BDNF; Sigma-Aldrich) for an additional week. Dorsal root ganglia (DRG) cultures were prepared from E13.5 embryos. DRGs were dissected and dissociated for 15 minutes at 37°C in 0.05% trypsin plus 0.53 mM EDTA (Invitrogen). After trypsinization, DRGs were rinsed with L-15 containing trypsin inhibitor (1 mg/ml; Sigma-Aldrich) and then triturated. Neurons were plated onto 4-well plates (LabTek) precoated with poly-γ-lysine and extracellular matrix (Sigma-Aldrich) and maintained in neurobasal medium supplemented with B-27 supplement plus 0.5 mM glutamine and 15 μg/ml NGF. Culture media were supplemented with penicillin/streptomycin (Sigma-Aldrich), and all cell cultures were maintained at 37°C in a humidified CO2 incubator.

In summary, we identified gigaxonin as the first E3 ligase adaptor that targets cytoskeletal IF proteins for degradation. Our findings provided insight into the mechanisms responsible for the degradation of several major types of IF proteins and provided a framework for understanding the mechanisms responsible for the progressive neurological disorder GAN. Based on these findings, it is possible that the abnormal regulation of gigaxonin may also be involved in the numerous other diseases known to involve accumulations of IF and/or their constituent proteins, such as Alzheimer’s disease and Parkinson’s disease (see Introduction and refs. 3, 7).
amplified by PCR and cloned into pLEX-MCS vectors between BamHI and NotI sites. Individual clones were confirmed by sequencing.

Lentiviruses were produced according to the manufacturer’s instructions by cotransfecting plasmids pLEX-MCS (Thermo Scientific), along with the helper plasmids pVSVG and pR2, into 293FT cells (Invitrogen) using Xfect Transfection Reagent (Clontech). Culture supernatant was collected 2 days later, and target cells were incubated with the viral supernatant supplemented with 8 μg/ml polybrene (Sigma-Aldrich) for 4–8 hours, after which the virus containing medium was replaced with the growth medium. This technique was used to establish 3T3 cell lines expressing FLAG-AN-VIM, FLAG-AC-VIM, and FLAG-rod-VIM constructs and Vim−/− cell lines expressing FLAG-AN-VIM, FLAG-AC-VIM, FLAG-rod-VIM, FLAG-Head-VIM, and FLAG-Tail-VIM constructs. In some cases, these lines were also infected with lentivirus expressing WT gigaxonin. Other lines used included BJ5ta, GAN 08F699, GAN F07476, 3T3, PC12, and SH-SY-5Y cells expressing WT gigaxonin and BJ5ta cells expressing SS2G gigaxonin.

Immunofluorescence, image acquisition, and electron microscopy. Cells were processed for indirect single and double immunofluorescence after fixation in methanol or 3.7% formaldehyde as previously described (34, 50). The fixed, immunostained cells were imaged using a Zeiss confocal LSM10 META (Carl Zeiss) microscope with oil immersion objective lenses (Plan-Apochromat, ×63 and ×100, 1.40 NA; Carl Zeiss) (54). Electron microscopy was performed as previously described (71).

SDS-PAGE and immunoblotting. Cells grown in 60- or 100-mm culture dishes were lysed in Laemmli sample buffer, and proteins were separated by SDS-PAGE and immunoblotted as previously described (34, 52).

Image processing. Image J (NIH) and Photoshop CS3 (Adobe Systems) were used for figure preparation. Immunoblots were quantified using Kodak molecular imaging network software (Kodak).

IP experiments. Cells for IP were prepared from BJ5ta cells expressing gigaxonin for 36 hours. 100-mm culture dishes were washed twice with ice-cold PBS and then lysed on ice in 600 μl RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0) containing protease (EDTA-free mini tablets; Roche) and phosphatase inhibitors (Cocktail sets I & II; Calbiochem). The lysates were centrifuged at 20,000 × g for 30 minutes, and the supernatants were removed in order to determine protein concentration using the BCA protein assay kit (Pierce; Thermo Scientific). The clarified supernatant was subjected to IP with protein A sepharose 4 fast flow resin (Amersham Biosciences) according to the manufacturer’s instructions. Briefly, 20 μg rabbit anti-gigaxonin IgG or anti-vimentin IgG was allowed to bind to approximately 100 μg of cell extract in 1 ml of 100 mM sodium phosphate buffer (pH 7.0) for 3 hours at 4°C and then mixed with protein A sepharose resin for another 16 hours at 4°C. For controls, 20 μg normal rabbit/mouse IgG was used. The resin was washed thoroughly, and samples were boiled in SDS-PAGE sample buffer. Mouse monoclonal anti-vimentin IgG or rabbit anti-gigaxonin IgG were used for immunoblotting after SDS-PAGE.

mRNA analysis. Total RNA was prepared from cells using TRIzol (Invitrogen) and further purified to remove any contaminating DNA (RNeasy kit; Invitrogen). For quantitative RT-PCR, 1 μg total RNA was used to generate cDNA with the SuperScript III First-Strand kit (Invitrogen), and 1 μl of the total reaction volume was used for quantitative RT-PCR. All amplifications were performed on a Mastercycler EP Realplex (Eppendorf) using a master-mix containing SYBR Green and Rox (Applied Biosystem). RNA expression levels were analyzed using the following primers: VIM forward, 5′-AAGAGAATTTGCCGTTGAA-3′; VIM reverse, 5′-GTGATGCTGAGAAGTTTCGT-3′; GPDH forward, 5′-CTGGCAGACCACTGTTGAG-3′; GPDH reverse, 5′-AGGTCCACCACTGTTGAG-3′; GAN forward, 5′-GGGATCCAGGAGATGTTTTGCTG-3′; GAN reverse, 5′-CGGATCCAGGAGATGTTTTGCTG-3′.

The ΔΔCt method was used for relative quantification, with GAPDH expression levels serving as reference (73). Each sample was run in triplicate, and any Ct values that diverged 4 or more cycles were excluded for subsequent analyses. Data represent average ± SD from 3 independent experiments.

ELISA. ELISA was carried out in 96-well Micro-test III assay plates (Falcon 3910; BD Labware) at room temperature on a Titer Plate shaker. Full-length vimentin and the vimentin rod domain (aa 103–411) were expressed in bacteria and purified as described previously (74). The wells of the microtiter plates were coated with 1 μg full-length vimentin or the rod domain in 100 μl PBS for 5 hours. The wells were then blocked 3 times with 0.1% Tween-20 in PBS (PBS-T; 300 μl/well) for 1 hour. To prepare cell extracts, FLAG-tagged gigaxonin expressing BJ5ta cells grown on a 100-mm dish were washed twice with 12 ml ice-cold Tris-buffered saline (TBS) and extracted with 1 ml PBS containing 1% Triton X-100, 0.5 M NaCl, 10 mM MgCl2, and protease inhibitors as above. The cell extracts (0–5 μg) in 100 μl of 0.5% BSA in PBS-T were overlaid and allowed to bind for 16 hours at room temperature. Wells were washed 3 times with PBS-T (300 μl/well) for 30 minutes, and then rabbit anti-FLAG IgG (125 μl of 0.5 μg/ml) in PBS-T was added and allowed to bind for 2 hours at room temperature. The wells were washed 3 times with PBS-T (300 μl/well) and incubated with 125 μl alkaline phosphatase–conjugated anti-rabbit IgG (1:5,000 dilution in PBS-T containing 0.5% BSA) for 1 hour. The plates were then washed 3 times with alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl2, pH 9.5; 300 μl/well) for 30 minutes, and 100 μl of 1 mg/ml p-nitrophenylphosphate disodium in the alkaline phosphatase buffer was added to each well. The developed color was read in a MRX Revelation microplate reader at 405 nm (Dynex Corp.). For controls, cell extracts were overlaid on BSA-coated wells (75).

In vitro ubiquitination assay. For substrate expression, we used the following constructs: pCDNA3-myc-VIM; pCDNA3–myc–Cullin-3 (Addgene catalog no. 19893; ref. 76); pet30A–WT gigaxonin. As a control, we used a construct encoding sic 60–barnase–DHFR fusion—consisting of 60 aa from the bacterial Sic1 protein (containing a PPXY Rsp5 ubiquitination motif), followed by a catalytically inactive (H102A) Bacillus anthracis subtilisin/barnase, with all lysine replaced by arginine, methionine, or alanine residues; followed by E. coli dihydrofolate reductase (DHFR)—that has previously been used for ubiquitination and unfolding studies (77). Radioactive vimentin and control substrates (in pGEM3zf+[+]) were expressed from a T7 promoter by in vitro transcription and translation using a TNT Coupled Reticulocyte Lysate System (Promega) or an E. coli T7 S30 Extract System (Promega) containing [35S]-methionine. The ubiquitination reaction mixture contained 100 nM UbE1, 200 ng UbeH5a (E2), 1 mg/ml ubiquitin, and 2 μM ubiquitin aldehyde (all from Boston Biochem); 20 mM creatine phosphate and 0.2 mg/ml creatine phosphokinase (both from Roche); 4 mM ATP (Sigma-Aldrich); and 1 μM DTT in ubiquitination buffer (25 mM Tris-Cl, pH 7.5; 50 mM NaCl; and 4 mM MgCl2), as described previously (78). In addition, in vitro–transcribed and –translated gigaxonin and Cullin-3 (supplemented with nonradioactive methionine) or Rsp5 (E3) were added to assay for the ubiquitination of vimentin or the control substrates (77, 78). The reaction was incubated at 30°C for 90 minutes, and then samples were placed in Laemmli sample buffer and analyzed by SDS-PAGE. Radioactive proteins were detected by autoradiography (Instant Image; Packard).

In vivo ubiquitination assay. In vivo ubiquitination was performed as described previously (79). In brief, HEK293T cells (100 mm) were cotransfected with pCI-6xHis-Ub and FLAG-tagged gigaxonin or with FLAG-tagged gigaxonin and pCDNA3.1 (empty vector). 24 hours after transfection, cells were incubated with 10 μM MG-132 for 4 hours, then washed twice with PBS. Cell were extracted in 2 ml lysis buffer containing 50 mM Tris-HCl (pH 7.4), 0.25 M NaCl, 0.1% Triton X-100, 1 mM EDTA, 50 mM NaF, 1 mM DTT, 0.1 mM Na2VO3, inhibitor cocktail (Roche), 0.1 mM PMSF, and 2 mM DTT.
N-ethylmaleimide (NEM); isopeptidase and deubiquitinase inhibitor). Next, 100 μl prewashed Talon magnetic beads (Clontech) were added to the cell extract to a total volume of 6 ml and rotated for 2 hours at 4°C. Beads were washed twice in lysis buffer followed by an additional wash with lysis buffer containing 10 mM imidazole. Next, protein was eluted with 250 mM imidazole in lysis buffer, added to Laemmli sample buffer, and loaded on SDS-PAGE for detection of vimentin by Western blot.

Statistics. Data represent average ± SD from at least 3 independent experiments. 2-tailed Student’s t-tests were carried out to assess the significance of the results. A P value of 0.01 or less was considered significant.

Study approval. The use and maintenance of mice was in accordance with the Guide of Care and Use of Experimental Animals of the Canadian Council on Animal Care. The present studies were approved by the Comité de Protection des Animaux du Centre Hospitalier Universitaire de Québec (CIPA-CHUQ), Québec, Canada.

Received for publication January 9, 2013, and accepted in revised form February 14, 2013.

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